Evaluation of antioxidant and anticancer properties of entomopathogenic fungi *Isaria* from Darjeeling Hills

A thesis submitted to

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In partial fulfilment of the requirements for the

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By

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This is to certify that the Ph.D thesis entitled **"Evaluation of antioxidant and anticancer properties of entomopathogenic fungi *Isaria* from Darjeeling Hills"** submitted to Sikkim University in partial fulfilment for the requirement of the degree of Doctor of Philosophy in Botany. This thesis embodies the research work carried out by **Mr. Abhjit Chhetri** at the Department of Botany, School of Life Sciences, Sikkim University. It is a record of *bona-fide* investigation carried out and completed by him under my supervision. He has followed all the rules and regulations prescribed by the University for the compilation of the thesis. The results are original and have not been submitted anywhere else for any other degree or diploma.

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List of Abbreviations

$ABTS^{\bullet +}$	2, 2´-azinobis-(3—ethylbenzothiazoline-6-sulphonate)
AHC	Agglomerative hierarchical clustering
BHT	Butylated Hydroxy Toluene
bp	Base pair
CFU	Colony forming unit
^{O}C	Degree centigrade
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediamine tetraacetic acid
EPF	Entomopathogenic fungi
FACS	Fluorescence activated cell sorting
FT-IR	Fourier Transform Infrared
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
g	Gram
HeLa	Henrietta Lacks cells
HPLC	High performance liquid chromatography
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HepG2	Hepatoblastoma cells
IC_{50}	Half-maximal inhibitory concentration
ITS	Internal transcribed spacer
LSF	Liquid static fermentation
MTCC	Microbial Type Culture Collection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mM	Millimolar
mm	Millimetre
ml	Millilitre
mg	Milligram
NaOH	Sodium hydroxide
•OH	Hydroxy radical
PC3	Prostate Cancer cells

PCR	Polymerase chain reaction
PCA	Principal component analysis
PBS	Phosphate buffer saline
PI	Propidium iodide
SD	Standard deviation
SE	Standard error
Tris	Tris-(hydroxymethyl-) aminomethane
UV-VIS	Ultraviolet Visible
w/v	Weight by volume
µg	Microgram
µl	Microlitre
µM	Micromolar
ZOI	Zone of inhibition
%	Percent

GLOSSARY

Anamorph	An asexual state of fungus
Aseptate	Lacking cross walls in the hyphae.
Clavate	Club shaped
Conidiophore	A specialized hypha upon which conidia develop.
Conidiospore	A nonmotile asexual spore formed on a conidiophore.
Conidiogenous cell	A cell that forms conidia.
Elliptical	Oval with a symmetric curve
Fusiform	Spindle shaped, tapering towards the end.
Hyaline	Colourless
Hypha	A single filament of a fungus.
Mycelium	The mass of hyphae making up the thallus of a fungus.
Phialide	A specialised conidiogenous cell that produces conidia in basipetal succession without increasing in length.
Septum	A cross wall in hypha.
Solitary	Alone
Somatic	Vegetative phase.
Synnemata	A group of erect conidiophores that are cemented together producing conidia at the apex and/or along the sides.
Stroma	A compact somatic structure in or which reproductive structures form.
Teleomorph	The sexual state of a fungus.

1. Introduction

Darjeeling is the northernmost hilly district of the Indian state of West Bengal, geographically lying between the latitudes of 26° 30’ 05" and 27° 27’ 10" N and the longitudes of 87° 59’ 30" and 88° 53’ E. The mountainous tract is contiguous to the west by Nepal and Bhutan towards the east, in the north the river Teesta forms the northern border with the state of Sikkim and in the south by the district of Jalpaiguri of West Bengal and Purnea in the state of Bihar. The borders of Nepal, Sikkim and Darjeeling meet at the peak of Phalut (3700m) forming a tri-junction with a similar tri-junction at Rachela (Tinsimana, 3100m) between the district of Kalimpong, Sikkim and Bhutan from where the river Jaldhaka flows down separating the countries of Bhutan and India (Lama, 2004). Excluding a part of the Siliguri subdivision the District of Darjeeling is exclusively a mountainous region with the altitudinal range of Darjeeling varying from ca. 132 m at Sukna to 3700m at Phalut/ Sandakphu. The study area of focus in this dissertation is located in the mountainous part of the Darjeeling District. The landscape is dominated by steep mountains, luxuriant vegetation and rain fed rivers.

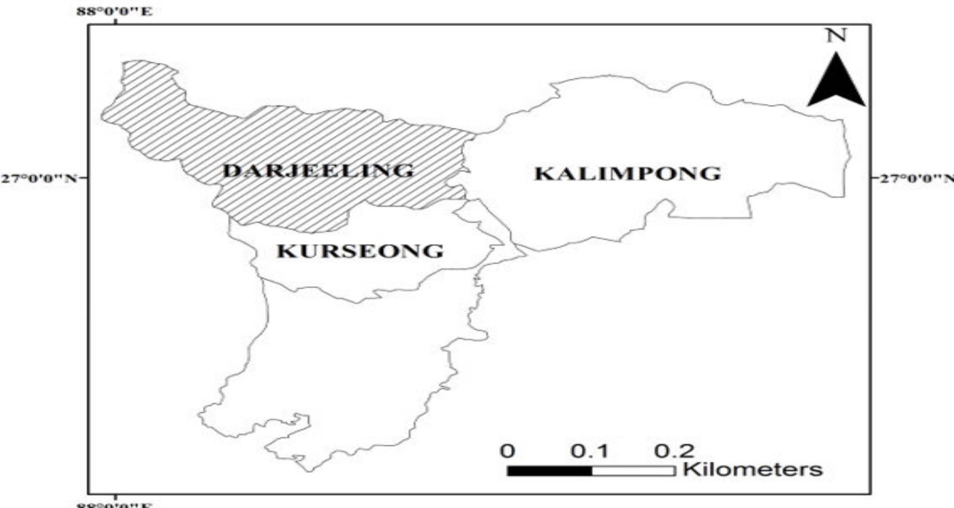


Figure 1.1: Map of Darjeeling hills (The Study Area)

Insect pathogenic fungi have fascinated man since times immemorial. Studies on entomopathogenic fungi (EPF) have become a significant topic to mycologists, entomologists, chemists and ecologists with mass attention due to their potent secondary metabolites production along with commercially viable mycomedicinal potential. They are considered as one of the most curious organisms to have evolved on this planet with highly unique life style involving complex chemical and biological interactions. The entomopathogenic fungi owing to their broad range of therapeutic benefits and practical traditional usage are becoming more and more important day to day and have been widely studied by various workers across the world. The area of research on any aspects of these fungi is currently blossoming with a goal to establish these organisms as environmentally safe, cheap to produce, reservoir of novel functional metabolites and efficient forms of pest control.

Although modern synthetic drugs are commonly available in the commercial market, however, these synthetic drugs are not generally regarded as safe (GRAS) due to their concern over side effects particularly drug resistance and sometimes they are often found to be detrimental for human health leading to great economic and social cost. On the other hand, there is still a gap in research on the distribution, mycelial biomass generation through artificial cultivation and screening of bio-constituents of the entomopathogenic fungi such as *Isaria* spp., on various cell lines for developing more efficacious and safer drugs with diminished or no side effects. Aside from their noted biological roles and function, the insect pathogenic fungi are found rarely in nature. Thus, these fungi are considered highly expensive and in the recent times interest in artificial cultivation has steadily grown to satisfy the large market demand. Among the entomopathogenic fungi, genus *Isaria* spp., from Darjeeling Hills which though used as an important traditional medicine in Asia but have not been studied so far is the major focus of this study. This fungus has been used in this thesis to address the gap in

literature by delving into its cultural characterization, phytochemical analysis, antioxidant activity, antibacterial susceptibility testing, cell viability testing and apoptotic effect analysis.

Fungi have the ability to grow and colonize a wide range of substrates which includes both vertebrate and invertebrate organisms. In case of latter, fungi are considered to play an important role in the natural regulation of insect populations (Evans, 1982a; 1982b). These insect attacking fungi are known as ‘entomopathogens’ and they can share either obligatory or facultative relationship with the insect host (Samson et al., 1988). According to Samson et al., (1988), the term ‘entomopathogenic fungi’ is restricted to those taxa of fungi, which are true pathogens of insects with their pathogenicity or for which circumstantial evidence exists concerning their pathogenicity. Hence the term “entomopathogenic fungi” have been used to denote the fungal species within the insect bodies or on the surface of their exoskeleton within the insects or those that may be pathogenic to them (Hung, 2010). In addition, the more flexible term ‘entomogenous fungi’ has not been used in the present study since that also relates to fungi growing on or colonising insect substrates, either facultatively or obligately, but which are strictly non-pathogenic (Samson et al., 1988). In general, entomopathogenic fungi are eukaryotic, unicellular or multicellular, filamentous, heterotrophic and saprophytic microorganisms. They can undergo reproduction via sexual or asexual spores, or both (Mora et al., 2017). This group of fungi are of global importance in terms of insect pathogens (Hyde et al., 2019). Entomopathogenic fungi plays an important role to balance the cycle of decomposition of organic matter in the environment and show specific pathogenicity towards insects without causing any harm or damage to human health (Moraga, 2020; Santos et al., 2021).

Apart from its traditional and biological control uses, *Isaria* spp., can be very useful for its secondary metabolites. Some of the major selected metabolites isolated

and identified from *I. fumosorosea* include; Beavurecin; A, E and J (cytotoxic), Beauverolides; C, F, I, L, Ja, L, La, M, and N (calmodulin inhibitors), Fumosorinone, A (PTP1B inhibitor), Trichocarane (Cytotoxic) (Weng et al., 2019), from *I. farinosa* include Cycloaspeptide, A, C, G and F (Cytotoxic), Militarinone, A, B, D, E and F (cytotoxic and antibacterial), Farinosone, A, B and C (neuritogenic and antibacterial) (Weng et al., 2019). Some of the potent metabolites reported from *I. cicadae* include; Cordycepin (immunomodulatory, antibacterial, antiviral, and antitumor), N-2-Hydroxyethyl adenosine (antioxidant), Cordycepic acid (bacteriostatic activity), Hercynine (antioxidant), ergothioneine (antioxidant); from *I. tenuipes* Penostatin A, B, C and J (PTP1B inhibitor), Isariotin, A, B, C, D, E, F, G, H, I and J (antimalarial and cytotoxic) (Zhang et al., 2019), from *I. japonica,* Isarin (cytotoxic), beauverolide I (calmodulin inhibitors) (Tuan et al., 2017).

Numerous species belonging to the members of entomopathogenic fungi have been shown to possess excellent antioxidant activity in multiple research studies. *Ophiocordyceps sinensis, Cordyceps militaris, Cordyceps cicadae* and *Isaria tenuipes*, as well as other species of entomopathogenic fungi are a good source of natural antioxidant, presenting in its composition phenolic compounds, alkaloids, nucleosides, cyclic peptides, polysaccharides followed by cordycepin and cordycepic acid (Das et al., 2021; Jiang et al., 2008; Li et al., 2001; Yan et al., 2014). The active principle molecule cordycepin ($C_{10}H_{13}N_5O_3$) is reported to exert multiple therapeutic effects that significantly increase the levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase activities in 6-OHDA-treated cells (Ashraf et al., 2020). It is believed that cordycepin prevents 6-OHDA-induced neurotoxicity in adrenal pheochromocytoma cells (PC12 cells) via its potent antioxidant mode of action (Olatunji et al., 2016). Cordycepin which is also found in *Isaria* spp., is considered as a potential antioxidant where the antioxidant capacity is almost comparable to that of

standard ascorbic acid (He et al., 2013; Ashraf et al., 2020). Antioxidant activity by extension is also related to anticancer activity and that's why this study is interesting.

The fungi of Darjeeling Hills surprisingly are an understudied research field since long time. Therefore, current knowledge about the exact diversity estimation is obscure in this region as there was very little effort made to complete these records. The mycological work on entomopathogenic fungi (EPF) is very limited at one end of the spectrum. Most of the work has been carried out in a quantitative survey where few species of EPF have been mentioned such as *Cordyceps militaris* and *Cordyceps nutans* (Pradhan et al., 2016). In the recent past few serious studies carried out by Chhetri et al., (2019 and 2020) on the genus *Isaria* have reported Darjeeling Hills as a treasure trove for entomopathogenic fungi.

1.1. Why this work was undertaken?

The Darjeeling Hills appear to be well explored in terms of floristic study but there is a lacuna on taxonomy based on morphological, microscopic, molecular DNA characters, and on the physiology and cultivation aspects of the genus *Isaria* spp. from Darjeeling Hills. The standardised protocol for bringing the taxa in axenic culture is limited for genus *Isaria* spp., of this region and there is a dearth of knowledge on nutritional requirements which is fundamental to understanding of physiology of entomopathogenic fungi. This further limits the current methods of artificial and viable cultivation. Furthermore, it was noted that no biochemical investigations have been carried out with respect to its antioxidant and antiproliferative bioactivities in the members of the genus *Isaria* spp., from Darjeeling Hills. The aforesaid genus was chosen as the target genus for this study. There is an urgent need to provide documentation for genus *Isaria* spp. The present research, therefore, was aimed at growing the isolates of *Isaria* spp., in artificial growth condition to produce biomass for testing the efficacy of the mycelial extracts on various cancer cell lines.

1.2. Research aims and objectives

1.2.1. General aim:

Based on the literature gap found in the genus *Isaria* spp., mainly with respect to nutritional, physiological, antioxidant and antiproliferative study, the broad aim of the thesis is to provide baseline information to the existing literature.

1.2.2. Specific objectives

The specific objectives proposed for the research were:

1. **To collect the fungal species of the genus *Isaria* from suitable locations in Darjeeling Hills.**

2. **To assess the taxonomy of the targeted genus *Isaria* based on morphological and molecular characterization.**

3. **To screen different *Isaria* species for their potential antioxidant activities.**

4. **To investigate the *in vitro* cytotoxic activity of the fungal extracts using cancer cell lines.**

Hypothesis: Anticancer activity of fungal extracts may exist due to their potent antioxidant compounds.

2. Review of Literature

2.1. Background

The literature pertaining to the taxonomy, physiology, molecular biology, biochemistry, pharmacology and biocontrol of entomopathogenic fungi is vast but relatively little is known about the genus *Isaria* spp., of this group from Darjeeling hills. It is not possible and is beyond the scope of this thesis to provide a complete yet comprehensive summary of all the available literature within the purview of this brief literature review.

Therefore, only the major works relevant to the present study was chosen and have been cited throughout. Information on *Isaria* spp., and allied members was obtained from the library textbook references, journal databases, PubMed, Scopus, Research Gate and Google Scholar. Some of the key words used for data collection were: *Isaria* spp., biological activities, biocontrol, traditional knowledge, culture and beavurecin. The extensive analysis and literature reviews of Fries (1821), Petch (1934), Brown & Smith (1957), Samson (1974), Gams et al., (2005), Luangsa-ard et al., (2005) Isaka et al., (2005), Sung et al., (2007), Kepler et al., (2017) Mongkolsamrit et al., (2018), Weng et al., (2019), (Zhang et al., 2019), and Chen et al., (2021) indicate the spectacular advances that have been made on the aspects of taxonomical and biochemical research on the genus *Isaria* over the years.

The purpose of this review is to present briefly the findings of previous workers in concord with the following lines: (a) historical perspective on entomopathogenic fungal research (b) the entomopathogenic fungus flora of India (c) the mycoflora of Darjeeling Hills (d) culture under lab condition (e) taxonomy and phylogeny (f) ecological conditions (g) secondary metabolites (h) pharmacological potential (i) infection mechanism and biocontrol attributes.

2.2. Historical perspective on entomopathogenic fungal research

Numerous entomopathogenic fungi have been reported invading and thriving on insects and the reviews of Steinhaus (1956, 1975) demonstrated early history of entomopathogenic fungi where he provides the historical hindsight of insect diseases notably muscardine silkworms infected with *Beauveria bassiana* circa 900 A.D. Probably it was the first observation made on entomophagy by sericulturists in the Orient, documented by the Japanese Royal family (Steinhaus, 1956; 1975). Most of the early documentations regarding insect fungi are associated with the species having indigenous ethnomycological uses (Cummings, 2009). The fungus which has received much attention is the lepidopteran pathogen *Ophiocordyceps sinensis* (Berk.) Sacc., known in Chinese as "Dong Chong Xia Cao" and in Japanese as "Tosukacho" which means literally "winter-worm, summer grass" particularly valued in Chinese medicine and may have been discovered and used by them for at least 2000 years (Lloyd, 1919; Cummings, 2009). However, the exact information regarding the earliest documentation of traditional use of entomopathogenic fungi first appeared in 'Lei Gong Pao Zhi Lun' (Lei's Treatise on Preparing Drugs) written by Xiao Lei (5th century AD) approximately 300 years before the first record of *Ophiocordyceps sinensis* (Zha et al., 2019). In addition, others like *Cordyceps sobolifera* was first recorded in Chinese literature as early as 300 AD (Wang, 1987). The silkworms infected with *Beauveria bassiana* were also prized for their medicinal properties in China, Japan, and Korea, with records dating back to 900 A.D. (Kikuchi *et al.,* 2004; Pemberton, 1999; Steinhaus, 1956, 1975, Cummings, 2009).

Although, for a long period of time the actual cause of infectious disease was not understood and remained a mystery for hundreds of years. A prevailing assumption of the time was that several environmental conditions during silkworm breeding caused the spontaneous or *de novo* development of the disease (Steinhaus 1956, 1975). For the first

time the work of Agostino Maria Bassi demonstrated that a fungus can cause a disease of insects (Egerton, 2012). He showed that the epidemic 'mark' or 'muscardine' disease of silkworm which was causing huge economic loses to the silk industry in northern Italy and France was caused by a fungus (Egerton, 2012). Bassi's lack of training in Cryptogamic botany was assisted by Giuseppe Balsamo-Crivelli who placed the fungus in the genus *Botrytis* and called it *B. paradoxa.* Later he changed the name to *Beauveria bassiana*, in honour of Bassi's achievements (Major 1944; Steinhaus 1956, 1975). It is also believed ironically that this classic investigation of Bassi on white muscardine disease of silkworms led to the establishment of the germ theory of diseases which postulated the involvement of certain microorganisms in case of infectious diseases (Samson, 1988). Moreover, in 1888 Elie Metchnikoff carried out mass production of *Metarhizium anisopliae* conidia and applied on field crops (Rajula et al., 2020). However, the early work of the nineteenth century on entomopathogenic fungi was carried out mostly by mycologists who were trained in plant taxonomy as a result of which most of the insect pathogenic fungi were recognized as plant pathogens. Many of the mycologists of time did not recognize that these fungi were pathogenic to insects. Several epithets used during that period of time mostly reflected the host plant on which they were found because there was a general assumption that these were plant pathogens (Rajula et al., 2020). This assumption and trend continued up to the time when Webber (1894) did a study on the fungi that relate with insects of *Citrus* in Florida and their infective ability towards insects was evident. Webber in his study recognized that *Aschersonia turbinata* infected *Ceroplastes floridensis* Comstock and that *Aschersonia cubensis* infected *Lecanium hesperidum* (=*Coccus hesperidum* L.). This work led to Webber (1894) while identifying *Aschersonia aleyrodis* Webber (on *Dialeurodes cirri* (Ricco), providing the first epithet which connected for the first time the fungi and the insect (Evans and Hywel-Jones, 1997). Thereafter, the study of

entomopathogenic fungi has received much mycological attention both with respect to means of combatting various diseases caused by them and with respect to taxonomic diversity, ecology and biochemistry. Subsequently, attempts have been made to study evolutionary relationship and genomics of entomopathogenic fungi with other members of fungal groups by means of molecular technique (Sung et al., 2007; Humber, 2008; Wang et al., 2016).

2.3. The entomopathogenic fungus flora of India

The first major attempt to provide a complete checklist of fungi reported from India by various workers was published by Butler and Bisby (1931). This list was further revised by Vasudeva (1960) who extended it to include all species reported from India up to the end of 1952 (Manjula, 1983). Although this list was considered to be one of the remarkable works of that time, but several additional lists have also been prepared by several other workers (Mundkur, 1938; Ramakrishnan & Subramanian 1952; Subramanian & Ramakrishnan 1958; Subramanian & Tyagi 1964; Tandon & Chandra 1964; Tilak & Rao 1968; Mukerji & Juneja 1974; Bilgrami et al., 1979; Manjula, 1983).

In recent years, there has been an increasing interest in study of entomopathogenic fungi from India for various scientific experiments. Fungi and insects comprise two of the largest groups of organisms on earth. Fungus–insect interactions have been reported to exhibit agonistic to mutualistic relationship (Martin, 1992; Biedermann et al., 2020). The evolutionary dynamics between fungi and insects are mostly studied for mutualistic systems (Zhang et al., 2014). However, the exact diversity of fungi is largely unknown and sparingly described (Cheek et al., 2020). Studies of entomopathogenic fungi in India are mostly confined to agricultural ecosystems. More than 3000 species of entomopathogenic fungi have been reported in association with insects, spiders and mites (Hywel-Jones, 2002). Among the reported species, approximately 750-1000, are

only described as entomopathogenic fungi placed in over 100 genera (Hibbett et al., 2007; St. Leger, & Wang, 2010; Hajek, & Eilenberg, 2018).

Further, based on the number of cryptic species being analysed using molecular phylogeny studies (Rehner, 2009), it is now clear that these estimates are very low (Vega et al., 2012). Thus, fungal entomopathogens constitute one of the largest numbers of taxa that are insect pathogens (Ignoffo, 1973). However, reported entomopathogenic fungal species represents less than 1% of the total number of described fungal species thus far (McLaughlin et al., 2009). Furthermore, **v**ery few entomopathogenic fungi have been described from India and most records have been from agricultural habitats occurring ubiquitously as pathogens of numerous diverse insects. Species such as *Beauveria bassiana, Nomuraea rileyi, Paecilomyces farinosus* and *Paecilomyces fumosoroseus* which infect a wide range of invertebrate hosts have been reported from forest areas and agricultural habitats (Thakur, & Sandhu, 2010). As per Subramanian, (1971), the first reliably recorded species of genus *Isaria* was *Isaria farinosa* (Dickson) Fr. from Mussorrie in India, reported by Hennings (1901). Further Indian records include *Isaria pulcherrima* berk and Br. **(**Sydow et al., 1937), *Isaria meliolae* Hansford (Thirumalachar & Lacy, 1951), *Isaria palmae* Stevans and King (Chona et al., 1956), *Isaria felina* Fries (Mishra, & Haque, 1959). The species recently reported from the genus *Isaria* in India includes; *Isaria sinclairii* (Berk. Lloyd), *Isaria tenuipes* (Peck), *Isaria japonica* (Yasuda), *Isaria farinosa* (Holmsk) Fr. (Sharma et al., 2015; Sharma, 2015).

The majority of species of an entomopathogenic fungi reported from India are members of the families Clavicipitaceae: *Metarhizium* (Sandhu et al., 2012; Dutta et al., 2013; Kaushik, & Dutta, 2016) and *Nomuraea* (Thakre et al., 2011), Cordycipitaceae: Beauveria (Das et al., 2011; Mudoi et al., 2019; Das et al., 2021), *Isaria* (Sharma et al., 2015; Chhetri et al., 2019; 2020), and *Lecanicillium* (Kumar et al., 2015), and

Ophiocordycipitaceae: *Hirsutella* (Agrawal et al., 2015), and *Ophiocordyceps* (Chhetri et al., 2019; Pradhan et al., 2016; Sharma, 2004). Though many new genera and species new to science are continuously being reported every year by different workers from different countries, very little records are available in the Indian context.

2.4. The mycoflora of Darjeeling Hills

The Mycoflora of Darjeeling Hills appeared to be very rich as its adjacent areas of Eastern Himalaya. It is believed that the starting point of mycological collections and their studies in this part of India, so far known to us, is by Sir J.D. Hooker, who did some pioneering work in exploring the macrofungal diversity of Assam, Darjeeling, Khasi hills and Sikkim. His monumental contribution was followed by recognisable and an interesting mycological publication by an English mycologist, Rev. M.J. Berkeley between 1850 and 1882 (Thatoi, & Singdevsachan, 2014).

In the recent times, when Pradhan et al., (2016) surveyed the macrofungal diversity from Darjeeling hills they noted that there is the presence of 98 species belonging to 72 genera and 47 families. Further, they have found that 55 genera have a unitary count of species and the rest had a count within the range of 2-9 species. In their field survey, Russulaceae was reported to be the dominant family representing the most species (9) recorded, followed by Marasmiaceae (4) and Coprinaceae (3). The species richness genera have been reported per specified collection areas and it was conceded that *Russula* (9), *Marasmius* (4), *Coprinus* (3) were predominantly found, while the rest had a unitary or binary distribution. A close look in distribution of species by trophic groups indicated niche heterogeneity where saprotrophic species (57) were most diverse, followed by ectomycorrhizal (17), parasitic (10), facultatively parasitic (5), putative ectomycorrhizal (4), coprophilic (4) and termite-associated (1) groups (Pradhan et al., 2016). The available literature on fungal diversity is scattered and the information

relating to fungal taxa from Darjeeling district has been well covered by fungal family Agaricaceae, Amanitaceae, Aricularіaceae, Ascobolaceae, Cantharellaceae, Hymenochaetaceae, Hypocreaceae, Hypoxylaceae, Inocybaceae and Polyporaceae (Roy and Acharya, 2019). In contrast, this is not the complete picture of the mycoflora from this region as many difficult terrains are yet to be explored for the determinable taxa exploration.

In the present context, no attempt can be made to estimate the number of entomopathogenic fungus flora of Darjeeling hills. Currently, very little is known of entomopathogenic fungi in Darjeeling hills with respect to taxonomic diversity and ecology, hence biodiversity of this important group is a huge pertaining issue.

2.5. Culture under lab condition

Atkinson (1894) carried out the pioneering cultural investigation on *Isaria tenuipes* Peck. The specimen used for raising pure culture was collected from the buried leaves having clavate sporophore arising from the host pupae. The distal half was flattened and densely covered with farinaceous white powder chiefly made up of oval to globose colorless conidia. On plate culture, germination resumed after 24 hours of incubation whereby the conidia appeared to be swollen giving rise to slender germ tubes which grew in sinuous line from both ends of the conidium (Petit, 1895). Sometimes the conidium separated from the base of germ tubes through septum formation or through constriction. The protoplasm was hyaline and homogenous and after 48 hours of incubation resulted in visible mycelial growth with circular tufts on the agar media, vacuole appeared by the end of three days of incubation becoming thick and irregularly placed (Atkinson, 1894; Petit, 1895).

During the late 1930, Taber and Vining isolated a strain of *I. cretacea* van Beyma from soil and powdery leaves of tomato plant. The white phototrophic

synnemata was described as an indeterminate synnemata as it bore spores on the lower portion while the tip maintained the capability of indeterminate growth (Taber & Vining, 1939). The wild type was designated as wild strain (A) and its variant as variant strain (B), they described that strain A could use ammonia, amino and nitrate for fulfilling nitrogen needs whereas various carbohydrates were utilized as carbon sources. Germination would take place only in presence of energy sources with little or no exogenous requirements of vitamins. For vegetative growth it was noted that it required exogenous biotin along with the pyrimidine moiety of thiamine. But biotin could be replaced with biotin-L-sulphoxide and biocytin and partially with desthiobiotin but not in presence of homobiotin, norbiotin, aspartic acids, pimelic acid, oleic acids, Tween 80 or with any combination of these sources. The strain A was found to produce variant strain (B) *de novo* which lacked the synnemata formation but has a higher growth rate with respect to parental strain. Moreover, it was also described that both the strains could enter into a heterokaryotic state which eventually suppressed the sporulation and formation of synnemata. Synnemata formation is influenced almost by the available carbon sources but not governed by the nitrogen sources (Taber & Vining, 1939).

Some species of the genus *Isaria* are known to produce fruiting bodies in culture under artificial laboratory conditions but the production of typical fruiting bodies in culture is still a problem. However, a few investigators have been able to produce synnemata of some species of *Isaria* in laboratory culture (Ben et al., 1998; Pham et al., 2020).

Mass production of synnemata is desirable if the fungi are to be exploited for harnessing the novel metabolite having medicinal properties. Yamanaka et al. (1998), in their study on *Isaria japonica* examined the fungus *in vitro* using liquid and solid media in order to produce fruit-bodies on a large scale. The mycelia grew well at 18–28°C on PDA medium with an initial pH of 7.0. The formation of fruit-bodies of *Isaria japonica*

was induced by lowering temperature to below 20°C in potato dextrose liquid medium. Such cold treatment was found to be stimulatory for developing fruiting bodies (Yamanaka et al., 1998).

It is demonstrated by Yamanaka and Inatomi (1997) that a sawdust-rice bran media supplemented with silkworm pupal powder markedly influenced the fresh weight of fruit bodies which increased with increasing percentages of pupal powder. They also reported that substitution of carbon rich grain (e.g., barley, millet, sorghum) in place of sawdust-rice bran also markedly increased the yield of fruit bodies. These results suggested that the grain media supplemented with pupal powder are most effective and stimulatory for mass production of fruit bodies of *Isaria japonica* (Yamanaka & Inatomi, 1997).

The works of Ban et al. (1998), was almost consistent with the findings of Yamanaka and Inatomi, (1997, 1998). Ban et al. (1998) reported that the optimum temperature range for mycelia production of *Isaria japonica* was 23-28°C on malt yeast glucose media with optimum pH requirement of 7.0 and the best temperature for the fruiting-body production being below 20°C under fluorescent light (Ban et al., 1998). They too indicated a profuse production of fruiting bodies on the media supplemented with silkworm pupal powder and the production increased gradually with increasing concentration of the same. These findings suggested that some chemical components might be present in the pupae of Lepidoptera moths which may be responsible for the triggering of fruiting body formation in *Isaria japonica* (Ban et al., 1998). Again the necessity of silkworm pupa for enhanced synnemata production for *I. tenuipes* was also shown by Kang et al., (2010) and Ji et al., (2011). Investigation for the search of liquid substrate from agro-industrial products for high conidia yield showed that the combinations of sugarcane molasses + rice broth, rice broth + yeast and sugarcane molasses + yeast + rice broth are suitable for high yield of conidia (Mascarin et al.,

2010). Further, in solid-state fermentation, the best conidia production was achieved with the soybean meal and broken corn for *I. farinosa*, and whole rice and broken rice for *I. fumosorosea* (Mascarin et al., 2010). Liu et al., (2018), showed that the optimal nutrition requirements for mycelial growth of *I. farinosa* are D-(+)-galactose and D-(−)-fructose as carbon resources and D-cysteine as well as yeast powder, peptone, and beef extract as nitrogen source at a carbon-to-nitrogen ratio of 1:1 to 1:7.

2.6. Taxonomic studies on entomopathogenic fungi

Insect pathogenic fungi are globally distributed and can be found in the following divisions of the true fungi, Microsporidia, Chytridiomycota, Entomophthoromycota (order: Entomophthorales), Basidiomycota, and Ascomycota (Araújo, & Hughes, 2016; Vega et al., 2012). The insect pathogenic fungal genus *Isaria* is of particular interest, and is the major focus of the present study. Studies by several researchers in the latter part of the nineteenth century laid the foundation for the current understanding of species diversity within the members of insect pathogenic fungi including genus *Isaria* (Samson et al., 1988). During this period increasing numbers of entomopathogenic fungi from around the world were examined mostly by European and American mycologists (Samson et al., 1988). By 1892, over 100 entomopathogenic species were named and many were placed into major groups like *Cordyceps* and *Isaria* (Cooke, 1892). Currently, more than 750 species of fungi from around 90 genera are known to be pathogenic to insects (Rajula et al., 2020).

Taxonomy and relationships among entomopathogenic fungal species were complicated in nineteenth century mycology with the discovery that many fungi can reproduce either sexually or asexually. Such fungi were referred to as pleomorphic and have two reproductive states. The whole fungus with both sexual and asexual states is referred to as ‘holomorph’ with the ‘anamorph’ being the asexual morph or state and the

‘teleomorph’ being the sexual morph or state (Kendrick, & DiCosmo, 1979; Seifert, & Samuels, 2000). The major complications arising out of two state reproductive characters were that the single teleomorph genus has anamorph species in different genera and several teleomorph species in different genera have anamorphs in the same genus (Reynolds 1993; Seifert & Gams 2001). Historically, it was important to prove teleomorph-anamorph connections either by producing mature stromata from an anamorphic isolate or produce conidia from cultures raised from teleomorph spores or tissues (King, 2006). Anamorphic forms have the ability to persist on a much broader substrate ranges than their sexual counterparts and are commonly isolated from soil lacking any definite host (Rossman, 1996). Most of the members of anamorphic fungi described from insect hosts in the nineteenth century were placed in the genus *Isaria* (Cummings, 2009). However, many of them were placed without direct evidence and later it became widely accepted that these were the conidial forms of *Cordyceps* species (Cooke 1892; Massee 1895).

Classical fungal taxonomy in the first half of the twentieth century based on morphology with emphasis upon microscopic characters has provided a basic framework to classify entomopathogenic fungi at most higher levels (Cummings, 2009). Morphological characters of the anamorphs of Hypocrealean fungi have been particularly useful in defining groups or genera of the teleomorphs, and these have been corroborated using molecular characters (O'Donnell, 1993; Spatafora and Blackwell, 1993; Rehner and Samuels, 1994; Rossman, 1996). Work by Petch between 1931 and 1944, addressed over 74 entomopathogenic species occurring in Sri Lanka, many of which are still valid (Hywel-Jones, 1997; Samson et al., 1988). Substantial contributions to the taxonomy of *Cordyceps* and allied species were also made by Kobayasi in Japan and Mains in North America and the scheme of classifications is generally accepted even today (Kobayasi, 1939, 1941; 1982; Mains, 1940, 1947, 1949, 1950).

In the late nineteenth century, Saccardo proposed the classification system of anamorphic fungi based on morphological characters such as general conidiomatal form, pigmentation, colour, shape, and septation, arrangement of conidia etc. (Goos 1956; Seifert & Gams 2001; Sutton 1996). In his *Sylloge Fungorum* (Saccardo, 1882-1931, 1972), Saccardo attempted to include descriptions of all fungal species based on the information from other authors (Rossman, 1996). Until the advent of molecular techniques, the circumscription of most genera in the Hypocreales was directly attributable to P.A. Saccardo. However, as with other morphological characters, certain exceptions are known for the multitudinous species. Therefore, discontent concerning the Saccardoan system of classification has arisen from the fact that the characteristics used to delimit taxa (i.e., spore color and septation, arrangement of the conidiophores, etc.) often results in the separation of morphologically similar genera, while at the same time placing together what seem to be unrelated genera resulting in an artificial, pigeon-hole taxonomic scheme (Goos, 1956; Rossman 1996). Later, Vuillemin in 1910-12, proposed a classification system based upon spore forms which he did by placing more taxonomic weightage on the morphology of conidiogenous cells and the method of conidium production (Humber 2000; Sutton 1996). Hughes (1953) reviewed the history of these higher taxa and integrated the earlier ideas into a revised classification system for anamorphic fungi. In his classification system, the mode of conidiogenesis was considered a phylogenetically informative character for separation of genera (Hughes, 1953).

From 1970s onward several researchers revised the generic concepts proposed by Hughes (Cummings, 2009). Most of these revisions are reflected in the monographs on important anamorphic genera including *Beauveria* (de Hoog, 1972), *Paecilomyces* (Samson, 1974), and *Metarhizium* (Tulloch, 1976). During this period extensive collection of fungi infecting insects and spiders in Ghana was made by Evans (1974).

He noted that many of the species were difficult to identify using available literature of the time with some of the previous collections lacking type specimens and inadequate descriptions (Evans, 1974). Subsequent work by various mycologists further described species in poorly known anamorphic genera including *Akanthomyces* (Samson & Evans 1974), *Gibellula* (Samson & Evans, 1973), *Hymenostilbe* (Samson & Evans 1975), and *Nomuraea* (Samson & Evans 1977). Collections and study by Evans in South America and the tropics included descriptions of many new species which provided much insight into the ecology of entomopathogenic fungi in tropical forests (Evans 1974, 1982; Evans & Samson 1982a, 1982b, 1984). Kobayashi, in his 1982 key to the genera *Cordyceps* and *Torrubiella,* recognized 282 species of *Cordyceps*, 59 species of *Torrubiella* and 75 species of other genera and this scheme of classification is still accepted (Kobayashi, 1982). Further, during this period, Kobayasi while working with Shimizu made substantial and valuable taxonomic contributions to both genera, describing 84 new species in *Cordyceps* and 27 in *Torrubiella* (Kobayasi 1982; Kobayasi & Shimizu 1982).

While surveying insect pathogenic fungi as source of biologically active novel fungal metabolites in the past two decades, has led to increasing interest in isolating these fungi from the tropics (Hywel-Jones, 2001; Hung, 2010; Isaka et al., 2005). The insect pathogenic fungi may have cosmopolitan distribution with little effort being put into describing members of these fungi from natural habitats (Hung, 2010). A study carried out in Southeast Asia by Hywel-Jones (2002), has reported that the highest number of insect pathogenic fungal species are found in Asia. Thailand has emerged as the richest source in terms of number of insect pathogenic fungal species, accounting for 321 species (Hywel-Jones, 2002). An important aspect of this study has been a focus on obtaining cultures and determining anamorph-teleomorph connections, which were often neglected by previous workers (Hywel-Jones 1995a, 1995b, 1996, 1997a; 1997b;

Hywel-Jones & Sivichai 1995; Cummings, 2009). Currently, Thailand alone is on record to have conducted long-running research on insect pathogenic fungi for over 20 years during which, about 400 species have been isolated from various habitats, identified and recorded according to the Atlas of Invertebrates-Pathogenic Fungi of Thailand Volume 1 | NSTDA (Rajula et al., 2020).

2.7. Molecular approaches on fungal nomenclature

The traditional approach to fungal taxonomy based on morphological characters because of the lack of usable characters has often been problematic in entomopathogenic fungi, especially in anamorphic species (Humber 2000; Inglis, & Tigano-Milani, 2006; Samson, 1995). Thus, the issue of species delimitation in fungi has long been confused with species concept and boundary (de Queiroz, 1998, 2007). Most of the anamorphic genera are easily distinguishable through their characteristic modes of conidiogenesis, however, only a limited range of morphological characters are used to separate species as many of them have simple morphology (Cummings, 2009). Moreover, these characters quite often display considerable morphological plasticity in the environment or in artificial culture which has led to taxonomic debates on species boundaries and resulted vagueness in number of described species. To mention, conidial shape and size are the only reliable morphological characters for species identification in *Beauveria* and *Metarhizium* (Glare *et at.* 1996a, 1996b; Mugnai *et at.* 1989; Rehner 2005; Rehner & Buckley 2005). However, in both genera spore dimensions demonstrate a high degree of intraspecific variability, especially in culture, and isolates may show characteristics which are intermediate between two different species resulting in a new type of species concept (Glare et al., 1996a, 1996b; Glare & Inwood, 1998; Mugnai et al., 1989). It has also been reported that in fungi the genetic basis for species concept is largely unknown (Rossman, 1996), and this often led to misidentification of taxa.

Taxonomic issues have occurred in the members of entomopathogenic fungi due to lack of spectacular morphological differences to delimit and to recognize species. Humber (2000), reported that relatively simple mechanisms associated with conidial production may often lead to similar morphologies and modes of development occurring in unrelated groups through the process of convergent evolution. Furthermore, sometimes with uniformous morpho characters it is not clear how to delimit and characterize taxa as well as whether the character gives a well-supported overview of monophyletic group or not. For example, in the classification of *Verticillium* based on morphological attributes (Gams, 1971) resulted in an unnatural grouping which included insect-pathogenic and plant-pathogenic species, with corresponding teleomorphs in two unrelated ascomycete families. A similar situation also arose in the classification of *Paecilomyces* by Samson (1974), with the inclusion of both thermophilic and entomopathogenic species in the genus, again with mention of each group having unrelated teleomorphs. Samson's classification of these fungi was primarily based on morphological characteristics, but was often highly subjective and leads to ambiguous identifications at the species level (D'Alessandro et al., 2014).

In the last three decades molecular identification techniques with respect to DNA analysis have become popular amongst mycologists for solving problems in fungal systematics across the world. Currently, it is one of the latest laboratory techniques available for systematic studies. Moreover, in the recent times it is well documented that molecular identification is rapidly becoming a major tool in fungal taxonomy. The popularity of this technique lies in its universal applicability, speed, and the presumption that it replaces taxonomic expertise, making this approach broadly applicable in many fields of mycology (Yahr et al., 2016). It is also stressed here that a common misconception in DNA barcoding technique is the assumption that existing reference data provide a definitive answer, either in species identification or to establish

whether a taxon is new to science (Lucking et al., 2020). Such an approach will definitely fail when reference data are incomplete or sequences are improperly labelled (Nilsson et al., 2006). Thus, ideally, not only morphological and molecular information but a broad array of characters needs to be quantitatively analysed in order to draw accurate conclusions in species delimitation and phylogeny for most effective identification (Sieber et al., 1998; Lucking et al., 2020). The nuclear ribosomal DNA of internal transcribed spacer (ITS) has been the most commonly sequenced region for fungal identification and systematics which is also selected as the universal barcode because it has a clearly defined barcode gap along with its wide usefulness for a vast range of fungi (Schoch et al., 2012). The ITS gene consists of two non-coding regions, called spacers, situated between the small subunit 18S, 5.8S and the large subunit 28s of ribosomal RNA (Lucking et al., 2020). The spacer regions and ribosomal genes both evolve at different rates so they can be informative at different taxonomic levels (Bruns et al*.,* 1991). The ITS region generally shows variation at around species level and thus it can be a powerful marker when dealing with phylogenetically more distant species (Kiss, 2012). However, the rate of divergence in ITS sequences may vary between different fungal groups and it often does not allow discriminating phylogenetically closely related species and does not resolve nodes at a higher phylogenetic level because of the frequency of indels (Lucking et al., 2020).

Apart from ITS regions, sequences from protein-coding genes typically provide greater taxonomic resolution and have been increasingly used to complement or replace ribosomal DNA sequences in phylogenetic analyses. Protein coding loci which are commonly used in fungal taxonomy include translation elongation factor 1-α, β-tubulin, ribosomal polymerase B, and mitochondrial ATPase6 (Bruns & Shefferson, 2004; Lutzoni et al., 2004). Genetic markers, such as internal transcribed spacer (ITS- rDNA), β-tubulin gene and ribosomal rRNA gene, have been extensively used for the molecular

characterizations and phylogenetic studies of entomopathogenic fungi (D'Alessandro et al., 2013; Rehner et al., 2011; Sosa-Gómez et al., 2009; Tigano-Milani et al., 1995; Yokoyama et al., 2006). However, when amplifying protein-coding genes as mentioned, amplification failure and sequencing success obstacles are often encountered in the experimental set up.

In the beginning of new millennium as a result of a global cooperative effort involving various mycologists across the globe to reclassify fungi according to phylogenetically sound principles based on the DNA sequence data for multiple genes has resulted in the publication of two monumentally important publications that completely reworked mycological systematics (Castrillo, 2020). One of the publications by James et al., (2006), was a phylogenetic overview of virtually all fungi and the second publication by Hibbett et al., (2007) provided many of the necessary taxonomic readjustments that were indicated by the phylogenetic studies (Castrillo, 2020). Based on molecular sequence data significant taxonomic revisions in entomopathogenic genera including *Cordyceps* and *Isaria* were undertaken (D'Alessandro et al., 2013; Chen et al., 2021; Johnson et al., 2009; Luangsa-ard et al. 2005; Sung et al. 2007; Kepler et al., 2017).

2.8. Genus *Isaria*

2.8.1. Nomenclature, taxonomy and phylogeny of *Isaria*

The genus *Isaria,* entomopathogenic anamorphic forms in the Hypocreales: Cordycipitaceae has had a complicated nomenclatural history. The name *Isaria* was for the first time used in the literature by Hill, in 1791 for describing three species of fungi that are now recognised as representing a myxomycete, a basidiomycete and a rust i.e. *Calocera viscosa* Fr., *Ceratiomyxa fruticulosa* (Müller) Mac Bride and a *Puccinia* species respectively (Petch, 1934). Persoon also introduced the name *Isaria* in 1794 for

the description of two species which were *I. mucida* and *I. agaricina*. However, in 1821 Fries did cite the name Pers., in the introduction of his Systema Mycologicum, without mentioning any species. In 1832, Fries did not refer to Persoon (1794) but to Hill (1791). In addition, he didn't mention Hill's species whereas both the original species described by Persoon, were listed. The first species mentioned by Fries (1832) was documented to be *Isaria terrestris* Fr., and it was chosen as the lectotype of *Isaria* by Petch (1934). Fries was the authority who established the genus *Isaria* based on the species *Isaria terrestris* Fr. (Fries, 1821). Later examination of the type material of this species however revealed features that the fungus consisted of sterile hyphae, probably belonging to an immature basidiomycete (de Hoog, 1972). Moreover, Petch (1934) also noted that in some cases the species were simply included in the genus exclusively on the basis of their association with insects. Many species were added to the genus, throughout the nineteenth and early twentieth century because of its turbulent nomenclatural histories, which eventually comprises of over 200 species mainly including the members of an entomopathogenic and mycoparasitic fungi (Hodge et al., 2005). The genus *Isaria* was used in the past to accommodate many insect pathogens and the classification of the species in the genus *Isaria* were mostly based on the presence of simple or branched synnemata producing one-celled hyaline conidia, with no preference given to differences existing in conidiogenous structures (Mains, 1955). Brown and Smith (1957) transferred some of the species described in *Isaria* Pers. and *Spicaria* Harting into genus *Paecilomyces*, which possess a conidiogenous structure similar to that of *Paecilomyces variotii* Bainier. In the nineteen seventies, De Hoog (1972) redescribed the genus *Isaria* and chose *Isaria felina* (DC.) Fr., as the lectotype and little discussion existed about its inclusion in the literature.

Samson (1974), monographed and redefined the genus *Paecilomyces* (type species: *P. variotii* Bainier) by delimiting the genus *Paecilomyces* to species with

verticillate conidiophores bearing divergent whorls of branches and phialides. The phialides were characterised by a cylindrical or inflated base tapering to a long distinct neck, producing chains of hyaline, one-celled smooth walled conidia in a basipetal chains. For more than 30 years *Isaria* was considered a subsection within the genus *Paecilomyces* sensu Samson (1974), who divided this genus into two sections: section *Paecilomyces* and section *Isarioidea*. Section *Paecilomyces*, included the type *P. variotii,* with mesophilic, thermotolerant and thermophilic species, accompanied often by a *Talaromyces, Byssochlamys* or *Thermoascus* ascigerous state, and colonies appearing yellow-brown to brownish in colouration. Section *Isarioidea*, included the mesophilic species without ascigerous states, and colonies appearing purple, pink, green or yellow in colouration. In this *Paecilomyces* section *Isarioidea,* Samson (1974) considered all entomogenous *Isaria* bearing flask-shaped phialides tapering abruptly to thin long necks and catenate conidia as *Paecilomyces* and proposed several further combinations that general (Gallou et al., 2016).

Hodge et al., (2005), redescribed the genus *Isaria* with the type species *Isaria farinosa* (Holmsk.) Fr. and most entomopathogenic mesophilic *Paecilomyces* species were transferred to *Isaria* (Hypocreales, Clavicipitaceae) (Chen et al., 2021). Following thorough nomenclatural history, formal conservation of the generic name *Isaria* was officially accepted in 2005 (Gams et al., 2005).

Molecular phylogenetic studies based on 18S nrDNA have reclassified the genus *Isaria* (Luangsa-ard et al., 2005; Sung et al., 2007). The polyphyletic nature of the genus *Paecilomyces*, including the section *Isarioidea* has been recognized several times by analyses of the large and small subunit rRNA genes (Luangsa-ard et al., 2004; Obornik et al., 2001). However, using the β-tubulin gene and the nuclear ribosomal internal transcribed spacer (ITS) region, Luangsa-ard et al., (2005) further investigated the phylogenetic relationships of *Paecilomyces* sect. *Isarioidea* species, and established

the existence of a monophyletic distinct clade named '*Isaria* clade', which includes: *Isaria amoenerosea* Henn., *Isaria cateniannulata* (Z.Q. Liang) Samson & Hywel-Jones, *Isaria catenioabliqua* (Z.Q. Liang) Samson & Hywel-Jones, *Isaria cicadae* Miq., *Isaria coleopterora* (Samson & H.C. Evans) Samson & Hywel-Jones, *Isaria farinosa* (Holmsk.) Fr., *Isaria fumosorosea* Wize, *Isaria ghanensis* (Samson & H. C. Evans) Samson & Hywel-Jones, *Isaria javanica* (Friederichs & Bally) Samson & Hywel-Jones, and *Isaria tenuipes* Peck (Gallou et al., 2016). Many of the reported *Isaria* strains have the ability to infect different insect orders in all developmental stages (D'Alessandro et al., 2013).

In the last decades, Sung et al. (2007), used multilocus sequence typing (MLST) to construct a phylogeny of the clavicipitaceous fungi, distributing the genus *Paecilomyces* among three families of the Hypocreales (i.e., Cordycipitaceae, Clavicipitaceae, and Ophiocordycipitaceae). Currently, some species from this genus were excluded from both *Paecilomyces* and *Isaria*, or still await transfer into appropriate genera (Gallou et al., 2016). For instance, Luangsa-ard et al., (2011) showed that *Paecilomyces lilacinus*, placed in the Ophiocordycipitaceae, was not related to *Paecilomyces* and proposed the new genus '*Purpureocillium'*.

In 2017, based on phylogeny-based nomenclature for Cordycipitaceae (Hypocreales), Kepler et al., proposed the rejection of *Isaria* in favour of *Cordyceps* and transferred *Isaria* species into *Cordyceps*. Until recently in 2018, Mongkolsamrit et al., introduced some *Isaria*-like species and the new genus *Samsoniella* Mongkols., Noisrip., Thanakitp., Spatafora, and Luangsa-ard. Further, Chen et al., (2018, 2020) reported four *Isaria*-like species: *Akanthomyces araneogenus* Z.Q. Liang, W.H. Chen, and Y.F. Han; *Samsoniella coleopterorum* W.H. Chen, Y.F. Han, and Z.Q. Liang; *Samsoniella hymenopterorum* W.H. Chen, Y.F. Han, and Z.Q. Liang; and *Samsoniella lepidopterorum* W.H. Chen, Y.F. Han, and Z.Q. Liang. In accordance with the most

recent system of classification, many species which were previously placed in the genus *Isaria* have been transferred to more appropriate genera (Chen et al., 2021). The delimitation of the genus *Isaria* has remained problematic throughout the century. Therefore, robust molecular phylogenetic analyses are still needed to draw conclusion for *Isaria*-like fungi to ensure accurate taxonomic identification with comparable results across different isolates and genotypes (Mongkolsamrit et al., 2018; Chen et al., 2021). The generic description of the genus *Isaria* is as follows:

Isaria Persoon 1794

Luangsa-ard, J.J., H.L. Hywell-Jones, L. Manoch and R.A Samson. Mycol.Res. 2005. 109 (5): 581

Classification: Fungi, Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Cordycipitaceae.

Conidiophores usually well-developed mono- or synematous, usually consisting of several verticillate branches, each bearing a dense whorl of phialides. Synnemata often branched with apical sporulating structure. Phialides consisting of a cylindrical or swollen basal portion, terminating in a thin often long neck, producing divergent conidial chains. Conidia one or rarely two celled, smooth walled, hyaline. Colonies bright coloured, white, yellow, pale green, pink, red or purple. Hyphae hyaline to slightly pigmented i.e. coloured, rough or smooth walled.

Teleomorphs: *Cordyceps* or *Torribiella,* often absent in culture.

Type species: *Ramaria farinosa* Holmsk. 1781.

Hosts: numerous diverse insects.

2.9. Host specificity

Naturally occurring EPF are very important in insect population regulation with recent estimates of 750 species found capable of parasitizing insects naturally, although the exact diversity of the EPF is not completely known (Shrestha et al., 2016). Some of the

more widespread fungi causing infection in insects include the members belonging to two diverse groups within kingdom Fungi, Entomophthorales (Phylum Entomophthoromycota, formerly Zygomycota) and Hypocreales (Phylum Ascomycota). These fungi are found to be distributed in wide range of terrestrial ecosystems including Arctic Circle and Antarctica (Shrestha et al., 2016).

Studies on insect pathology has revealed that the majority of the species of EPF show considerable variation with respect to host specificity and include both fastidious pathogens with restricted host ranges and opportunistic pathogens having a broad host-range (Fargues, & Remaudiere, 1977). Entomophthoralean pathogens are generally characterized by narrow host range and are distributed mostly in temperate forests with rare reports from tropical regions and in general are more susceptible towards foliar insects and mites (Evans, 1982; Pell et al., 2001). Hypocrealean pathogens, on the other hand, have narrow to very broad host range and are dominantly distributed in humid tropical forests (Shrestha et al., 2016). The insect group belonging to the members of Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, Aranae, etc., are more susceptible in comparison to the members of other groups of insects (Hung, 2010).

Additionally, some of the reported taxa are capable of infecting only particular groups of non-insect arthropods, *e.g. Gibellula* species are specific pathogens of hunting spiders (Hywel-Jones, 2001), while diverse species of *Hirsutella* are narrow pathogens to acarine hosts (Minter et al., 1983; Samson et al., 1980). Furthermore, it should be kept in mind that the species of *Beauveria bassiana* and *Metarhizium anisopliae* display diverse host ranges. *Beauveria bassiana* is an insect pathogen which has been recorded from over 700 host species belonging to 15 insect orders and is also recognized to infect mites (Acari) (Li, 1988). Similarly, the host records of *M anisopliae* include over 200 species belonging to 11 insect orders (Zimmermann, 2007b).

In the genus *Isaria,* majority of the species has a relatively wide host range with the most dominant being Lepidoptera, but this host range is relatively narrow when compared to *B. bassiana* (Zimmermann, 2007a). The record of target hosts of the genus also comprises other insect orders including Hymenoptera, Diptera and spiders etc. (Zimmermann, 2008). *Isaria* included within Cordycipitaceae is an entomopathogenic fungal genus having a wide host range of more than 100 species (http://www.mycobank.org/). This genus plays important roles in biodiversity conservation and is utilized in medicines and agriculture (Luangsa-ard et al., 2005). The host of *C. militaris* is Lepidopteran pupa and the color of its fruiting bodies is yellow or orange, while *O. sinensis* host is Hepialu larva, and the color of its fruiting bodies is dark brown (Chiu et al., 2016).

Table 2.1: Host range of species accepted in *Isaria* spp., (Based on Luangsa-ard et al., 2005).

Species	Host range	References
***I. amoenerosea* Henn.**	Chelonethida, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera	Samson 1974; Samson & Evans 1977
***I cateniannulata* Samson & Hywel-Jones**	Coleoptera, Diptera, Lepidoptera, Hymenoptera	Liang 1981; Shimazu 2001
***I. cateniobliqua (*Z.Q. Liang)**	Lepidoptera	Liang 1981
***I. farinosa* (Holmsk) Fr.**	Acari, Aranea, Coleoptera, Chelonethida, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Opiliones, Thysanoptera.	Humber and Hansen 2005
***I. fumosorosea* Wize**	Acari, Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Neuroptera, Thysanoptera.	Humber and Hansen 2005; Smith. 1993.
***I. javanica* Friederichs & Bally**	Coleoptera, Lepidoptera, Aranaea	Samson 1974.
***I. ghanensis* Samson & Evans**	Lepidoptera	Samson 1974; Samson & Evans 1977
***I. tenuipes* Peck**	Coleoptera, Lepidoptera	Samson 1974; Luangsa-ard et al., 2004

2.10. Diversity and distribution

The general diversity and distribution of any entomopathogenic fungi depend on environmental conditions, health of insect hosts, its number and the physical factors favouring the growth of particular taxa (Hajek & St. Leger., 1994). The distribution of *O. sinensis* in China is from Central Yunnan Plateau to the Qilian Mountains in Qinghai Province, and from Mount Daloushan in Guizhou Province to the wide areas of the Himalaya (Negi et al., 2015; Negi et al., 2020). *Cordyceps militaris* has a worldwide natural distribution and is widely reported from East Asian countries, mainly China, Japan, Korea, Thailand, Nepal, Bhutan and India (Shrestha et al., 2010). Genus *Isaria* also has a widespread geographical distribution (Gams et al., 2005), but nowhere abundant in any part of their range. The catalogue of the United States Department of Agriculture, Agriculture Research Service (USDAARS) collection of Entomopathogenic Fungal Cultures (ARSEF) enlists more than 1000 *Isaria* strains from different countries in North, Central, and South America, Europe, Africa, Australia, and Asia. In Asia, Thailand is the richest in terms of number of entomopathogenic fungal species identified and described (Hung et al., 2010). In addition, *Isaria* strains can infect different insect orders in all developmental stages, and are commonly isolated from soil (D'Alessandro et al., 2013; Gallou et al., 2016). A number of species such as *Isaria cicadae* and *Isaria sinclairii*, are reported to be distributed in southern Asia, including China, Taiwan, India (Sharma et al., 2015; Sharma et al., 2021), Indonesia, Japan, Nepal (Shrestha, 2011), South Korea, Sri Lanka, Thailand and Vietnam. They are also found in Oceania (Australia and New Zealand), South America (Brazil, Guatemala and Mexico), North America (Canada) and Africa (Madagascar) (Zha et al., 2019). Furthermore, different species of the genus *Isaria* which includes members like *Isaria sinclairii* (Berk.) Lloyd, *Isaria tenuipes* Peck, *Isaria japonica* Yasuda and *Isaria farinosa* (Holmsk) Fr. have also been reported from India by various workers (Chhetri et al., 2020; Sharma et al., 2015; Sharma et al., 2021).

Figure 2.1: World natural distribution of *Isaria* spp., in USDA- ARS collection of entomopathogenic fungal culture (Castrillo, 2020) (outline map: not to scale).

2.11. Nutritional value

Compounds with nutritional value from natural sources have some very interesting properties that can be exploited for the design of innovative novel feed formulations. *Cordyceps* spp. and *Isaria* spp. provides a diverse spectrum of essential minerals and vitamins to our body. It possesses several minerals like iron, potassium, magnesium, calcium, besides other essential nutrients like vitamins and antioxidants. All these nutrients constitute excellent dietary supplement since they are easily absorbed by our body for being naturally available in *Isaria* spp.

The proximate composition and biochemical profiling of natural fruiting bodies of *I. cicadae* has been worked out by a number of workers (Sharma et al., 2015; Sharma et al., 2021). Carbohydrates have been documented as a major constituent (50.2%), while the protein content (4.46%) crude fat (6.6%) and crude fibre (1.55%), were recorded to be minor constituents of the fruiting bodies of *Isaria cicadae* (Sharma et al., 2021). Vitamin

D content of *I. cicadae* was found to be 3,605.84 IU/g (Sharma et al., 2021). The proximate compositions of the fruiting bodies of *Isaria tenuipes* showed the moisture content (57.56 ±0.07 %), crude fat (21.76±0.00%), crude fibre (6.20±0.26%) and crude protein (6.83±0.02%), (Hong et al., 2007). The soluble sugars in this fungus were found to be composed of glycerol, glucose, mannitol, sucrose etc., and the total carbohydrates content was 24.00±3.14 mg/g dry weight of the fruiting bodies. The other species within the genus viz., *Isaria sinclairii* (Berk.) Lloyd, *Isaria tenuipes* Peck, *Isaria japonica* Yasuda and *Isaria farinosa* (Holmsk) Fr. have been reported to contain substantial amount of nutritional as well as nutraceutical components (Sharma et al., 2015). All of these species were reported to be pf low fat content and rich in protein, fibre, ash, and carbohydrates. Mineral elements such as Fe, Mg, Cu, Mn, and Ca were reported to be detected in appreciable amounts. All three types of fatty acids (saturated, monounsaturated, and polyunsaturated) as well as bioactive compounds like ascorbic acid, β-carotene, lycopene, phenolic compounds, and polysaccharides were detected for each species (Sharma et al., 2015). *Cordyceps militaris* is an analogues of genus *Isaria* in nutritional content. High amounts of amino acids (69.32 mg/g in fruiting body and 14.03 mg/g in corpus), unsaturated fatty acids (70%), adenosine (018% in fruiting body and 0.06% in corpus) and cordycepin concentration (0.97% in fruiting body and 0.36% in corpus) are reported from *Cordyceps militaris* (Hur, 2008). Similarly, in *Ophiocordyceps sinensis,* it has been reported to contain amino acids in the range of 29-33% (Hsu et al., 2002), 70% unsaturated fatty acid (Hyun, 2008), cordycepic acid in a range of 7-29**%** **(**Jiang, 1987), thymine, adenosine and cordycepin are found to be major nucleoside compounds in a range from 138.5– 174.2, 79.6–186.5 and 31.3–91.2 μg/g, respectively (Xie et al., 2010).

2.12. Traditional uses

Most of the Asian Countries are culturally rich with multilingual and multi-ethnic populations, possessing vast repository of traditional medical knowledge (TMK). This traditional knowledge has been developed through years of observations which were traditionally passed down from one lineage system to another by means of oral or in rarity with written documentary tradition. Indigenous peoples and local communities residing in different parts of Asia have substantial amount of empirical knowledge related to harvesting and preparing fungal material, as well as appropriate knowledge about the use of many species of economically important group of fungi. Thus, this information on TMK for different EPF can be invaluable, not only to the indigenous peoples who have used fungal medicines since time long-established, but also for any mycological researchers to use medicine outside of its traditional environment.

According to the traditional Chinese medicine, fruit bodies of entomopathogenic fungi are highly valued as medicinal herb. Many natural entomopathogenic fungi are used in traditional Chinese medicines in China, Japan, Korea and other Asian countries (Zhu et al., 1998). The word Chanhua is originated from ancient Chinese culture. As a part of Traditional Chinese Medicine, Chanhua has a long history of existence. The earliest documentation of traditional use of entomopathogenic fungi appears in 'Lei Gong Pao Zhi Lun' (Lei's Treatise on Preparing Drugs) written by Xiao Lei (the Southern and Northern Dynasties, 5th century AD) who recorded it as a valuable myco-medicinal tonic for reducing internal toxicity that is some 300 years older record than the first record of *O. sinensis* ('Worm in winter, herb in summer', 'Dongchong Xiacao') (Zha et al., 2019). Chanhua literally means 'flower of cicada', as it grows on cicada nymphs and resembles flowers like appearance. Based on its literal meaning and the Chinese ancient records aforementioned, Chanhua should only be considered to *Isaria*-like fungi that grow on

cicada nymphs (Zha et al., 2019). EPF, *Isaria cicada,* the asexual type of *Cordyceps cicadae,* is one of the popular medicinal mushrooms. The synnemata of this fungus is called snow-flake DongChoongHaCho in Korea because of its white appearance (Kang et al., 2010). The fruiting body or the synnemata of this fungus is widely used in expensive traditional medicines and health products in East Asia (Dong et al., 2015; Yue et al., 2013). As noted in Chinese Pharmacopoeia, *I. cicadae* have been applied in therapies for fatigue, night perspiration, fever, childish convulsion, palpitation, and dizziness since long time (Chunyu et al., 2019). In China, *I. cicadae* is used as a food and tonic supplement to treat chronic kidney diseases, and for children with seizures (Hsu et al., 2015; Li et al., 2019).

Among the many typical forms of the fungus DongChoongHaCho (Tochukaso), Cicada DongChoongHaCho the EPF, *Isaria sinclairii* has been widely used in a powdered form as a promising Korean crude drug for the improvement of stamina and treatment of various illness including cancers (Ahn et al., 2013; Oh et al., 2001). Moreover, the fruiting bodies of *Isaria* spp. have been used for treatment of cancer patients in Korea (Ahn et al., 2004; Oh et al., 2001). Drink products containing materials of *I. sinclairii* are commercially available in Korea, and this fungus is considered a candidate nutraceutical agent or supplement for diabetic and cancer patients (Ahn et al., 2004). Furthermore, *Isaria tenuipes* has also been used in traditional medicinal practices. The fruiting body is mostly used in the production of health foods and traditional medicine with its multiple functionality such as lowering blood glucose, antitumor, antibacterial, anti-depression, antioxidation, and anti-aging activity, lowering blood fat and immunoregulation (Zhang et al., 2019).

2.13. Pharmacological potential of the chemical components of *Isaria* spp.

It is estimated that more than 200 secondary metabolites have been isolated and identified from both *Isaria cicadae* and *Isaria tenuipes* (Zhang et al., 2019). Many of these

secondary metabolites (SMs) are the functional components exhibiting anti-viral, anti-bacterial, and anti-tumour activity and few of them involved in an immunity regulation (Hsu et al., 2015; Weng et al., 2002). Moreover HEF, SMs are considered as important drug resources (Zhang et al., 2019). For example, Fingolimod, a novel type of immunosuppressive agent, is a new medicine approved by the FDA in September, 2010, and the European Medicines Agency in March, 2011, derived from Myriocin (ISP-I) a SM of the HEF *Isaria sinclairii*, which is used for treatment of multiple sclerosis, renal cancer, and asthma (Strader et al., 2011). In general, a great variety of biotherapeutic compounds of immense biological activity have been extracted and purified from the different species of the HEF genus *Isaria*.

Overall, the spectrum of pharmacological activities and its application demonstrated by diverse members of insect pathogenic fungal genera *Isaria* spp in particular is extremely broad. Modern pharmacological studies carried out on *Isaria* spp have shown to possess multiple biological and therapeutic functions including; anti-inflammatory, anti-wrinkle, antitumor, antibacterial, antifungal, antiviral, antiparasitic, against paediatric convulsion, dizziness, night perspiration, palpitation as well as immunomodulatory, hepatoprotective and sedative properties (Chunyu et al., 2019; Dai et al., 2021; Li et al., 2020; Mapook et al., 2022; Prommaban et al., 2022). In addition, they are also involved in regulation of blood pressure and cardiovascular disorders, such as hypercholesteremia along with other chronic diseases like diabetes, neurodegenerative diseases, Alzheimer's disease, and cancers (Chunyu et al., 2019; Dai et al., 2021; Li et al., 2020; Prommaban et al., 2022; Zeng et al., 2021). The multiple pharmacological effects of *Isaria* spp., can be attributed to a wide range of biologically active chemical metabolites including polysaccharides, myriocin, ergosterol peroxide, and nucleosides (Chunyu et al., 2019; De Silva et al., 2013; Reis et al., 2017).

2.13.1. Cytotoxic effects

The water-soluble intra-polysaccharide and extra-polysaccharides isolated from *Isaria farinosa* B05 demonstrated significant antitumor activity against sarcoma 180 in mice (Jiang et al., 2008). These polysaccharides were able to inhibit sarcoma 180 tumour growths in mice by 61.63% at 200mg/kg intra-polysaccharides and 55.93% at 200mg/kg extra-polysaccharides (Jiang et al., 2008). The mode of action of these carbohydrates has been demonstrated to enhance the mediated mechanism of the host and not be directly cytocidal (Wang et al., 1995; Seong et al., 2000). Polysaccharides extracted from the mycelium of fungus constitute one of the main bioactive agents which has the potential to exhibit multiple pharmacological activities including antitumor, anti- inflammatory, immunopotentiation, hypoglycaemic, hypocholesterolaemia, protection of neuronal cells against free radical- induced cellular toxicity, steroidogenesis and antioxidant activities (Huang, 2001; Jiang et al., 2008; Koh et al., 2003; Sharma et al., 2015; Yan et al., 2014; Zhong et al., 2009). Moreover, characterization of metabolites from *Isaria farinosa* has demonstrated the antisenescence, antitumor and others effects such as auxin and cytokinin like properties (Jiang et al., 2008).

A bioactive compound, 4-acetyl-12, 13-epoxyl-9-trichothecene-3, 15-diol (AETD), isolated from the methanol extract of fruiting bodies of *Isaria japonica* Yasuda showed apoptosis-inducing activity and IC_{50} value of 10 nmol/l on human leukaemia cells (HL-60) (Oh et al., 2001). An investigation into the mechanism of action of AETD induced apoptosis of HL-60 cells was reported to be associated with the formation of intracellular reactive oxygen species (ROS) and the resulting depletion of intracellular glutathione (GSH) followed by downstream event of caspase-3 activation (Pae et al., 2003). Caspase-3 activity was determined by using fluorometric immunosorbent enzyme assay where the fluorescence intensity of 10nM AETD treated HL-60 cells was recorded to be 33.1 (Pae et

al., 2003). Studies by Shin et al., (2003) have indicated that fungal extract from *Isaria japonica* could significantly decrease tumour weights and volumes, in mice inoculated with Sarcoma-180 tumour cells. Further, it was reported that the fungal extract at 50mg/kg caused inhibition of the tumour volume by 28.1% (3749 ± 279.3 mm^3) and a decrease in the tumour weights by 42.3% (5.1 ± 0.4 g), as compared to clinically available positive drug, at 30 mg/kg, inhibited the tumour weight by 74.4% (2.3 ± 0.1 g) against that of the control (8.8 ± 0.3 g) (Shin et al., 2003). The methanolic extracts from fruiting body of *I. tenuipes* DGUM 32001 has been reported to possess significant cytotoxicity against human cancer cell lines: MCF-7 and HepG2 (Shim et al., 2001). While the ethyl acetate fraction of this fungus showed the highest cytotoxicity against HepG2 and MCF-7 with $IC_{50\ values}$ of 40 and 9.6 µg/mL, respectively (Shim et al., 2001). Nam et al., (2000) isolated and evaluated for the first time the ergosterol peroxide and acetoxyscirpenediol from methanol extract of *Isaria tenuipes*. They found that the 50% inhibitory concentrations of ergosterol peroxide against human gastric tumour cell line (SNU-1), human hepatoma cell line (SNU-354), human colorectal tumour cell line (SNU-C4) and murine sarcoma-180 were 18.7, 158.2, 84.6 and 74.1 mM, respectively. Whereas the IC_{50} values of acetoxyscirpenediol against SNU-1, SNU-C4, SNU-354 and sarcoma-180 were 1.2, 4.0, 2.2 and 1.9 mM, respectively. Further it was revealed that the cytotoxic activities of acetoxyscirpenediol were about 4.0 – 6.6 times stronger than those of cisplatin which is used clinically for treating cancer patients (Nam et al., 2000). Different isariotin compounds mainly G, H, I, J and F of *I. tenuipes* have been found to exhibit cytotoxic activities against cancer cell lines like KB, BC, MCF-7, and NCI-H187 and non-malignant Vero cells (Bunyapaiboonsri et al., 2009; 2011). Recently, similar findings have been demonstrated on MCF-7 breast carcinoma cell line where the maximum inhibition was found to be 44.83 ± 6.88% with ethyl acetate extract of *Isaria tenuipes* VHI-2 fruiting bodies (Pham et al., 2020).

Beauvercin (BEA) is reported to have a potent cytotoxicity towards multi drug resistant human cancer cell lines such as human retinoblastoma Y79, HaCaT, HepG2 and HepG2/ADM cells (Cheng et al., 2009; Wang et al., 2014). BEA also demonstrated significant activity towards NIH/3T3 and CT-26 murine cell lines (Cheng et al., 2009). At 10μM concentration BEA can induce significant toxicity in TM-Luc 102 and Caco-2 cells (Fernández-Blanco et al., 2016). BEA inhibits migration of the metastatic breast cancer (MDAMB-231), prostate cancer ((PC-3M) cells and exhibits antiangiogenic activity in HUVEC-2 cells (Zhan et al., 2007). The metabolite can bring about apoptosis activating mitochondrial pathways, including decrease of ROS generation, loss of mitochondrial membrane potential, release of cytochrome c, and activation of Caspase-9 and−3 (Tao et al., 2015). The most notable biological function of this compound is associated with its inhibitory activity to protein tyrosine phosphatase 1B (PTP1B) with an IC_{50} value of 0.59 M (Heilos et al., 2017; Wang et al., 2014; Zhang et al., 2017). The compound generally forms complexes with cations and thereby increases the permeability of biological membranes (Toman et al., 2011; Wätjen et al., 2014; Lu et al., 2016; Wu et al., 2018). Functionally, it transports divalent cations across biological membranes and acts as an ionophore leading to increase in cytoplasmic Ca^{2+} concentration, causes ATP depletion and eventually activates calcium-sensitive cell apoptotic pathways (Gibson et al., 2014; Li et al., 2017). During *in vitro* studies carried out by various workers, beauvericin has been reported to reverse the multidrug-resistance (MDR) phenotype in yeast and potentiates the fungicidal activity of fluconazole against fluconazole-resistant *Candida albicans* at sub-cytotoxic concentrations and known cytotoxic agents against multidrug-resistant (MDR) cancer cell lines. Beauvericin also hinders directional cell motility of cancer cells (Gibson et al., 2014). Despite several bioactivities, beauvercin is one of the most predominant mycotoxins and has obvious toxic effects towards humans and animals which can pose health risk at certain concentrations (Mallebrera et al., 2018; Ojuri et al., 2018; Panasiuk et al., 2018).

In a continuing search for pharmaceutical active compounds from insect pathogenic fungi a novel pyridone alkaloid compound, named fumosorinone has been isolated from *I. fumosorosea* (Liu et al., 2015). Fumosorinone after 24 h treatment, exhibited potent cytotoxic activity against human cancer lines, including HeLa (IC_{50} = 11µg/ml), MDA-MB-231(IC_{50} = 30µg/ml), and MDA-MB-453(IC_{50} = 31µg/ml) cell lines (Chen et al., 2018). Further investigation by the same author's revealed fumosorinone displayed highest cytotoxicity towards HeLa cells in a time- and dose-dependent manner. They found that this compound exhibited potent cytotoxicity against the HeLa cell line over 48 h (IC_{50} 5 µg/ml) and 72 h (IC_{50} 3 µg/ml) (Chen et al., 2018). The mechanism of action involves the inhibition of cell migration by reducing the phosphorylation of focal adhesion kinase (FAK) at tyrosine (Tyr) 861 and marginally increasing the phosphorylation of FAK at Tyr397 without affecting the phosphorylation of FAK at Tyr576 or Tyr925 (Chen et al., 2018). Moreover, this compound acts synergistically with 5-FU and p38 inhibitors which could potentially enhance the anticancer bioactivity (Chen et al., 2018). Protein tyrosine phosphatase 1B (PTP1B), is an intracellular phosphatase involved in the insulin signalling cascade which can dephosphorylate the insulin receptor (IR) as well as insulin receptor substrate (IRS) (Asante-Appiah, & Kennedy, 2003). The overexpression of PTP1B results in insulin resistant states and it has been considered as a promising insulin-sensitive drug target for the prevention and the treatment of T2DM (Huijsduijnen et al., 2002; Liu et al., 2015).

Ergosterol peroxide isolated from methanol extract of *I. tenuipes* showed high potential against human gastric tumour cell line (SNU-1), human hepatoma cell line (SNU-354), human colorectal tumour cell line (SNU-C4) and murine sarcoma-180 (Nam et al., 2001). Ergosterol peroxide has been reported to exhibit significant cytotoxicity against DU-145, PC-3 and M-2182 prostate cancer cells at a concentration range of 25-200 µm/ml (Han et al., 2014). The mechanism of action of ergosterol peroxide is mostly

by inducing apoptosis via activation of death receptor 5 and caspase 8/3 in DU 145 prostate cancer cells (Han et al., 2014).

Cycloaspeptides F and G isolated from the genus *I. farinosa* (Zhang et al., 2009) exhibited potent cytotoxic effects and inhibited the growth of MCF7 cells, with GI_{50} values of 18.7 and 15.2 μM (Zhang et al., 2009). Cycloaspeptides F and G also displayed modest cytotoxicity towards HeLa cells (Zhang et al., 2009). In case of known cycloaspeptides A and C, the cytotoxic effects against HeLa cells were reported with GI_{50} values of 31.2µM and greater than 63.4 µM whereas with respect to MCF7 cells the GI_{50} values was found to be 23.4µM and 31.9 µM respectively (Zhang et al., 2009).

Militarinone A has been reported to exhibit significant cytotoxic activity against the human lung carcinoma cell line A549, with IC_{50} value of 1.75 μM and antimicrobial activity with IC_{50} value >150.0µM (Ma et al., 2011). In addition, this compound also displayed neurotrophic effects on PC-12 cells at 10.0µM (Schmidt et al., 2002). On the other hand, militarinone B was reported to exhibit moderate cytotoxicity against human lung carcinoma cell line A549, but displayed a good antimicrobial activity against *S. aureus S. pneumoniae* and *C. albicans* (Ma et al., 2011). The novel militarinone E was reported to be significantly cytotoxic against human lung carcinoma cell line A549, with IC_{50} value of > 1.59 µM and militarinone C was comparatively less effective with an IC_{50} value of > > 120.0 µM. However, both the compounds E and C displayed moderate antimicrobial activity against *S. aureus S. pneumoniae* and *C. albicans* (Ma et al., 2011).

Isariotin F isolated from *I. tenuipes* demonstrated significant activity against the malaria parasite *P. falciparum* K1 as well as cytotoxic activities against cancer cell lines (KB, BC, and NCI-H187) and non-malignant (Vero) cells (Bunyapaiboonsri et al., 2009).

Some of the alkaloids isolated from *Isaria* spp., such as isariotins G, H, I and J displayed cytotoxic activities. Thus compound isariotin G displayed significant cytotoxic

activities against KB, MCF-7, NCIH187 and Vero cells with respective IC_{50} = 6.19, 9.21, 22.18, and 26.97 µg/ml. Isariotin H exhibited cytotoxic activities against KB, MCF-7, NCIH187 and Vero cells with respective IC_{50} values of 8.26, 18.56, 37.36, and 22.64 µg/ml. Isariotin I showed potential cytotoxicity against KB, MCF-7, NCIH187 and Vero cells and the IC_{50} values were 5.12, 5.37, 2.25, and 26.85 µg/ml. Isariotin J exhibited cytotoxicity towards KB, MCF-7, NCIH187 and Vero cells with respective IC_{50} values of 2.98, 4.37, 44.86, and 1.77 µg/ml (Bunyapaiboonsri et al., 2011).

2.13.2. Antioxidant activity

The radical inhibition of various extracts, natural powdered materials, active principle and polysaccharides from several isolates of *Isaria* spp. have been reported in both *in vitro* and *in vivo* models. N-(2-Hydroxyethyl) adenosine (HEA) isolated as one of the active principles from n-BuOH fraction of *Isaria sinclairii* showed strong DPPH radical scavenging effect with IC_{50} values of 5.08 mg/ml (Ahn et al., 2008). Study carried out on uronic acid bound water-soluble exo-polysaccharides and intrapolysaccharides from the fermentation broth-mycelia of *Isaria farinosa* B05 have exhibited strong hydroxyl radical, superoxide radical and H_2O_2 scavenging activities. Chelated Fe^{2+} had great reductive ability (0.8-6.4 mg) and higher anti-lipid peroxidation ability at low concentration (≤0.64mg) (Jiang et al., 2005). Jiang et al. (2008), conducted an experiment to study the effect of water soluble intra and extra-polysaccharides from the fermentation and mycelial broth of *I. farinosa* B05 on SOD and CAT activities of tumour-bearing mice. From this study it was found that in comparison with the control group, intra-polysaccharides exhibited elevated antioxidative activities both in hepatocytes and erythrocytes. The SOD activity in intra-polysaccharides was observed to be significantly higher in hepatocytes and erythrocytes ($p<0.05$) at a dose of 200 mg/kg and the CAT activity in intra-polysaccharides at a dose of 200 mg/kg was found to be significantly higher in hepatocytes ($p<0.05$) (Jiang et al., 2008). Whereas, with the extracellular polysaccharides,

the SOD activity at a dose of 400 mg/kg was observed to be significant higher in erythrocytes ($p<0.05$) and the CAT activity at a dose of 200 mg/kg was observed to be significantly higher in hepatocytes ($p<0.05$) (Jiang et al., 2008).

Again, extracellular polysaccharides and intracellular polysaccharides from *Isaria sinclairii and Isaria tenuipes* have shown to exhibit significant DPPH radical scavenging activities, ABTS radical scavenging activities, reducing power, Iron chelating activities, scavenging activities of superoxide anion radicals, ferric reducing antioxidant power, as well as inhibition rate of peroxidation of polyunsaturated fatty acid from lipoprotein (Sharma, 2015). Administration of powdered fruiting bodies of *Isaria tenuipes* at 3% (w/w) level has increased plasma total antioxidant potential and decreased level of lipid peroxidation in eight-week-old male Sprague-Dawley rats (Park et al., 2006). Similarly, Dongchunghacho (*Isaria tenuipes*) rice at 13.6 % (w/w) level has been shown to possess potent erythrocytic antioxidant enzyme activities comparable to *Cordyceps militaris* at 13.6% (w/w) level (Park et al., 2011). *Isaria japonica* Yasuda, has exhibited significant 2, 2-diphenyl-1-picrylhydrazil (DPPH) scavenging activity (49%), hydroxyl radical scavenging activities (37%), and superoxide radical inhibition properties (43%), at 0.1mg/ml (Yahagi et al., 1999). A novel antioxidative pseudo-di-peptide, 3, 4-diguanidinobutanoyl-DOPA known as hanasanagin was isolated from fruiting body of fungus *Isaria japonica* (Sakakura et al., 2005). This compound demonstrated a potent DPPH scavenging activity with IC_{50} value of 8.1µM and SOD activity was found to be 152.0 SOD unit/ µmol (Sakakura et al., 2005). Recently, the extract from *I. tenuipes* fruiting body (IF) was found to show potent *in vitro* antioxidant activity of ABTS inhibition, DPPH scavenging and lipid peroxidation inhibition (Prommaban et al., 2022).

2.13.3. Anti-diabetic potential

A large number of animal studies, using both normal and diabetic animals, have demonstrated that intake of *Isaria* spp has proven to have a glucose lowering effects.

Pharmacological potency of powdered extracts of *Isaria sinclairii* (5-10% w/w) using microarray technique has showed to possess hypoglycaemic activity against diabetes-inducing compounds in obese and diabetic animal models.

The hypoglycaemic effect has been observed and investigated with either the fruiting body or mycelia of *Isaria* spp. in both *in vitro* and in animal models. The powdered materials have been found to exhibit hypoglycaemic properties but the exact molecules involved is still unidentified. Ahn et al., (2010), demonstrated that powdered, *Isaria sinclairii* (IS) at 10% w/w, in the diet was significantly effective in reducing blood glucose levels and body weight gain in C57BL/6 obese (ob/ob) mice after twenty-six weeks of treatment. The mechanism of hypoglycaemic activity of powder the IS, is possibly through the upregulation of glucokinase (Gk-rs1) and downregulation of hydroxy prostaglandin dehydrogenase (Hpgd 15), both associated with suppression of diabetes (Ahn et al., 2010). An aqueous extract of the *Isaria farinosus* G30801 fermented medium showed hypoglycaemic potential in streptozotocin T1DM and T2DM, induced rats, although the mechanism of action was not specified by the authors (Lu et al., 2010). Additionally, in growing *Isaria tenuipes,* the inclusion of 13.6% of *Dongchunghacho* in polished rice was found to exhibit hypoglycaemic, hypocholesterolemic, and antioxidant potential in streptozotocin (STZ)-induced diabetic rats (Park et al., 2011). Protein tyrosine phosphatase 1B (PTP1B) is generally regarded as a negative regulator of insulin receptor (IR) signaling and a potential drug target for the treatment of type II diabetes and other associated metabolic syndromes (Chen et al., 2014). Penostatin derivatives are a novel kind of PTP1B inhibitors isolated from solid cultures of the entomogenous fungus *I. tenuipes*. PTP1B at 35ng/mL exhibited the best potential for the treatment of type II diabetes based on PTP1B induced insulin receptor (IR) inhibitory activity (Chen et al., 2014).

Fumosorinone isolated from *I. fumosorosea* compound has been implicated as a negative regulator of insulin receptor signaling and a potential drug target for the treatment of type II diabetes and other associated metabolic syndrome (Liu et al., 2015a). Liu et al., (2015b) through *in vivo* study using type 2 diabetic KKAy mice showed that fumosorinone increased glucose uptake and improved insulin resistance by down-regulating the expression of PTP1B and activating the insulin signaling pathway involved in T2DM. Recently, Qian et al., (2021) study has revealed that myriocin an active ingredient of *Isaria cicadae* act on diabetic nephropathy (DN) through different targets such as AKT1, MAPK8, and TP53 and other targets, which can help to develop innovative drugs for effective treatment of DN.

2.13.4. Anti-inflammatory activity

The anti-inflammatory action of *Isaria* spp. has been mostly reported in *I. sinclairii*. Adjuvant induced arthritis in rats is a chronic disease which develop into two distinct phases of diseases activity, characterized by an acute periarticular inflammation followed by soft tissue inflammation (Jacobson et al., 1999). The results of the investigation carried out using water and methanol extracts from *Isaria sinclairii* showed profound anti-paw edema effects and also remarkably inhibited UV-mediated upregulation of NF-κB activity in transfected HaCaT cells (Ahn et al., 2013).

Glycosaminoglycan (IS GAG) isolated from *Isaria sinclairii* also produced a noticeable anti-edema effect (Ahn et al., 2013). This IS GAG with increasing concentration inhibited the pro-inflammatory cytokine levels of prostaglandin E2-stimulated lipopolysaccharide in LAW 264.7 cells, cytokine TNF-α production in splenocytes, and atherogenesis cytokine levels of vascular endothelial growth factor (VEGF) production in HUVEC cells. Furthermore, the histological analysis revealed that the LV dorsal root ganglion, including the articular cartilage linked to the paw-treated with IS GAG, was repaired markedly against CFA (Complete Freund's Adjuvants)-induced

cartilage destruction. It was further observed that the combined treatment with Indomethacin® (5 mg/kg) and IS GAG (10 mg/kg) was found to be more effective in inhibiting CFA-induced paw edema at different time intervals revealing IS GAGs potential anti-inflammatory activity (Ahn et al., 2013). Autoimmune thyroiditis (AIT), also known as Hashimoto or chronic lymphocytic thyroiditis, is an autoimmune disease characterized by inflammatory destruction of the thyroid tissue and is caused by high titers of circulating antithyroid antibodies, represents the most common cause of hypothyroidism worldwide (Yang et al., 2021). Recently, the thyroid anti-inflammatory effect of *I. felina* (IF) has been investigated using a mouse model. The result of the study demonstrated that mice fed with higher dose IF preparation alleviates thyroid inflammation and cell apoptosis on experimental autoimmune thyroiditis (EAT) (Yang et al., 2021).The authors has provided substantial evidence of IF's ability to ameliorates EAT in mice, where they found that IF significantly reduced follicle destruction and lymphocyte infiltration in the thyroid gland along with decreased levels of serum TSH, thyroid antibodies, and cytokines(Yang et al., 2021).

Polysaccharides isolated from *Isaria cicadae* has been reported to markedly decrease LPS-induced inflammatory cytokine levels in NRK-52E cells and TGF-β1-induced fibroblast activation in NRK-49F cells (Yang et al., 2020). *Cordyceps cicadae* is the recognized synonym of *Isaria cicadae* (Luangsa-Ard et al., 2005). The powdered preparation of *I. cicadae* at 15-30g/day could down-regulate the expression of major fibrosis markers (α-SMA and fiber-fibroin) and key fibrogenic molecules TGF-β1 and CTGF, and upregulate the expression of IFN-γ, which has antifibrotic effects that maintains, matrix metalloproteinase and tissue inhibitor of metalloproteinases-1 (MMP/TIMP) balance (Wang et al., 2022). The result of the study by Wang et al., (2022) demonstrated that oral preparation of *I. cicadae* can prevent the development and

progression of trinitrobenzene sulphonic acid (TNBS) induced colonic fibrosis in mice which could be useful drug in treatment of colonic fibrosis.

2.13.5. Antigenotoxic capacity

The oxidative stress to the living system could also be brought by exposure to genotoxic chemicals. The protective effect of the extract of *Isaria tenuipes* cultivated on soybeans against the dimethylhydrazine induced genotoxicity or DNA damage and oxidative stress in male F344 rats for nine weeks was found to be highly effective in protecting colonic DNA damage events in rats. The effect of anti-genotoxicity has been attributed to increased antioxidant activity which was noticeable in the form of reduced plasma lipid peroxidation (Park et al., 2007). The fruiting body extract of *Isaria sinclairii* (IS) was assessed for genotoxic evaluation and it was reported that these extracts did not produce any mutagenic response in a *Salmonella typhimurium* assay (Ahn et al., 2004). There was also no significant genotoxic effect on Chinese hamster ovary (CHO) cells compared with control in the chromosome aberration (CA) test and the micronuclei (MN) test showed no significant change in the occurrence of micro nucleated polychromatic erythrocytes in male ICR mice intraperitoneally administered with IS extracts at doses of 15, 150, or 1500 mg/kg, suggesting it has anti-mutagenic potential in the *in vitro* and *in vivo* systems (Ahn et al., 2004).

2.13.6. Antiobesity effects

Obesity is one of the most prevalent nutritional disorders observed in the developed world and is mainly associated with other disorders such as hypertension, diabetes, and arteriosclerosis (Hall et al., 2002), premature aging (Slawik, & Vidal-Puig, 2006), and cancer (Jee et al., 2006). The powdered form of *Isaria sinclairii* (IS) showed a significant antiobesity effect in an animal model experiment. It was reported that obese (fa/fa) Zucker rats fed with powdered IS at a concentration of 5 or 10% (w/w) levels over 4 months resulted in significant decrease in rate of body weight gain in a dose-dependent manner

(Ahn et al., 2007). Weights of abdominal adipose tissues surrounding the epididymides were found to be markedly reduced by this powder in parallel with an attenuated body weight gain. Total cholesterol, triglycerides, bilirubin, and low-density lipoprotein (LDL) in the serum were found to be significantly lowered in the powder administered rats after 17 weeks of treatment. Obese (*fa/fa*) Zucker rats displayed markedly elevated serum leptin levels (>24.6%) suggesting that *I. sinclairii* powder can exert an antiobesity effect in Zucker obese rats (Ahn et al., 2007). In a different experiment, *Isaria sinclairii* showed an increase in the level of poly unsaturated fatty acid (PUFA) which was also true with reduced fat (7%) accumulation in adipose tissue indicating overall anti-obesity activity in rat model (Ahn et al., 2010). Lipid metabolism generally maintains a subtle balance between synthesis and degradation. When the balance of lipid metabolism is disturbed, hyperlipidaemia may occur. Hyperlipidaemia is defined as elevations of fasting total cholesterol concentration which may or may not be associated with raised levels of triglyceride concentration (Nelson, 2013). Hyperlipidaemia is a dominant risk factor represented by raised levels of triglyceride or cholesterol which in turn can cause atherosclerosis, hypertension and diabetes (Esmaillzadeh, & Azatbakth, 2008; Nelson, 2013). Inhibition of pancreatic lipase may inhibit fat absorption and prevent the condition of obesity and hyperlipidaemia. Lee and colleagues (2006), reported that *Isaria tenuipes* grown on egg yolk (PTE) can improve lipid profiles and lipid peroxidation. Its probable mechanism of action is to modulate physiological functions, such as various atherogenic lipid profiles and antioxidants in hypercholesterolemic 8-week-old male Sprague-Dawley rats fed a high fat/high cholesterol diet (Lee et al., 2006). Park and his co-workers reported moderate suppression of hyperlipidaemia by Dongchunghacho (*Isaria tenuipes*) rice supplementation in STZ-induced diabetic rats (Park et al., 2011). The hypolipidemic effects of *Isaria* spp. have been investigated by previous workers but the exact molecules involved in it is still lacking and awaits further investigation.

2.13.7 Antihypertensive effect

The antihypertensive and related activity of *Isaria* spp. and its derivatives has also been studied. Ahn et al., (2007), assessed the hypertensive effects of methanol extracts of *I. sinclairii* in the spontaneously hypersensitive rats. In their study it was showed that the methanol extract could be used for the treatment of metabolic circulatory disorders where an alcohol extract of this fungus was shown to produce anti-hypersensitive effects. Further, active principle of the extract in the form of an adenosine $5^{/}$ -acetate which was suggested to be a vasoactive substance that may be helpful in exhibiting blood pressure lowering effects comparable to standard vasorelaxing activity of adenosine (Ahn et al., 2007). The same group reported that glycosaminoglycan **(**GAG) from the same species produced an antihypertensive effect, which was found to be more effective than the positive control captopril. In the spontaneously hypersensitive rat animal model, a fall of 19% in body weight was observed in the group that received GAG, indicating that GAG derived from *I. sinclairii* may be a potent, naturally occurring antihypertensive agent (Ahn et al., 2013).

2.13.8. Immunomodulatory activity

The immunomodulators are defined as a substances or compounds that help to control the immune system of the body (Das et al., 2021). There are a number of compounds well documented and recognized in the *Isaria* spp. that has the immunomodulatory activity. The major immunomodulating molecule, fingolimod (FTY720) is the first oral immunomodulating drug (0.5mg) from *I. sinclairii* approved for relapsing–remitting multiple sclerosis (Khatri, & Kramer, 2011; Khatri, 2016). It is generally regarded as a blockbuster drug with sales of US $ 2.48 billion in 2018 (Palmer, 2018). It is a high-affinity non-selective mimetic of the S1P1 and is also the first immunomodulator class: sphingosine 1-pohosphate receptor modulator obtained by

chemical modification of a natural product isolated from *Isaria sinclairii* cell supernatant (Chiba et al., 2011; Fujita et al., 1994). It is orally active and predominately metabolized in the liver by sphingosine kinase to the active metabolite fingolimod-phosphate (Novartis Pharmaceutical Corporation, 2011). If it is taken orally, the drug prevents the development of neurological signs in both monophasic and relapsing remitting (RR) models of MS (Penton-Arias, & Haines, 2016). The most striking feature of this wonder drug is that it acts as a safe and potent immunomodulator by inducing marked decrease in peripheral lymphocyte numbers, especially CD4-positive cells and IL-2R-positive cells at doses that display an immunosuppressive activity (Chiba, & Adachi, 2012; Downes et al., 2007; Ueda et al., 2005). It has been comprehensively reported that reduction of circulating lymphocytes by FTY720 is because of the sequestration of lymphocytes into the secondary lymphoid organ (Chiba et al., 2011). The immunomodulatory properties of FTY720 come from its novel mechanism of action, which is to sequester autoreactive B and T cells in lymph nodes and hinder their trafficking towards central nervous system (CNS) thereby preventing them from provoking multiple sclerosis relapses (Khatri, & Kramer, 2011). These immunomodulatory properties have been confirmed in various autoimmune disease models such as lupus nephritis, adjuvant- and collagen-induced arthritis, EAM, autoimmune diabetes, and especially in the experimental autoimmune encephalomyelitis (EAE) model, showing higher efficacy than beta interferon (Penton-Arias, & Haines, 2016).

Furthermore, *Isaria sinclairii*, as the anamorph stage of *Cordyceps cicadae* have demonstrated that its mycelium or culture broth extracts have multiple therapeutic activities (Fukatsu et al., 1997). Takano and his colleagues reported immunomodulating activity of *I. japonica* crude broth and demonstrated that this crude broth augmented anti-sheep red blood cell IgM plaque-forming cells response upon oral administration at 10 and 30 mg/kg/day for 4 consecutive days in mice (Takano et al., 1996). According to the *in*

vivo and *in vitro* studies, the polysaccharides isolated from *Isaria sinclairii* was reported to show the activation of macrophages through the TLR4 signaling pathway, increase of interferon IFN-ϒ production by Peyer's patch cells and immunomodulatory function by RAW 264.7 cells (Takano et al., 2005; Cheng et al., 2012). The works presented by Cheng and his colleagues using the intrapolysaccharides isolated from *Isaria sinclairii* showed that RAW264.7 macrophage cell cultures could stimulate nitric oxide production significantly at doses between 12.5 and 100 µg/ml, thereby demonstrating an immunomodulating activity (Cheng et al., 2012). Shin et al., (2003), had similarly demonstrated that the fungal extract from *Isaria japonica* has shown significant immunostimulant activity as well as a significant anti-tumour activity in mice inoculated with Sarcoma-180 tumour cells. These workers demonstrated that the fungal extract exhibited phagocytosis enhancing effects with, the phagocytosis index (PI) value of 2.0 which was approximately the same as with zymosan (PI=2.0), but far better in compassion to extract from *Ophiocordyceps sinensis* (PI=1.0). Thus, this fungal extract was reported to have anti-tumour activity along with immunostimulant activity which was comparable and better than the effects brought by *Ophiocordyceps sinensis* (Shin et al., 2003).

2.13.9. Neuro-protective effects

Tsushima et al., (2010), in their study based on histochemical observation showed that an extract from a fungus *Isaria japonica* grown on silkworm pupae (*Bombyx mori*) was capable of reversing astrogliosis in the hippocampal CA3 area and was reported to improve memory deficits in ageing mice (Tsushima et al., 2010; Suzuki et al., 2017). Further, their findings suggested that the extract of *I. japonica* fungus grown on silkworm was associated with the ability to recover from central nervous system (CNS) deficits (Robel et al., 2011). Thus, they represent a promising astrocyte-targeted modulator (Ishiguro et al., 2021). Furthermore, the fungal powder administered as a nutraceutical to Alzheimer's patients was found to significantly increase the acetylcholine concentration in

the cerebrospinal fluid which is a target for symptomatic improvement in Alzheimer's disease (Ishiguro et al., 2021; Terayama et al., 2016). Spirotenuipesine A and B isolated form fungus *I. japonica* fruiting body at a 1 μM level and paecilomycine A at a level of 10nM showed significant biological activity in neurotrophic factor biosynthesis in 1321N1 human astrocytoma cells (glial cell lines) and the released neurotrophic factors was believed to promote neuronal differentiation of PC-12 cells (Kikuchi et al., 2004a; 2004b; 2004c). Recently, the novel cyclic peptide naturido was isolated from a medicinal fungus *Isaria japonica* grown on domestic silkworm (*Bombyx mori*) which has been reported to be a promising glia–neuron modulator for the treatment of not only senescence, but also for Alzheimer's disease and other neurodegenerative diseases (Ishiguro et al., 2021). Thus, *I. japonica* may have potential as a source of new candidate drug for the treatment of senescence and neurodegenerative diseases.

2.14. Entomopathogenicity and infection of insect pests by *Isaria* spp.

2.14.1 Infection mechanism

Entomopathogenic fungi (in addition to nematodes) are the only insect pathogens capable of bringing about infection on their host by adhering to the surface of the cuticle and directly penetrating the external cuticle (Bedding et al., 1982; Ortiz-Urquiza et al., 2013).

The fungal pathogens mostly gain access to nutrients in the arthropod haemocoel by direct penetration of the host cuticle (Payne et al., 1988; St. Leger, 1991). Structurally, the insect cuticle is a complex insoluble multi-layered matrix and the outermost surface, the epicuticle, plays a key role in protecting insects against fungal infection and preventing the predator to reach haemocoel (Crespo et al., 2000; Kaczmarek et al., 2021; Pedrini et al., 2007). Moreover, the cuticle and alimentary canal are considered to be important barriers against infection as they are the site of pathogen/parasite entry into the haemocoel.

The multi-layered matrix of epicuticle is composed of three major components; lipids, proteins and chitins along with presence of phenolic compounds that accelerate or inhibit fungal growth, and their presence partially determines whether an adherent fungus develops (Kaczmarek et al., 2021). In order to successfully establish within the insect host, the invading fungi must proliferate within the haemocoel. This is generally achieved in a sequential manner when the entomopathogenic fungus adheres to the host by nonspecific hydrophobic and electrostatic interactions between the conidia and the insect cuticle (Mannino et al., 2019). After spore attachment, the spores must remain in contact with the host cuticle for a sufficient length of time to allow subsequent germination and penetration (St Leger, 1991). The growing hyphae then penetrate the cuticle and enter the host body for proliferation by using a combination of mechanical pressure and the production of cuticle-degrading enzymes (Mannino et al., 2019; Ortiz-Urquiza et al., 2013). To evade detection, the fungus during this stage of infection, grows as hyphal bodies that generally lack cell walls or any surface specific residues and these hyphae are recognized by the insect host (King, 2006). However, the success of the infection process is highly dependent on several factors, such as the structure and composition of the cuticle, the presence of antifungal compounds in the exoskeleton, as well as the efficiency of the cellular and humoral defence reactions of the insect after invasion (Mora et al., 2017).

The composition of the cuticle also strongly influences conidia germination, and this variation often results in differences in susceptibility between insect species (Ortiz-Urquiza et al., 2013). Once the penetration is complete then the fungus will establish itself on the insect host and thereafter death may take place rapidly or can take much longer (King, 2006).

Death is thought to be facilitated by the depletion of nutrients, physical obstruction or invasion of vital organs and toxicosis. Following the death of insect host, the fungus switches from hyphal to mycelial growth and grows saprotrophically, subsisting on the

remaining nutrients on the insect cadaver (Inglis et al., 2001). The fungus then reproduces via asexual means producing conidia arising directly from the mycosed cadaver or on synnemata.

However, if the environmental and host conditions are favourable, ascomycetous entomogenous fungi may produce a teleomorphic form and reproduce via ascospores produced within perithecia usually formed on an aboveground stroma (King, 2006). The infectious spores or spore's fragments are then dispersed to other insect hosts and the cycle repeats itself. A generalised mechanism of infection of entomopathogenic fungi is presented in figure (Figure 2.2).

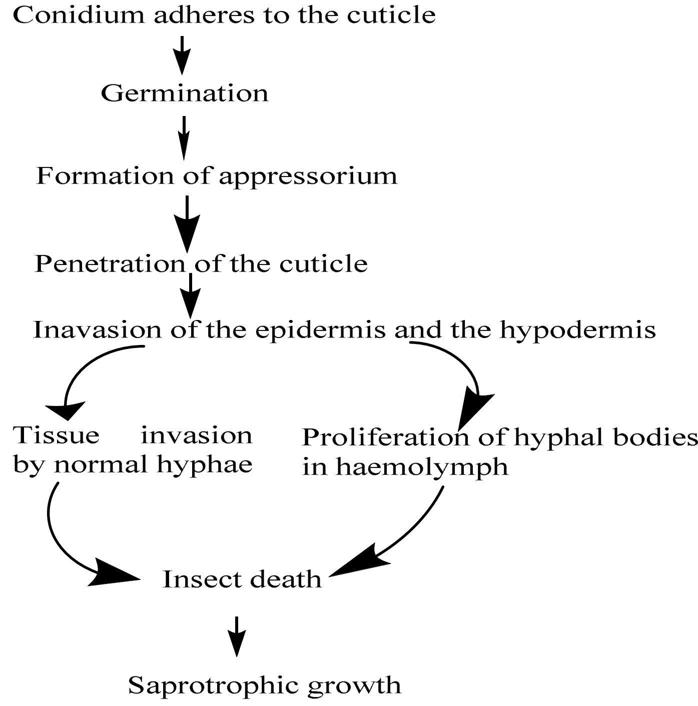


Figure 2.2: A summary of the general infection mechanism of insects by entomopathogenic fungi (Based on Clarkson, & Charnley, 1996; Matthew et al., 2007).

2.15. The Biological control of insect pests by *Isaria* spp.

Biological control can be defined as the "utilisation of natural or modified organisms, genes or gene products to reduce the effects of pests and diseases" (Cook,

1988). The vast majority of the insecticides and pesticides are indispensable in use but they have certain negative impact on nontarget species like honey bees, fish and wildlife both within and outside agro-ecosystems (Pedigo and Rice, 2014). In addition, these chemical compounds have deleterious effects on the environment, increased reports of insecticide resistance and ever-increasing costs of insecticide production (Khachatourians, 1986; Charnley, 1989; Kaya & Lacey 2007). To circumvent the deleterious effects of chemical pesticides on environment worldwide an alternative approach to use entomopathogenic fungi (EPF) is gaining momentum due to its eco-friendly nature, effectiveness, specificity and harmlessness to non-target organisms (Das et al., 2021; 2022). Entomopathogenic fungi have been widely employed for the control of major insect pest and the study reveals that 700 species from approximately 90 genera are pathogenic to insects (Charnley, 1989; Suganya, & Selvanarayanan, 2010). 1000 species of EPF under phyla Entomopthoromycota, Blastocladiomycota, Mycosporidia, Basidiomycota and Ascomycota have been documented to regulate insect populations (Das et al. 2019). Some of the most important members that are most intensively investigated fungi for preparing 687 mycoinsecticide are *Verticillium lecanii* (Zimm), *Isaria fumosorosea* (Wize), *Beauveria bassiana* (Vuill), *Metarhizium anisopliae* (Metschinkof), *Hirsutella thompsonii* (Fisher) and *Nomuraea rileyi* (Farlow) (Das et al., 2019) for controlling various pest species of Coleoptera, Lepidoptera, Hemiptera, Tysanoptera, Isoptera, and Orthoptera and Acarina (Hussein et al., 2016). Most of the species belonging to these fungi vary widely with respect to ease of mass production, host range, environment friendliness, and the ability to initiate epizootics (Barta et al., 2019). Therefore, they provide a broad range of organisms with potential for development as pest control agents (Roberts & Humber, 1984). Currently, the most used species of entomopathogenic fungi globally in biological control are *Beauveria bassiana* (33.9%), *M. anisopliae* (33.9%) *Isaria fumosorosea* (5.8%), and *Beauveria brongniartii* (4.1%) out of the humongous numbers isolated, identified and tested species (de Faria and Wraight, 2007; Rajula et al., 2020).

2.15.1 Biocontrol of Citrus pests

The citrus rust mite, *Phyllocoptruta oleivora* (Ashmead) (Acari: Eriophyidae), is a major pest of citrus in tropical regions (Aghajanzadeh & Mallik, 2007; Robles-Acosta, 2019). *Phyllocoptruta oleivora* can infest leaves and green branches of citrus. Damage occurs when *P. oleivora* feed on the developing fruit; penetration of epidermal cells by the mite's stylet causes dark brown areas to develop on the fruit surface (McCoy & Albrigo, 1975). The principal control method for *P. oleivora* is the use of broad-spectrum insecticides (Alves et al. 2005). However, the use of chemicals compounds as pesticides enhances the risk of insecticide resistance and environmental degradation of agroecosystems (Robles-Acosta, 2019). The use of entomopathogenic fungi for pest control is an alternative strategy, particularly for dealing with the problems of resistance (Omoto et al., 1994) and environmental pollution, thereby improving the economic and biological sustainability of agroecosystems (Alves et al., 2005).

The members of fungi that are reported to contribute towards natural regulation of insect and mite populations are from the orders Hypocreales and Entomophthorales (Dolinski & Lacey, 2007). The latter are difficult or impossible to mass-produce, and therefore have not been produced commercially or applied on a large scale. In contrast, several species of Hypocreales are commercially available for control of insect and mite pests; some of these belong to the genera *Beauveria, Metarhizium, Isaria, Aschersonia, Hirsutella,* and *Lecanicillium* (Inglis et al. 2001; Goettel et al. 2005: Robles-Acosta, 2019). The fungi *Isaria fumosorosea* (Wize) have been extensively studied as biological control agents (Das et al., 2022). Robles-Acosta, et al., (2019), reported that *I. fumosorosea* could be used as a biocontrol agent. However, its efficiency under experimental conditions never exceeded 50 % against *P. oleivora* (Robles-Acosta, 2019).

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) has emerged as a major insect pest species responsible for damages of citrus (*Citrus* spp.) fruit

quality and tree health (Corallo et al., 2021). This insect is a vector of the phloem-limited bacterium *Candidatus liberibacter asiaticus*, a putative causal agent of citrus greening disease known as Huanglongbing (HLB), considered one of the most destructive diseases of citrus orchards worldwide (Bove, 2006; Stauderman et al., 2012). Disease management is primarily based on vector control using pesticides which can affect natural enemies that play an important role in pest control (Corallo et al., 2021). Entomopathogenic fungi play an important role in the natural regulation of insect populations and are good microbial candidates for control of phloem feeding pests because they penetrate the cuticle directly (Hajek & St. Leger, 1994; Goettel et al., 2010).

The literature survey reveals that several entomopathogenic fungi are known to infect naturally or remain associated with *Diaphorina citri* (Stauderman et al., 2012). Of them, include *Isaria fumosorosea* (*Paecilomyces fumosoroseus*) Wize (Hypocreales: Cordycipitaceae) is a prominent one (Meyer et al., 2008). *I. fumosorosea* (*Ifr*) is a promising candidate for pest management since it is produced commercially and demonstrate pathogenicity against *Diaphorina citri* and other citrus pests (Avery et al. 2009, 2011; Hoy et al., 2010; Hunter et al., 2011). The morphological and molecular characteristics of *Ifr* strains used as biocontrol agent has been evaluated and compared to a reference strain to develop better strategies for psyllid pest management (Meyer et al., 2008).

Treatment of blastospores of *Isaria fumosorosea* under lab conditions against psyllid has resulted in the mortality of the pathogen (Avery et al., 1999). Studies carried out by Stauderman et al., (2012), confirms the potential of *Isaria fumosorosea* to be used in IPM strategies for *D. citri*. The broad-spectrum potential of *Isaria fumosorosea* (*Ifr*) on insect pests such as *H. vitripennis, T. citricidus* and *D. abbreviatus* were found to provide a potential biological control agent for these and other pests of agricultural crops (Hunter et al., 2011). Lezama-Gutiérrez et al., (2012), reported that *I. fumosorosea* (*Ifr-4*) was found

to exert positive biocontrol effect against the nymphs and adults of *D. citri* under field conditions.

2.15.2. Biocontrol of Conifer pests

The Western Conifer Seed Bug (WCSB), *Leptoglossus occidentalis* Heidemann (Heteroptera: Coreidae), is a cone and seed insect native from Western North America where it is considered relatively serious pest of conifer seed orchards (McPherson et al., 1990; Bates et al., 2000). It can feed on several species of conifers seeds using its mouth parts in piercing the cone and sucking out the endosperm resulting in a high rate of conelet abortion thereby impacting the seed viability in different plant genus - *Pinus, Pseudotsuga, Tsuga, Picea, Abies, Cedrus, Juniperus* and *Pistacia* (Bernardinelli & Zandigiacomo, 2001). This pest was observed feeding on Stone pine cones in several regions in Europe impacting the overall production of profitable Stone pine edible nuts (Bracalini et al., 2013; Farinha et al., 2018). There have been reports suggesting an important impact of this bug on the profitable Stone pine edible nuts (Farinha et al., 2018). Information on the biocontrol of these insect pests is well documented and laboratory evaluation of WCSB susceptibility to hypocrealean insect-pathogenic fungi particularly *Isaria fumosorosea* Wize and *Beauveria bassiana* (Balsamo) Vuillemin has shown their potential of possible use in biocontrol of this invasive insect (Barta 2009; Barta 2010). Since *Isaria fumosorosea* isolates exhibited excellent virulence against Western Conifer Seed Bug and was reported to reach the lowest median lethal concentrations (Barta, 2010).

2.15.3. Biocontrol of Mosquito

Insecticide resistance due to the overuse of chemical substance is seriously undermining efforts to eliminate the prevalence of medically threatening malaria arthropods. In response, research on alternatives to the use of chemical insecticides against adult mosquito vectors has been increasing day by day. Fungal entomopathogens

formulated as biopesticides have received much attention and have shown considerable potential in enhancing the susceptibility (Blanford et al., 2012).

The Asian malaria vector, *Anopheles stephensi* when exposed for six hours to conidial suspension of *I. farinosus* at standard dose rate of $1x10^9$ spores ml^{-1} could bring about marginal decline with modest virulence with a percent biting risk at day 14 was 52.9, responding to the feeding stimulant (Blanford et al., 2012). Study carried out by Karthi et al., (2020), found that the conidial suspension of *I tenuipes* at a level 1.0×10^5 conidia/mL could heavily damage the internal gut cells and external physiology of the dengue larvae compared to the control. The authors also reported that the mode of action of *I. tenuipes* is target-specific and the sub-lethal dosage at a level 1.0×10^4 conidia/mL displayed a significant oviposition deterrence index and also blocked the fecundity rate of dengue mosquitos in a dose-dependent manner (Karthi et al., 2020).

2.15.4. Biocontrol of Livestock pests

Ticks have gained considerable attention as they are one of the main economic threats to the cattle industry worldwide affecting productivity, health and well-being (Alonso-Díaz et al., 2021). It has been estimated that more than around 80% of cattle population worldwide is exposed to tick infestations (Snelson, 1975; Giles et al., 2014), where the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini), *R. (B.) annulatus* (Say), and *Amblyomma mixtum* (Koch) are considered the most important livestock ticks in country like Mexico (Alonso-Díaz et al., 2021). The desirability for alternative methods to control tick populations is prompted by the high prevalence of multiresistant tick strains to the main chemical acaricides and their ecological consequences. Biological control using entomopathogenic fungi (EPF) is one of the most promising alternative options (Alonso-Díaz et al., 2021). The EPF of the genus capable of bringing about infection against ticks both in natural and experimental settings includes *I. farinosus* against *I. ricinus* (Kalsbeek et al., 1995; Polar, 2007), *I. fumosoroseus* against *I. ricinus* (Kalsbeek et

al., 1995), *R. sanguineus* under experimental set up (Samish et al., 2001), and *I. tenuipes* against *R. microplus* naturally (Polar, 2007). Laboratory experiments have demonstrated positive sign of EPF efficacy to control susceptible and resistant/multiresistant tick populations. It was demonstrated that fungal strains chiefly belonging to *I. fumosorosea* CLO55 and PFR 97 at dose of 10^{11} conidia m^{-2} at 20± ^{O}C against adult midges resulted in LT_{50} (time taken in days to kill 50% population of midges) value of 2.74 and 3.22 days respectively (Ansari et al., 2011). Whereas the reported LT_{90} (time taken in days to kill 90% population of midges) for these two strains was 5.76 and 5.99 days (Ansari et al., 2011).

2.16. Major secondary metabolites produced by *Isaria* spp.

Members of the Hypocrealean genus *Isaria* have been reported to possess rigorous secondary metabolisms through different operative metabolic pathways, including non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS) and terpenoid synthetase (TS), which produce diverse spectrum of secondary metabolites (SMs), such as ribosomal peptides (NRPs), polyketides (PKs), terpenoids of miscellaneous types (Lu et al., 2017; Wang et al., 2018; Zhou et al 2011). Members belonging to Hypocrealean Entomopathogenic Fungi (HEF) are generally considered to be a rich source of secondary metabolites (Gibson et al., 2014). Most of the members of HEF are known to produce a large number of structurally diverse spectrums of secondary metabolites (SMs) with a remarkable range of bioactivities having potential applications in human and veterinary medicine and agricultural production (Molnar et al., 2010; Gibson et al., 2014).

Based on last forty years of research published in various scientific journals with the exclusion of patents, it is estimated that more than 200 SMs have been isolated and identified from both *Isaria cicadae* and *Isaria tenuipes* (Zhang et al., 2019). Many of these SMs are the functional components exhibiting anti-viral, anti-bacterial, and anti-tumour activity and few of them involved in an immunity regulation (Hsu et al., 2015; Weng et

al., 2002). Moreover HEF, SMs are considered as important drug resources (Zhang et al., 2019). For example, Fingolimod, a novel type of immunosuppressive agent, is a new medicine approved by the FDA, and the European Medicines Agency, derived from Myriocin (ISP-I) a SM of the HEF *Isaria sinclairii* (Strader et al., 2011). Myriocin is also referred to as ISP- I for immunosuppressant from Isaria. In general, a great variety of biotherapeutic compounds of immense biological activity have been extracted and purified from the different species of the HEF genus *Isaria*. A comprehensive list of the isolated potent bioactive compounds from *Isaria* species are presented in Table 2.2.

Nucleosides are the major class of bioactive compound reported from the genus *Isaria*. Studies have shown that cordycepin an important nucleoside isolated from *Isaria cicadae* having a broad-spectrum pharmacological activity comparable with the metabolite from *Cordyceps* spp. Cordycepin shows immunomodulatory, antioxidant, antidepressant, antibacterial, antiviral, and anti-tumour activity on human cell lines (Zhang et al., 2019). As a tonic supplement this compound has shown to enhance male reproduction by stimulating *in vitro* and *in vivo* steroidogenesis in mouse Leydig cells by activating the PKA pathway (Chen et al., 2017). US Food and Drug Administration (FDA) in 2007, has characterized cordycepin as an orphan drug for the treatment of TdT-positive acute lymphocytic leukaemia (https://www.fda.gov/). Interestingly, cordycepin along with pentostatin was subjected to a clinic trail (phase 1/phase 2) in patients with refractory TdT-positive leukaemia in 2008–2010 (https://www.clinicaltrials.gov/). In China, cordycepin is used for clinical adjuvant treatment of non-small cell lung cancer and nephropathy of type 2 diabetes mellitus (Zhang et al., 2019).

The nucleoside adenosine was reported to be higher in mycelium of *I. tenuipes* than its fruiting body part (Prommaban et al., 2022). The natural extract of *I. tenuipes* has been reported to display significant antioxidant and anti-wrinkle properties that could be used in pharmaceutical, cosmeceutical, and nutraceutical industries (Prommaban et al., 2022). In

addition, adenosine derivative, N-(2-hydroxyethyl) adenosine reported from *Isaria cicadae* demonstrates potent antioxidant activity (Ahn et al., 2008).

Cordycepic acid (D-mannitol), an isomer of quinic acid with a polyol structure, isolated from *Isaria cicadae* sporoderm-broken spore powders demonstrates pharmacological functions (Zhang et al., 2019). It is used as an important index of quality control for *O. sinensis* products. It exhibits potent diuretic and prophylaxis action against postoperative acute renal failure, relieving cough and asthma, and anti-free radical activities. The compound was found effective to provide protection or treatment for patients with cerebral ischemia and trauma where it helps in improving cerebral microcirculation and cerebral blood flow (Lin et al., 2016).

Cordysinin A, a diketopiperazine (DKP) isolated from the mycelia of *C. cicadae* (Lu et al., 2017), is a cyclic dipeptide formed from two amino acids with or without further structural modifications in diketopiperazine nucleus. It is reported to inhibit the proliferation of human glioma U87-MG and U251 (Xuewei et al., 2016). Natural DKPs have attracted attention in the field of drug discovery owing to their rigid structure, chiral nature, varied side chains and diverse bioactivities including neuroprotective, anti-oxidative, anti-hyperglycaemic, anti-inflammatory, antitumour, antiviral, antifungal and antibacterial activities (Wang et al., 2013).

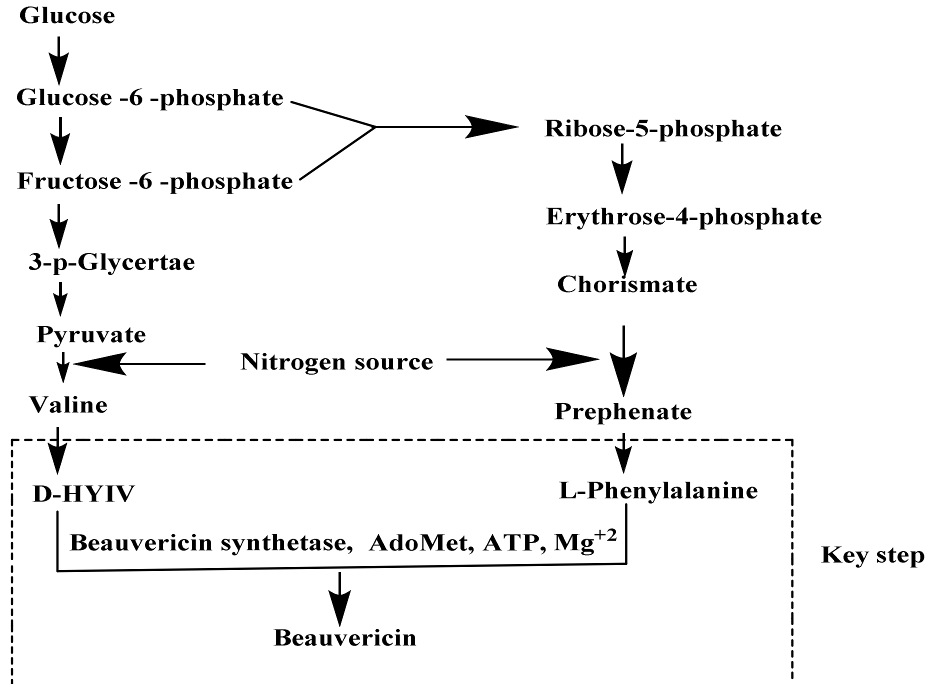
Some of the species belonging to the genus *Isaria* have been reported to produce a cyclic peptide referred to as the cyclooligomer depsipeptide beauvericin (BEA). Beauvericin's are a class of cyclohexadepsipeptides with core structures made up of three N-methyl-L-phenylalanine units that are connected alternately with three 2-hydroxy-D-isovaleric acid (D-Hiv) residues (Hamill et al., 1969). The chemical structure consists of alternating 2-hydroxy-3-methylbutanoic acid and three aromatic amino acid N-methyl-L-phenylalanine (N-Me-Phe) (Wang et al., 2018). They were first reported and isolated from the culture of the insect-pathogenic fungus *B. bassiana* (Hamill et al., 1969). Beauvericin

is widely produced by the members of family Cordycipitaceae which include; *B. bassiana* and other *Beauveria* spp., as well as by *I. fumosorosea, I. japonica, I. tenuipes* and *I. cicadae* (Chhetri et al., 2020; Wang et al., 2018). Beauvericin has moderate antibacterial, antifungal, and insecticidal activities but potent cytotoxic activity against human cancer cell lines (Wang et al., 2012; Lu et al., 2016; Tao et al., 2015; Wätjen et al., 2014; Weng et al., 2019; Gibson et al., 2014; Wu et al., 2018).

2.16.1. Biosynthesis pathway of Beavurecin

The necessary components in BEA biosynthesis are amino acid L-phenylalanine (L-Phe), the hydroxy acid D-hydroxyisovaleric acid (D-HYIV), ATP/Mg^{2+}, and S-adenosyl-methionine (AdoMet), which is the source of the methyl group for the L-phenylalanyl residues and the beavurecin synthetase (Wang, & Xu, 2012; Zobel et al., 2016). Nitrogen source could be any amino acid that offers nitrogen to L-phenylalanine or valine by transamination metabolic pathway and a hexose or a pentose could be the carbon source instead of glucose. However, glucose is the most effective for beauvericin biosynthesis, as reported by Xu et al., (2011). The beauvericin synthetase is a multifunctional enzyme that catalyzes depsipeptide formation. It is a single polypeptide chain with a molecular mass of approximately 250 kDa and a calmodulin binding motif (Peeters et al., 1988; Wang, & Xu, 2012). It catalyzes beauvericin biosynthesis via a nonribosomal, thiol-templated mechanism (Kopp, & Marahiel, 2007; Steiniger et al., 2017; Xu et al., 2008). Beauvericin synthetase can interact with Ca^{2+} sensor calmodulin (CaM) in a Ca^{2+}-dependent manner (Kim, & Sung, 2018). According to review by Wang and Xu (2012), there are five intermediates key step of beauvericin biosynthesis. First, two beauvericin synthetase modules are activated by the corresponding L-Phe and D-HYIV, which are covalently attached to the enzyme-bound 4'-phosphopantetheinyl arm as thioesters. In the second step, the L-phenylalanyl residues are N-methylated by AdoMet. In the third step, a peptide bond is formed. Fourth, a linear hexadepsipeptide intermediate is synthesized and in the

final step, the linear hexadepsipeptide is cyclized to make beauvericin (Wang, & Xu, 2012). The optimum pH requirement for beauvericin formation was found to be pH 7.2, and the optimum temperature recorded was 25-27 °C and the beauvericin synthetase was reported to be inactive if the temperature exceeded 30 °C (Peeters et al., 1983; 1988). The schematic representation of the BEA biosynthesis is shown in Figure 2.3.



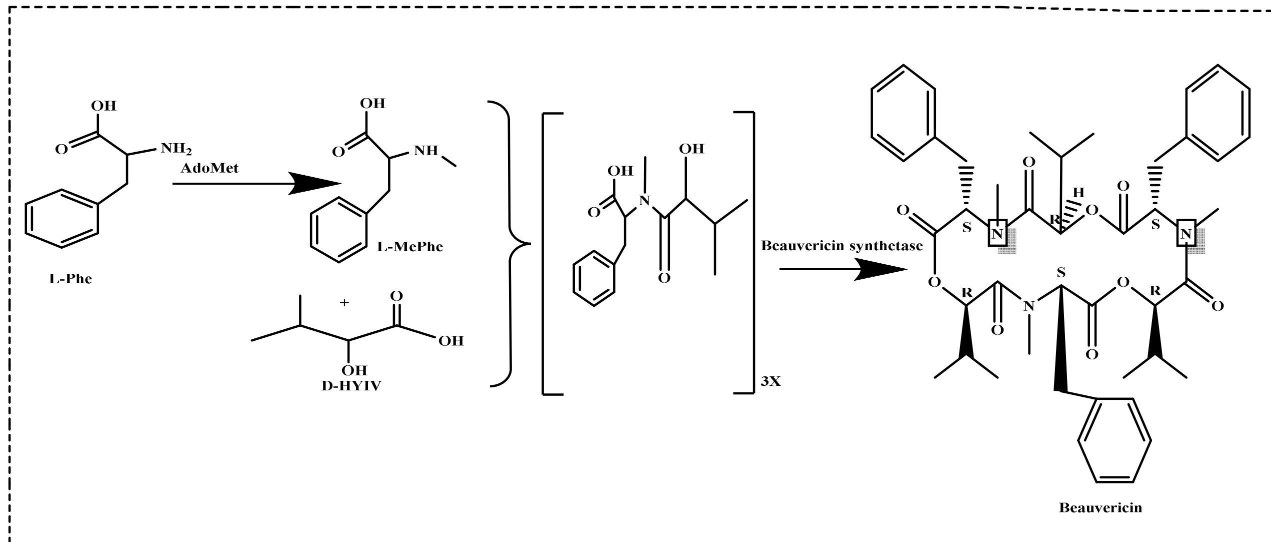


Figure 2.3: Beauvericin biosynthesis pathway based on Wang and Xu, 2012; Wu et al., 2018.

The entomopathogenic fungus *Isaria fumosorosea* was reported to produce cyclotetradepsipeptides, namely beauverolides C, F, I, Ja, L, M, and N (Madariaga-Mazón et al., 2015). Beauverolides are known mostly to represent a group of lipophilic and neutral cyclotetradepsipeptides containing linear and branched C9- or C11-*β*-hydroxy acid residues (Kuzma et al., 2001). All these cyclotetradepsipeptides exerted a high affinity activity towards Calmodulin (CaM), with a dissociation constants (Kd) value ranging from 0.078–3.440 μM. The only tryptophan containing beauverolide Ja showed the highest affinity to CaM with Kd, value of 0.078 μM (Madariaga-Mazon et al., 2015). Calmodulin (CaM) can modify its function in several ways, either inhibiting or altering the interaction with other proteins (Madariaga-Mazón et al., 2015). Some of these compounds may be used as antipsychotics, muscle relaxants, antidepressants, minor tranquilizers, and local anesthetics (Johnson, 2006; Menyhárd et al., 2009). Beauverolide L displayed anti-immunity activity against the greater wax moth, *G. mellonella* (Vilcinskas et al., 1999). Fumosorinone has been reported from the culture broth of *Isaria fumosorosea*. It is a new 2-pyridone alkaloid and its structure was confirmed by HRESIMS 1D and 2DNMR (Liu et al., 2015a). Structurally fumosorinone resembles tenellin and desmethylbassianin but it differs widely in chain length and degree of methylation. Functionally it is well characterized by a classic noncompetitive inhibitor of protein tyrosine phosphatase 1B with IC_{50} value of 14.04 μM (Liu et al., 2015a).

Genome sequencing and molecular experimental studies have revealed that the gene cluster of fumosorinone biosynthesis includes a hybrid polyketide synthase–nonribosomal peptide synthetase gene, two cytochrome P450 enzyme genes, a trans-enoyl reductase gene and other two transcription regulatory genes (Liu et al., 2015). Fumosorinone A is a natural alkaloid isolated from the fermented broth culture of *I. fumosorosea*. The structure was determined through HRESIMS and has found to be structurally similar to fumosorinone (Zhang et al., 2017).

2.16.2. Putative synthesis pathway of fumosorinone

The structure of fumosorinone is highly resembles tenellin. The genetic basis for the biosynthesis of tenellin is well established and on the basis of tenellin research a putative biosynthetic pathway and the reactions involved in the biosynthesis of fumosorinone has been proposed (Liu et al., 2015a). Polyketide synthase (PKS) produce polyketide chains with different reduction and methylation patterns and non-ribosomal peptide synthetase (NRPS) catalyses the fusion of the polyketide chain to an amino acid (tyrosine) (Boettger et al., 2012) and an offloading domain for release (Du and Lou, 2010).

In the primary steps acetate is extended seven times with seven unites of malonate by PKS modules, programmed C-methylations occur after the first three and the sixth extensions, and cycles of full reduction occur after the first two extensions (Fisch et al., 2011). In the next steps ORF3 (fumosorinone A) encodes a cytochrome P450 monooxygenase which is believed to catalyse an unprecedented oxidative ring expansion of prefumosorinone A to form the 2-pyridone core of fumosorinone (Halo et al., 2008). Then ORF4 (fumosorinone B) encodes an unusual cytochrome P450 monooxygenase which may be involved in the hydroxylation of the nitrogen of prefumosorinone B but not of acyltetramic acid prefumosorinone A to form fumosorinone (Liu et al., 2015a).

Figure 2.4: Putative biosynthesis of fumosorinone based on Lieu et al., 2015.

The two novel spirocyclic trichothecane derivatives, spirotenuipesine A and B were isolated form fungus *I. japonica* fruiting body grown in barley grain and the structure of these compounds was elucidated with the help of HREIMS and NMS spectroscopy (Kikuchi et al., 2004). Both the compounds spirotenuipesine A and B at a 1 μM level displayed potent activity in neurotrophic factor biosynthesis in glial cells and the released neurotrophic factors promote neuronal differentiation of PC-12 cells (Kikuchi et al., 2004b). Therefore, these compounds are active in neurotrophic factor biosynthesis, suggesting that both may be lead compounds in drug synthesis for serious neuronal disorders such as Alzheimer's disease. Continuing investigation into secondary metabolites from cultivated fruiting bodies of *Isaria japonica* led to isolation of three novel trichothecane-type sesquiterpenoids, paecilomycine A, B and C and the structures of

these compounds were deduced from their spectroscopic data and their absolute configurations were elucidated by preparing their MPA esters (Kikuchi et al., 2004c). Paecilomycine A displayed potent biological effects. Tenuipesine A, a novel trichothecane with an unprecedented carbon-migrated skeleton including a cyclopropane ring was isolated from fungus *I. japonica* by the same co-workers. However, the biological activity of tenuipesine A was not tested by these workers and this compound however, may be a promising therapeutic one with biological novelty (Kikuchi et al., 2004a).

The two-novel cyclic pentapeptides cycloaspeptides F and G along with the two known cycloaspeptides A and C, have been isolated from the crude extract of the fungus *I. farinosa* (Zhang et al., 2009). The structures of these metabolites were elucidated primarily by NMR and MS methods. Cycloaspeptides represent a class of ABA-containing cyclic pentapeptides that are generally reported from fungi.

The novel N-hydroxypyridones, militarinones E and F along with two known militarinones A and B were isolated from the fermented culture of the fungus *I. farinosa* (Ma et al., 2011). The structures of the novel militarinones were elucidated by spectroscopic methods and confirmed by X-ray crystallography. An N-hydroxypyridone derivative represents a group of compounds with a rich diversity of structures and bioactivities. Two new pyridone alkaloids, farinosones A and B were isolated from the mycelial extract of fungus *I. farinosa,* together with a known compound farinosone C (Cheng et al., 2004). The structures of these novel compounds were characterized through NMR spectroscopy. Farinosones A and C at 50 μM concentration displayed neurite outgrowth in the PC-12 cell line, while farinosone B did not show any activity. Farinosones are more or less related to militarinones in chemical structure, belonging to the terpene group of compounds (Weng et al., 2019).

From the culture broth of *I. tenuipes* a novel penostatin J together with three known penostatins A, B and C were isolated and the structure of the novel compound was elucidated using HRESI-MS (Chen et al., 2014). These compounds (A, B, C & J), were

found to be a potent protein tyrosine phosphatase 1B (PTP1B) inhibitors (Chen et al., 2014).

Isariotins A, B C and D alkaloids possessing a unique bicyclo [3.3.1] nonane ring were isolated from the culture broth of fungus *I. tenuipes* (Haritakun et al., 2007). The structures of these compounds were elucidated primarily by NMR and through mass spectroscopic analyses. Compounds A, C and D had moderate activity against the *M. tuberculosis* $H_{37}Ra$ and their MIC values were 486.0, 488.0 and 544.0 μM respectively, but the activity of compound B was not specified (Haritakun et al., 2007). Continuing investigation into secondary metabolites from fermentation culture broth of *I. tenuipes* led to the isolation of new spirocyclic and bicyclic hemiacetals, isariotins E and F, together with TK-57-164A and their structural formula was established by HRESIMS (Bunyapaiboonsri et al., 2009). Fermentation of *I. tenuipes* with minimal salt medium in a liquid static condition produced four new alkaloids isariotins G, H, I, J and their structures were elucidated on the basis of NMR spectroscopic and mass spectrometry data (Bunyapaiboonsri et al., 2011). All these compounds were reported to exhibit potent antimalarial and cytotoxic activities.

Table 2.2: Enumeration of phyto-pharmaceutical metabolites from different species of *Isaria* spp., depicting chemical structure and pharmacological activities. (Chemical structures were prepared using Chem Draw Professional 15.0).

SL No	Source	Trivial Name/Mol. Formula/Mol. Mass (g/mol)	Structure	Study/activity/ results	Study model	References
1.0	*I. sinclairii*	N-(2-Hydroxyethyl)adenosine /$C_{12}H_{17}N_5O_5$ /428.4		Antioxidant activity (IC_{50} value of 5.08 mg/ml in the fraction containing N-(2-Hydroxyethyl) adenosine.	HUVEC endothelial cells/DPPH assay	Ahn et al., 2008
2.0	*I. sinclairii*	Myriocin/ $C_{21}H_{39}NO_6$ / 401.5		Potent inhibitor of lymphocyte proliferation in mouse allogeneic mixed lymphocyte reaction (MLR) with IC_{50} value = 8.0nm.	Mouse model	Chiba, 2020
3.0	*I. farinosa*	Farinosone A/$C_{25}H_{27}NO_4$ / 405.5		Induced neurite outgrowth in the PC-12 cell line at concentration of 50 μM but exhibited no cytotoxicity.	PC-12 cell line	Cheng et al., 2004
4.0	*I. farinosa*	Farinosone B/$C_{25}H_{27}NO_5$ /421.5		No cytotoxicity was observed against PC-12 cell line at tested concentration.	PC-12 cell line	Cheng et al., 2004
5.0	*I. farinosa*	Farinosone C / $C_{19}H_{25}NO_5$ / 347.4g		Induced neurite outgrowth in the PC-12 cell line at concentrations of 50 μM and exhibited no cytotoxicity.	PC-12 cell line	Cheng et al., 2004

6.0	*I. farinosa*	Cycloaspeptide A/ $C_{36}H_{43}N_5O_6$ /641.8		Cytotoxic effects against HeLa (GI_{50} 31.2µM) and MCF7 cells (GI_{50} 23.4µM).	HeLa and MCF7 human cancer cell lines	Zhang et al., 2009
7.0	*I. farinosa*	Cycloaspeptide C/ $C_{35}H_{41}N_5O_6$ / 627.7		Cytotoxic effects against HeLa (GI_{50} >63.4 µM) and MCF7 cells (GI_{50} 31.9 µM).	HeLa and MCF7 human cancer cell lines	Zhang et al., 2009
8.0	*I. farinosa*	Cycloaspeptide F/ $C_{42}H_{53}N_5O_{11}$ / 803.9g/mol		Cytotoxic effects demonstrated on HeLa (GI_{50} 49.8 µM) and MCF7 cells (GI_{50} 18.7 µM).	HeLa and MCF7 human cancer cell lines	Zhang et al., 2009
9.0	*I. farinosa*	Cycloaspeptide G/$C_{36}H_{43}N_5O_7$ /657.8g/mol		Cytotoxic effects demonstrated on HeLa (GI_{50} 30.2 µM) and MCF7 cells (GI_{50} 15.2 µM).	HeLa and MCF7 human cancer cell lines	Zhang et al., 2009

10	*I. farinosa*	Militarinone A /$C_{26}H_{37}NO_6$ / 459.6		Significant cytotoxic activity against the human lung carcinoma cell line A549, with IC50 value of 1.75 μM. Antimicrobial activity with IC_{50} value >150.0µM. Exhibited neurotrophic effects on PC-12 cells at 10.0µM	Human lung carcinoma cell line A549. PC12 cells. *S. aureus S. pneumoniae* and *C. albicans*	Ma et al., 2011: Schmidt et al., 2002
11	*I. farinosa*	Militarinone B /$C_{26}H_{33}NO_5$ / 439.5		Exhibited moderate cytotoxic against human lung carcinoma cell line A549, with IC_{50} value of > 120.0 μM. Significant antimicrobial activity against *S. aureus S. pneumoniae* and *C. albicans,* IC_{50} value of 2.11., 2.60., & 43.4 μM respectively.	Human lung carcinoma cell line A549. *S. aureus S. pneumoniae* and *C. albicans*	Ma et al., 2011
12	*I. farinosa*	Militarinone E /$C_{26}H_{35}NO_5$ / 441.6		Exhibited significant cytotoxic against human lung carcinoma cell line A549, with IC_{50} value of > 1.59 μM. Moderate antimicrobial activity against *S. aureus S. pneumoniae* and *C. albicans,* IC_{50} value of >150.0 µM respectively.	Human lung carcinoma cell line A549. *S. aureus S. pneumoniae* and *C. albicans*	Ma et al., 2011

13	*I. farinosa*	Militarinone F /$C_{26}H_{35}NO_5$ / 441.25		Exhibited moderate antimicrobial activity with IC_{50} values of >150.00 µM against *S. aureus S. pneumoniae* and *C. albicans.* Comparatively demonstrated moderate cytotoxic activities against human lung carcinoma cell line A549, with IC_{50} value of > 120.0 μM.	Human lung carcinoma cell line A549. *S. aureus S. pneumoniae* and *C. albicans*	Ma et al., 2011
14	*I. fumosorosea / I. cicadae*	Beauvericin/$C_{45}H_{57}N_3O_9$ / 783.9		Potent cytotoxicity towards Human retinoblastoma Y79, HaCaT, HepG2 and HepG2/ADM cells with IC_{50} value 0.4-4.0, 2.7, 3.12 and 2.40 µM. Against, NIH/3T3 and CT-26 murine cell lines with IC_{50} values of 1.2 and 1.4 µM respectively. Inhibitor of PTP1B with IC_{50} value of 0.59 µM.	Human retinoblastoma Y79 cells. HepG2 and HepG2/ADM cells	Cheng et al., 2009; Wang et al., 2014; Heilos et al., 2017; Zhang et al., 2017
15	*I. cicadae*	Cordycepin / $C_{10}H_{13}N_5O_3$ / 251.24		Antitumor effects, it induces apoptosis *via* ADORA2A receptor-p53-caspase-7-PARP pathway in C6 glioma cells. It also inhibits the proliferation and progression of NPC by targeting the	C6 glioma cells; C666-1 cells	Chen et al., 2014; Zhou et al., 2022; Prommaban et al., 2022

				MAPK/ERK and β-catenin pathways at 750µM. Displayed potent anti-wrinkles activity.		
16	*I. cicadae*	Cordysinin A/ $C_{11}H_{18}N_2O_3$ / 226.27		Inhibit the proliferation of human glioma U87-MG and U251 cells with IC_{50} values 14.5 and 29.4 µM respectively.	Human glioma U87-MG and U251 cells	Xuewei et al., 2016
17	*I. cicadae*	Beauvericin A/ $C_{46}H_{59}N_3O_9$ /798.0		Cytotoxic against multidrug resistant HepG2; IC_{50} 2.81µM and HepG2/ADM cells IC_{50} 2.93 µM.	HepG2 and HepG2/ADM cells	Wang et al., 2014
18	*I. cicadae*	Beauvericin E/$C_{41}H_{57}N_3O_9$ /735.9		Cytotoxic against multidrug resistant HepG2; IC_{50} 13.67 µM and HepG2/ADM cells IC_{50} 14.48 µM.	HepG2 and HepG2/ADM cells	Wang et al., 2014
19	*I. cicadae*	Beauvericin J/$C_{45}H_{57}N_3O_{10}$ /799.9		Cytotoxic against multidrug resistant HepG2; IC_{50} 5.04 µM and HepG2/ADM cells IC_{50} 2.67 µM.	HepG2 and HepG2/ADM cells	Wang et al., 2014

20	*I. fumosorosea*	Beauverolide C/$C_{35}H_{49}N_3O_5$ / 591.8	Calmodulin (CaM) inhibitor with affinity constant Kd, value of 0.300 µM	CaM-Biosensor *h*CaM 124C-AF350	Madariaga-Mazón et al., 2015
21	*I. fumosorosea*	Beauverolide F/$C_{33}H_{45}N_3O_5$ / 563.7	Calmodulin (CaM) inhibitor with affinity constant Kd, value of 0.400 µM	CaM-Biosensor *h*CaM 124C-AF350	Madariaga-Mazón et al., 2015
22	*I. fumosorosea*	Beauverolide I	Calmodulin (CaM) inhibitor with affinity constant Kd, value of 0.190 µM	CaM-Biosensor *h*CaM 124C-AF350	Madariaga-Mazón et al., 2015
23	*I. fumosorosea*	Beauverolide Ja	Calmodulin (CaM) inhibitor with highest affinity constant Kd, value of 0.078 µM	CaM-Biosensor *h*CaM 124C-AF350	Madariaga-Mazón et al., 2015
24	*I. fumosorosea*	Beauverolide L /$C_{29}H_{45}N_3O_5$ / 515.7	Calmodulin (CaM) inhibitor with affinity constant Kd, value of 1.660 µM	CaM-Biosensor *h*CaM 124C-AF350	Madariaga-Mazón et al., 2015
25	*I. fumosorosea*	Beauverolide M	Calmodulin (CaM) inhibitor with affinity constant Kd, value of 3.440 µM	CaM-Biosensor *h*CaM 124C-AF350	Madariaga-Mazón et al., 2015

26	*I. fumosorosea*	Beauverolide N/$C_{27}H_{41}N_3O_6$ /503.6		Calmodulin (CaM) inhibitor with affinity constant Kd, value of 1.740 µM	CaM-Biosensor *h*CaM 124C-AF350	Madariaga-Mazón et al., 2015
27	*I. fumosorosea*	Fumosorinone/$C_{29}H_{35}NO_5$ /477.6		Non-competitive inhibitor of PTP1B with IC_{50} value of 14.04 µM. Antidiabetic activity.	p-nitrophenyl phosphate (pNPP) as substrate. Type 2 diabetic KKAy mice	Liu et al., 2015a: Liu et al., 2015b.
28	*I. fumosorosea*	Fumosorinone A		Inhibitor of PTP1B with IC_{50} value of 3.24 µM.	p-nitrophenyl phosphate (pNPP) as substrate	Zhang et al., 2017
29	*I. fumosorosea*	CAF-603/ $C_{15}H_{26}O_2$ / 238.37		Cytotoxicity against MDA, MCF-7, SKOV-3, Hela, A549, and HepG2, with an IC50 of 0.1–6.0 µg/mL.	Tumor cell lines: MDA, MCF-7, SKOV-3, Hela, A549, and HepG2	Zhang et al., 2009; Weng et al., 2019
30	*I. tenuipes*	Isariotin A/ $C_{21}H_{33}NO_7$ / 411.5		Moderate activity against the *M. tuberculosis* $H_{37}Ra$ with the MIC value of 486 µM	*Mycobacterium tuberculosis*	Haritakun et al., 2007
31	*I. tenuipes*	Isariotin B /$C_{21}H_{31}NO_8$ / 425.5		Not specified	Not specified	Haritakun et al., 2007

32	*I. tenuipes*	Isariotin C/$C_{21}H_{31}NO_7$ / 409.5		Moderate activity against the *M. tuberculosis* $H_{37}Ra$ with the MIC value of 488 µM	*Mycobacterium tuberculosis*	Haritakun et al., 2007
33	*I. tenuipes*	Isariotin D/$C_{19}H_{29}NO_6$ /367.4		Moderate activity against the *M. tuberculosis* $H_{37}Ra$ with the MIC value of 544 µM	*Mycobacterium tuberculosis*	Haritakun et al., 2007
34	*I. tenuipes*	Isariotin E /$C_{21}H_{31}NO_5$/ 377.5		Not evaluated due to sample limitation by the authors	Not applicable	Bunyapaiboonsri et al., 2009
35	*I. tenuipes*	Isariotin F/$C_{21}H_{32}ClNO_5$ /413.9		Inhibition of *Plasmodium falciparum* K1 with an IC50 value of 5.1 μM and cytotoxic activities against cancer cell lines (KB, BC, and NCI-H187) and nonmalignant (Vero) cells with respective IC50 values of 15.8, 2.4, 1.6, and 2.9 μM.	*Plasmodium falciparum* K1, Cancer cell lines KB, BC, and NCI-H187.	Bunyapaiboonsri et al., 2009
36	*I. tenuipes*	Isariotin G/ $C_{21}H_{32}ClNO_6$ /429.9		Antimalarial activity; IC_{50} = 5.51 µg/ml. Cytotoxic activities against KB, MCF-7, NCIH187 and Vero cells with respective	*Plasmodium falciparum* K1, and three cancer cell lines (KB, MCF-7, and	Bunyapaiboonsri et al., 2011

				IC_{50} = 6.19, 9.21, 22.18, and 26.97 µg/ml.	NCIH187) and nonmalignant Vero cells	
37	*I. tenuipes*	Isariotin H /$C_{21}H_{30}ClNO_5$ /411.9		Antimalarial activity; IC_{50} = >10.0 µg/ml. Cytotoxic activities against KB, MCF-7, NCIH187 and Vero cells with respective IC_{50} = 8.26, 18.56, 37.36, and 22.64 µg/ml.	*Plasmodium falciparum* K1, and three cancer cell lines (KB, MCF-7, and NCIH187) and nonmalignant Vero cells	Bunyapaiboonsri et al., 2011
38	*I. tenuipes*	Isariotin I/$C_{21}H_{30}ClNO_6$ /395.9		Antimalarial activity; IC_{50} = 3.81 µg/ml. Cytotoxic activities against KB, MCF-7, NCIH187 and Vero cells with respective IC_{50} = 5.12, 5.37, 2.25, and 26.85 µg/ml.	*Plasmodium falciparum* K1, and three cancer cell lines (KB, MCF-7, and NCIH187) and nonmalignant Vero cells	Bunyapaiboonsri et al., 2011
39	*I. tenuipes*	Isariotin J/$C_{22}H_{33}NO_6$ / 407.5		Antimalarial activity; IC_{50} = 2.19 µg/ml. Cytotoxic activities against KB, MCF-7, NCIH187 and Vero cells with respective IC_{50} = 2.98, 4.37, 44.86, and 1.77 µg/ml.	*Plasmodium falciparum* K1, and three cancer cell lines (KB, MCF-7, and NCIH187) and non-malignant Vero cells	Bunyapaiboonsri et al., 2011

40	*I. tenuipes*	Penostatin A/ $C_{22}H_{32}O_3$/ 344.5	Protein phosphatase 1B inhibitors, IC_{50} value = 15.87µM.	*p*-nitrophenyl phosphate (*p*NPP) as a substrate	Chen et al., 2014
41	*I. tenuipes*	Penostatin B/ $C_{22}H_{32}O_3$ / 344.5	Protein phosphatase 1B inhibitors, IC_{50} value = 33.65µM.	*p*-nitrophenyl phosphate (*p*NPP) as a substrate	Chen et al., 2014
42	*I. tenuipes*	Penostatin C/ $C_{22}H_{30}O_2$ /326.5	Protein phosphatase 1B inhibitors, IC_{50} value = 0.37 µM	*p*-nitrophenyl phosphate (*p*NPP) as a substrate	Chen et al., 2014
43	*I. tenuipes*	Penostatin J /$C_{22}H_{30}O_3$ /342.5	Protein phosphatase 1B inhibitors, IC_{50} value = 12.53 µM	*p*-nitrophenyl phosphate (*p*NPP) as a substrate	Chen et al., 2014
44	*I. tenuipes*	Ergosterol Peroxide/ $C_{28}H_{44}O_3$/428.6	Antioxidant activity with 46.3% lipid peroxidation 690µM concentration. Induces apoptosis via activation of death receptor 5 and caspase 8/3 in DU 145 prostate cancer cells.	Rat liver microsomes, DU 145 prostate cancer cells	Kim et al., 1999; Han et al., 2014
45	*I tenuipes*	TK-57-64A/ $C_{22}H_{35}NO_6$ / 409.5	Inactive towards KB, BC, and NCI-H187 and nonmalignant Vero cells	KB, BC, and NCI-H187 and nonmalignant Vero cells	Bunyapaiboonsri et al., 2009
46	*I japonica*	Tenuipesine A /$C_{17}H_{24}O_6$ / 324.15	No biological activity tested.	—	Kikuchi et al., 2004a

47	*I japonica*	Spirotenuipesine A / $C_{15}H_{22}O_4$ / 266.33		Potent neurotrophic factor biosynthesis activity in glial cells at 1µM.	1321N1 human astrocytoma cells and PC-12 cells	Kikuchi et al., 2004b
48	*I japonica*	Spirotenuipesine B /$C_{15}H_{22}O_5$ /282.33		Potent neurotrophic factor biosynthesis activity in glial cells at 1µM.	1321N1 human astrocytoma cells and PC-12 cells	Kikuchi et al., 2004c
49	*I japonica*	Paecilomycine A/ $C_{15}H_{22}O_4$ / 266.14		Paecilomycine A enhanced the extension of neurite outgrowth of PC-12 cells at 10nM	1321N1 human astrocytoma cells and PC-12 cells	Kikuchi et al., 2004c
50	*I japonica*	Paecilomycine B/ $C_{15}H_{22}O_5$ / 282.14		Displayed no biological activity at tested concentration of >1µM.	1321N1 human astrocytoma cells and PC-12 cells	Kikuchi et al., 2004c
51	*I japonica*	Paecilomycine C/ $C_{15}H_{20}O_4$ / 264.13		No biological activity tested due to insufficient amount of sample	—	Kikuchi et al., 2004b
52	*I. japonica*	Hanasanagin/$C_{15}H_{23}N_7O_5$ / 381.39		Antioxidant activity, EC_{50} value of 8.1 µM.	DPPH radical,	Sakakura et al., 2005.
53	*I japonica*	(3R,6R)-4-Methyl-6-(1-methylethyl)-3-phenylmethyl-perhydro-1,4-oxazine-2,5-dione/ $C_{15}H_{19}NO_3$ /261.32		Induces apoptosis of HL-60 cancer cell lines at 5.0-100 µg/ml.	Human HL-60 leukemia cell	Oh et al., 2002

54	*I. japonica*	4-Acetyl-12,13-epoxy-9-trichothecene-3,15-diol/ $C_{17}H_{24}O_5$ / 308.4		Cytotoxic against human cancer cells HL-60, U-937, HeLa, MCF-7, and HepG2 with respective IC50 value = 10, 22, 45, 53 and 170 nmol/l.	Human cancer cell lines; HL-60, U-937, HeLa, MCF-7, and HepG2	Oh et al., 2001; Pae et al., 2003
55	*I. japonica*	Naturido/ $C_{26}H_{37}N_4O_{10}$-H/ 566.2588		Glia–neuron modulator at 0.03 to 1.0 µM concentration. Hair anti-ageing effect.	Hippocampal neurons, Senescence-accelerated mice (SAMP8).	Ishiguro et al., 2021
56	*I. felina*	Isarfelin A/ C36H55O7N5/669		The IC_{50} value of antifungal activity was 3.1µg/mL against *R. solani.* Insecticidal activity with EC_{50} value = 75.99µg/mL	*R. solani* and *Leucania separata*	Guo et al., 2005
57	*I. felina*	Isarfelin B / C35H53O7N5 /655		The IC_{50} value of antifungal activity was 3.1µg/mL against *R. solani.* Insecticidal activity with EC_{50} value = 75.99µg/mL	*R. solani* and *Leucania separata*	Guo et al., 2005

3. MATERIALS AND METHODS

3.1. Chemical and Reagents

The solvent methanol (99.9%) was of HPLC grade from HiMedia (India). Beauvercin, gallic acid, tannic acid and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO, USA) and SD Fine Chemical Limited (India) respectively. Ferrozine, EDTA (Ameresco), potassium ferricyanide (HiMedia), ferric chloride (SRL), trichloroacetic acid (SRL 90544) and 2, 4, 6-tri [2-pyridyl]-s-triazine (Sigma) used were of the highest purity grade that was obtained from different vendors. Dulbecco's modified Eagle's medium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution were purchased from HiMedia, India. All other reagents and solvents used in this study were of analytical grade and obtained from reputed Indian suppliers. Glassware used in all experiments were washed in cleaning solution (potassium dichromate 100 gm: sulfuric acid 500ml: water: 1000 ml) to remove contaminating trace elements by soaking for twelve hours, followed by rinsing with tap water several times and finally in distilled water. For routine cleaning of glassware, cleaning solution HiSpark from HiMedia, India was used. Water was treated in a Milli-Q water purification system.

3.2. Study sites

The field trip for the collection of an entomopathogenic fungi were conducted in different ecological regions of Darjeeling Hills between the first week of June and the first week of October in 2016 and 2017. The entomopathogenic fungal samples were collected from seven sites throughout the temperate forest of Darjeeling Hills. The major collection sites noted in the present study covers mainly the hilly regions of Darjeeling District which are rich in natural vegetation. Search of entomopathogenic fungi was carried out in all possible microhabitats in the natural forest systems such as moss-

covered trunk of forest trees, underside of leaves and the leaf litter on forest floor. However, special attention was always given to sites close to rivers and springs for their high humidity. Natural forests dominated by *Castanopsis* sp. were the most abundant collection sites. Ecological records of the collecting sites, like climate, vegetation and soil texture, was critical for the collection of fungal samples (Lama, 2004). The collecting localities were divided into five main regions: Lebong; Jalapahar: Tiger Hill; 3rd Mile; 6th mile; Takdah Cantonment: and Ragayrung.

3.3. Collection of *Isaria* spp.

Mycoses insect cadavers were carefully photographed covering the natural habitat of the entomopathogenic fungi *Isaria* spp along with the disease symptoms and vegetation pattern of the location. Photographs are linked to each fresh specimen by a unique reference number (Young, 2005). Precautions were taken to avoid collection of over-mature; premature, or badly insect-damaged specimens because some characters are lost in damaged specimens (Hyde et al., 2010). Wherever possible, more than one specimen was collected. This is generally done for replication a purpose which is necessary to confirm the accuracy of the size, availability and morphology of the fungal structures (Senanayake et al., 2020). Important morphological, ecological and physiological data were noted on the field site using the fresh material. Once the on field preliminary investigation is completed the specimen was collected and cleaned gently using soft paint brushes in the field and placed in an individual presterilized transparent plastic container lined with sterilised dry tissue paper or cotton wool so as to avoid moisture accretion and deterioration followed by growth of unwanted moulds. The containers containing *Isaria* samples were properly field labelled comprising certain tentative information about the samples.

Table 3.1 The sites of sample collection of the genus *Isaria* spp., from different ecological regions of Darjeeling hills.

Strain No.	Scientific Name	Date of Collection	Location	Latitude (N)	Longitude (E)
IDAR-01	***Isaria javanica* Frieder & Bally**	16/08/2016	Mungpoo, Darjeeling, India	26º58′26.55″	88º20′22.99″
IDAR-02	***Isaria farinosa* (Holmsk). Fr.**	12/09/2016	PNHZP, Zoo, Darjeeling, India	27º03′31.00″	88º15′15.86″
IDAR-03	***Isaria tenuipes* Peck**	7/09/2016	3rd Mile, Darjeeling, India	27º0′39.61″	88O17′34.6″
IDAR-04	***Isaria tenuipes* Peck**	12/08/2017	Ragayrung, Darjeeling, India	27º2′9.62″	88º15′45.63″
IDAR-05	***Isaria farinosa* (Holmsk). Fr.**	16/09/2017	Jalapahar, Darjeeling, India	27º3′34.48″	88º15′3″
IDAR-06	***Isaria tenuipes* Peck**	21/08/2016	Lebong, Darjeeling, India	27º4′27.22″	88º13′53.45″
IDAR-07	***Isaria tenuipes* Peck**	23/09/2017	6th Mile, Darjeeling, India	27º3′41.57″	88º16′35.51″
IDAR-08	***Isaria farinosa* (Holmsk). Fr.**	12/10/2017	Tiger Hill, Darjeeling	26º59′48″	88º17′40″
IDAR-09	***Isaria fumosorosea* Wize**	23/10/2017	Mongmaya, Darjeeling, India	27º03′36.00″	88º25′48.00″

The specimens lacking synnemata were brought to the laboratory and were examined under an inverted microscope to confirm fungal infection. A number of the collected specimens could not be reliably identified to any arthropod group. Often these were small larval stages or in an advanced state of decomposition and lacking any readily identifiable features. The specimen was handled carefully and processed as quickly as possible for axenic culture. The, collected specimens were stored at 4°C for up to a week wherever necessary before isolation of cultures.

3.4. Fungal isolation into pure culture

One of the essential steps needed to be performed while working artificial fungal cultures is to separate the disease-producing organism from the mixed cultures (mostly bacteria and fungi) and to grow the desired fungus in pure culture (Petit, 1895). To achieve this, agar plate cultures were made with manual nutrient agar preparation. In the present research, potato dextrose agar (PDA) has been used, as preliminary studies showed that all the isolates were capable of germination upon inoculation of serial diluted conidia. In most of the cases, a pure culture was directly obtained from the insect by touching a flamed inoculating needle containing a loopful of streptomycin solution (30µg/ml) to the spore bearing synnemata and then touching gently onto a potato dextrose agar media under sterile conditions. The conidia were inoculated into three equidistant points on 90 cm Petri plate containing 12 ml sterile PDA media in triplicate at 20°C. In majority of the cases, this technique often leads to the isolation of pure cultures. However, in some cases, i.e., in case of heavy contaminations a loopful of spores were serially diluted in sterile peptone water up to the level of 10^{-3} dilutions and the diluted contents were poured into a sterilized PDA plates followed by subculturing of the desired fungal colony. Culture plates were examined every 24 hours after inoculation to confirm germination of the conidia and also to check for the development of contaminating fungi.

For short term use, the cultures were stored as agar discs in sterile distilled water at 4°C. Additionally, stock cultures were accordingly maintained on PDA slants and were held in storage at 4°C, until used (Alexopoulus & Beneke, 1964).

3.5. Morphological observation of the isolated fungi

Isolated fungi were microscopically observed to determine if they were the target fungi. For preliminary identification of fungal species, conidiogenous structures were mounted in lacto phenol cotton blue (staining solution for fungi) and the fungi were identified using the micro-morphological characters and taxonomic keys (Luangsa-ard et al., 2007; Lacey, 2012). Twenty-five conidia were randomly measured from each spontaneous isolate Furthermore, the cultured mycelia on potato dextrose agar plates that were maintained for a few weeks at 20°C were observed in terms of colony color and hyphal morphology. A piece of mycelium from each isolate was transferred onto a glass slide with a drop of lactic acid (60%) without color dye and lactophenol blue were used as the mounting media. The preparation was observed under a binocular research microscope with high magnification (Olympus CX21i) for precise morphological observation at genus level.

3.6. DNA sequencing analysis of isolated fungi

To confirm the identity of fungal isolates as *Isaria* spp. preliminary identifications were complemented by a GenBank BLAST search using ITS nrDNA sequences. For molecular identification, genomic DNA was extracted from pure axenic cultures grown on potato dextrose agar (PDA) for one week at 25°C, by a simple and rapid DNA extraction protocol (Aamir et al., 2015) using FasPrep 24 tissue homogenizer (MP Biomedical GmbH, Eschwege, Germany). The DNA was resuspended in 50 µl TE (Tris-EDTA; pH= 8.0) buffer and analyzed quantitatively as well as qualitatively by 1 % agarose gel electrophoresis. The nuclear ribosomal internal transcribed spacer (ITS) gene

was amplified by PCR using primer pair ITS4 & ITS5 (White et al., 1990) in a reaction volume of 50 µl. The reaction mixture contained 32 µl PCR grade water (Sigma, St. Louis, MO, USA), 5µL PCR buffer (10×), 4µL of 10 mMdNTPs mix (Sigma-Aldrich), 1 µl of each primer (20 pmol/µl), 1 µL (5 U/µl) of Taq polymerase (Sigma-Aldrich) along with 20–50 ng of template DNA. Amplification was done using an Applied BiosystemsProFlex PCR System (Applied Biosystems, Waltham, MA, USA) following standard cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 90 seconds, primer annealing at 52°C, primer extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The amplified products were analyzed on 1.2% agarose gel containing ethidium bromide. The PCR products were purified using an Axygen PCR clean-up kit (Axygen Scientific, CA, USA). Sequencing reactions were performed with a BigDye terminator cycle sequencing kit, ver. 3.1/1.1 (Applied Biosystems). All the sequencing reactions were purified and analyzed on an ABI Avant 3100 automated DNA sequencer (Applied Biosystems).

Table 3.2 PCR and sequencing primers used in this study.

Region	Primer	Sequence	Reference
ITS	ITS4	5' TCCTCCGCTTATTGATATGC 3'	White *et al.,*1990
ITS	ITS5	5'-GAAGTAAAAGTCGTAACAAGG	White *et al.,* 1990

Table 3.3 Composition of nutrient media used for determination of mycelial growth characterization of *Isaria* spp., from Darjeeling Hills

	Composition of media (g/L)													
Nutritional reagents	**PDA**	**SDA**	**MEA**	**CMA**	**OMA**	**MPDA**	**CDA**	**HAM-1**	**LAM**	**MSA**	**TA**	**GPA**	**MCM**	**HAM-2**
Dextrose	20	20	20			10		10				10	20	50
Maltose									10					
Asparagine									2					
Sucrose							30							
Malt extract			20						3	100		15		
Potato infusion	200													
Oat meal					60									
Corn meal				17										
Peptone		5	6			5						15	2	
$MgSO_4.7H_2O$						0.5	0.5	0.5	0.5				0.5	0.5
KH_2PO_4						1		0.5	0.5				0.46	
K_2HPO_4							1						1	

KNO_3								2	2					
$NaNO_3$							3							2
NaCl										100				
$CaCL_2$														0.10
KCl							0.5							
$FeSO_4.7H_2O$							0.01							
Yeast extract												10	2	
Tryptone											20			
Agar	20	20	20	20	20	20	20	20	20	20	20	20	20	
H_2O	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

CDA: Czapek Dox Agar., CMA: Corn Meal Agar, GP: Glucose Peptone Agar, HAM-1`: Hennerberg Agar., HAM-2: Hopkins Agar., ME: Malt Extract Agar, MCM: Mushroom Complete Medium, MPDA: Martin Peptone Dextrose Agar Medium, MSA; Malt Salt Agar, NA: Nutrient Agar, OMA: Oat Meal Agar, PDA: Potato Dextrose Agar, SDA: Sabouraud Dextrose Agar, TA: Tryptone Agar

3.7. Growth and long-term preservation of fungal cultures

For long term storage, the pure culture was incubated at 20°C in an incubator under dark conditions with periodic observation under the inverted microscope to check for mycelial growth and unwanted contamination. Once the axenic culture reaches a diameter of 30 mm, they were cut into small sized agar discs ($9mm^2$) aseptically using sterile corkborer. Mature healthy cultures agar discs (3-7 per vial) were then transferred in 30 ml Borosil glass vial covered by 15 mm of mineral oil (liquid paraffin specific gravity 0.830-0.890). The mineral oil vials were thoroughly sterilised by autoclaving twice, 36 hours apart, at 121°C for 20 minutes. The storage vials were stored at cool room temperature for long term storage. All freezing culture was stored at Department of Microbiology, St Joseph's College Darjeeling. The cultures were later recovered by draining excess oil from inoculum as possible and more than one subculture was made so as to reduce the adhering oil prior to any experimental work. However, the purity of the cultures was ensured periodically by checking culture plates under the inverted microscope regularly for mycelial growth and contamination from undesired microbes (Alexopoulus & Beneke, 1964).

3.8. Preservation of specimen's herbarium or spontaneous specimen

After proper aseptic isolation of the living culture the clean fruit body of spontaneous specimens were air dried. Silica gel (blue) was taken in glass bottles capped with aluminium foil and sterilised in an oven for 3 hours at 180°C. After sterilisation the silica gel crystal were allowed to cool down and filled aseptically ($1/3^{rd}$ volume) to a 70 % alcohol presterilized plastic container. The air-dried specimens were aseptically transferred to a container containing sterilised dry coarse self-indicating blue silica gel. The specimen containers were then sealed with cello tape and preserved at 4°C (Alexopoulus & Beneke, 1964).

3.9. Cultural techniques.

3.9.1 Media used for the cultivation of mycelia

Preliminary studies showed that potato dextrose agar medium was suitable for the isolation and growth of the isolates under study. Therefore, it was used as the basic culture medium. Stock cultures were accordingly maintained and preserved on potato dextrose agar at 4°C until use for the following plate inoculation experiment. The liquid medium used for evaluation of the mycelial growth and biomass yield for biochemical assay was modified by the addition of 0.01% yeast extracts to potato dextrose broth. In the present study following media were used for the isolation of genus *Isaria* members, characterization of the isolates and production of biomass for carrying out experiment on biological activity. All the media used were sterilised in an autoclave at 15, p.s.i., for 15 minutes. Compositions of the fifteen-culture media used based on Alexopoulus & Beneke, 1964; Booth, 1971 and Shrestha et al., 2006 has been presented in Table 3.3.

3.9.2. Preparation of the inoculum

Each strain was inoculated onto basic potato dextrose agar [2% (w/v); 20ml] medium in a 90 mm Petri dish and was cultured for 10 days at 20°C in the dark. Mycelial disks with a diameter of 5mm were punched out aseptically from the actively growing tip of mycelia culture with a presterilized corkborer. Then three-point inoculations were carried out in 90 mm Petri dish by placing the mycelial discs at an equidistant position to each other on a given nutrient media, followed by incubation at a given conditions (mentioned in the text). For biomass yield, each mycelial disk was inoculated into 30 ml basic medium in a 100-ml Erlenmeyer flask and grown in a static culture (seed culture) at 25°C in the dark for 14 days. Hereafter, for large scale mycelial growth each primary seed culture was transferred into a 2 L Erlenmeyer flask containing 300 ml of the basic culture medium, and incubated at 20°C for

21 days under liquid static conditions. The experiment was carried out in three biological replicates for maximum extractions.

3.9.3. Incubation

All the basic nutritional and growth experiments for biomass generation were incubated in a constant temperature at 20°C, in an incubator without artificial illumination for five, ten and fifteen days. Since rather low temperature and dark/ light condition prove to be necessary for the fungus to grow well in artificial liquid static culture. In experiments on adequate mycelial biomass generation the cultures were incubated on the bench in the laboratory, where the temperature was 18-20°C in summer and where the cultures were exposed to the natural day light.

3.9.4. Determination of the mycelial growth in culture media

Radial growth of each fungal colony was calculated from the mean of three diameters intersecting at right angles in plate cultures to the nearest mm. To evaluate the mycelial growth in various liquid media, 2-5 mycelial discs were inoculated into 30ml liquid medium in a 100-ml Erlenmeyer's flask and grown in a static culture at 20°C for 14 days in the dark. Each condition was tested in four replications. After cultivation, the mycelium was collected on filter paper (Whatman No. 2), rinsed free of nutrients with sterilised cold distilled water and then gently squeezed to remove excess water. Each mycelium was then transferred on a preweighed piece of aluminium foil and dried in a drying oven for 2 days at 70°C, which was sufficiently long to dry the amounts of mycelia obtained to constant dry weight before being weighed. The average mycelial growth was calculated from the value of four replicate flasks for each medium and analysed statistically using ANOVA with $P < 0.05$ (Shrestha et al., 2006).

3.10. Effects of physical environmental and nutritional factors on vegetative growth

3.10.1 Effect of Temperature

The influence of temperature on the growth of nine isolates was studied using petri dishes each containing - approximately 20 ml. of 2% potato dextrose agar. To screen the optimum temperature for the mycelial growth of these isolates, 5 different temperatures (12, 16, 20, 24, and 30°C) were studied. Three mycelial discs were inoculated onto agar media as mentioned earlier. The medium was adjusted to pH 7.0 and incubated for 14 days at 12, 16, 20, 25 and 30°C separately. Radial growth of mycelia on each Petri dish was measured as described previously. To calculate final mean value of mycelial growth of each strain three colony replications were used (Shrestha et al., 2006).

3.10.2. Effect of pH

The ability of nine isolates of *Isaria* to grow on different hydrogen ion concentrations of the medium was studied using the potato dextrose agar medium. The medium was adjusted to pH 4, 5, 6, 7, 8 and 9 with the addition of 1 N NaOH or HCl before autoclaving, and incubated for 14 days at 20°C. The measurement of mycelial growth was performed following same technique as mentioned earlier (Shrestha et al., 2006).

3.10.3. Effect of Carbon Source

The ability of nine isolates of *Isaria* to use different organic compounds as sources of carbon and energy was tested in a basal nutrient solution of simple composition.

Peptone	2.0g
KH_2PO_4	0.5g
$MgSO_4.7H_2O$	0.5g
Distilled water	1000.ml

The above basal medium was supplemented separately with different carbon-source at the rate of 1% weight/volume and organic acids at the rate of 0.01% respectively. The carbon source included dulcitol, D- glucose, fructose, galactose, mannose, raffinose, sodium acetate, starch,

trehalose and xylose. The organic acid carbon source included citric acid, fumaric acid, maleic acid, oxalic acid and tartaric acid. All media for carbon nutrition determination were prepared into two parts, the desired carbon source being autoclaved separately and afterwards added aseptically to the basal medium. Basal medium without carbon served as control (Shrestha et al., 2006).

3.10.4. Effect of Nitrogen Source

To study nitrogen source utilization variety of nitrogen sources was added to the basal media with slight modification of Shrestha et al., 2006. The concentration of each nitrogen source added separately in the media was set at 0.5% (w/v) level. The basal medium employed had the composition. The nitrogen source included casein, peptone, yeast extract, urea, asparagine, cysteine, glycine, glutamic acid, calcium nitrate, potassium nitrate, ammonium sulphate, sodium nitrate. Basal medium without nitrogen served as control.

Glucose	10.0g
KH_2PO_4	0.5g
$MgSO_4.7H_2O$	0.5g
Thiamine hydrochloride	500g
Distilled water	1000.0ml

3.10.5. Effects of C/ N Ratios on Mycelial Growth

A defined basal salt medium had a composition.

$NaNO_3$	2.0g
KH_2PO_4	0.5g
$MgSO_4.7H_2O$	0.5g
$CaCl_2$	0.3g
$FeSO_4$	0.01g
Distilled water	1000.0ml

This basal salt medium was used in all C: N ratios medium preparations. Glucose and proteose peptone (SRL-41406) were used as the carbon and nitrogen sources, respectively. The medium was autoclaved for 15 minutes at $121^{O}C$. Stock solutions of glucose (100 g l^{-1}) and proteose peptone (10 g l^{-1}) were autoclaved separately. To this sterilized basal salt media glucose and proteose peptone was added separately as required to achieve various C: N ratios. The C: N ratios were calculated as carbon and nitrogen present in the glucose (40% carbon) and proteose peptone (14% nitrogen). The basal salts medium was mixed with glucose at a rate of 1, 2, 3, 4, 5 and 6 % Carbon l^{-1}. Finally, the C: N ratios (Glucose versus Proteose peptone) were adjusted to 10:1, 20:1, 30:1, 40:1, 50:1 and 60:1. The basal media were adjusted to pH 7 before autoclaving by adding 1 N sodium hydroxide or 1 N hydrochloric acid. After incubation on the media for 14 days at 25°C, the colony diameter was measured to nearest mm (Shrestha et al., 2006).

3.10.6. Effect of Macro and micro nutrients

Macro elements: In this experiment, method of Sung et al., 2010 was followed with slight modifications. To determine the effect of potassium, (KH_2PO_4) calcium ($CaCl_2$), sodium (NaCl), Magnesium ($MgSO_4.7H_2O$) were added separately to Martin's peptone dextrose agar (MPDA) at a rate of 0.05% (w/v). Trace elements: Similarly, Iron ($FeSO_4.7H_2O$), Manganese ($MnSO_4.7H_2O$), Copper ($CuSO_4.5H_2O$) and Zinc ($ZnSO_4.7H_2O$) were added as above at the rate of 0.01%. MPDA without $MgSO_4.7H_2O$ and KH_2PO_4 was used as control (Shrestha et al., 2006).

3.10.7. Effect of Vitamins

The simple basal media was used to study the effect of vitamins and had the following composition:

Glucose	10.00gms
Asparagine	1.00gms
KH_2PO_4	0.5gms
$MgSO_4$	0.5gms
Distilled water	1000.00ml

The vitamins included riboflavin, pyridoxine, nicotinic acid, biotin, ascorbic acid and pyridoxine was sterilized aseptically using a micro-syringe filter (0.22 micron) and subsequently added to a sterilized basal media at a rate of 0.5µg/ml and inositol at a rate of $5mgl^{-1}$. The basal media without vitamin was used as a control (Shrestha et al., 2006).

3.11. Inoculum Preparation and mycelial growth for biomass yield

For seed culture, the agar was cut into plugs (6mm in diameter with the help of cork-borer) and three plugs from the growing tip was inoculated into 250 ml Erlenmeyer flasks containing 25 mL (n=3) of improved potato dextrose broth (PDB supplemented with 0.01g yeast extracts per liter). The seed culture flask was incubated on a rotary shaker at 100 rpm and 25°C for 5 days. Hereafter, for large scale mycelial growth each primary seed culture was transferred into a 2 L Erlenmeyer flask containing 3000 ml of the seed culture medium, and incubated at 25 °C for 30 days under liquid static conditions. The experiment was carried out in three biological replicates for maximum extractions (Shrestha et al., 2006).

3.11.1 Preparation of the fungal extract

After 12 days of liquid static growth, the mycelium pellets were separated by filtration with muslin cloth and washed thoroughly with deionized H_2O. The mycelium pellet was oven-dried at $60^{O}C$ temperature for three days and then pulverized. The pulverized fungal material (10 g) was extracted with analytical grade methanol and double distilled water (70:30) using a magnetic stirrer at a low speed for 16 hours, then the mixture was centrifuged at 3000 × *g for* ten minutes and the supernatant was decanted. The decanted methanol extract was filtered

twice through the Whatman No. 1.0 filter paper and the process was repeated three times to ensure no mycoconstituents were remained in the residues. All the methanol extracts obtained in each phase were combined in a round bottom flask. The methanol was then removed by rotary evaporation to dryness under reduced pressure to produce aqueous methanol extract of nine isolates (IDAR-01 to IDAR-09 ME). The IDAR-01to IDAR-09 ME residue was redissolved in methanol at a concentration of 100 mg/mL to make a stock solution. The stock solution was sterilized by filtration through a 0.2-micron syringe filter prior to its use for antimicrobial and antiproliferative assays. The experiment was performed using methanol (vehicle) control at the same dilution as a negative control. The % yield of Fr-ME extract was calculated using the formula as: Percentage yield = (crude dried extract/dried sample) x 100 % (Awang et al., 2021).

3.12. Qualitative Analysis of the Phytochemicals of the mycelia

Preliminary phytochemical screening was carried out on the highly concentrated methanolic extract solution of pulverized mycelia from nine isolates using standard procedures as described by Ejikeme et al., (2014), Ezeonu and Ejikeme (2016), Harborne, (1973), Sofowora (1993), and Trease and Evans (1989).

3.12.1. Test for Tannins

To 5 ml of the extract, 3 drops of 0.1% ferric chloride was added gently. A brownish green or a blue-black colouration was recorded as a positive test (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016).

3.12.2. Test for Phlobatannins

Each extract (10 ml) of each powder sample was boiled with 5 ml of 1% aqueous hydrochloric acid. Deposit of red precipitate was indicative of a positive test (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Trease & Evans, 1989).

3.12.3. Test for Saponins

A mixture of distilled water (5 ml) and extract (10 ml) was agitated vigorously for a stable persistent froth. The formation of emulsion on addition of three drops of olive oil was indicative of a positive result (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Trease & Evans, 1989).

3.12.4. Test for Steroids

Extract (5 ml) was added 2 ml acetic anhydride followed with 2 ml of concentrated sulphuric acid. A violet to blue or green colour change in sample(s) indicates the presence of steroids (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Trease & Evans, 1989).

3.12.5. Test for Terpenoids

Chloroform (2ml) was mixed with concentrated sulphuric acid (3ml) which was then carefully added to 5 ml of each extract to form a layer. The presence of a reddish-brown colouration at the interface indicated positive results for the presence of terpenoids (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Trease & Evans, 1989).

3.12.6. Test for Flavonoids

10 ml of concentrated extract was hydrolyzed with 5 ml of 1.0 M dilute ammonia solution followed by the addition of 5 ml of concentrated sulphuric acid. Appearance of yellow colouration which disappeared on standing showed the presence of flavonoids (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Trease & Evans, 1989).

3.12.7. Test for Alkaloids

The extract was made alkaline using 5 ml of 28% ammonia solution (NH_3) in a separating funnel. Equal volume of chloroform (5.0 ml) was used in further solution extraction in which chloroform solution was extracted with two 5 ml portions of 1.0 M dilute sulphuric acid. This final acid extract was then used to carry out the following test: 0.5 ml of

Dragendroff's reagent (Bismuth potassium iodide solution) was mixed with 2 ml of acid extract and precipitated orange colour indicated the presence of alkaloid (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Harborne, 1973; Trease & Evans, 1989).

3.12.8. Test for phenols

1 ml of concentrated methanolic extract was taken and 2 ml of distilled water and 10% ferric chloride was added in it. The formation of green or blue color indicated the presence of phenols (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Harborne, 1973; Trease & Evans, 1989).

3.12.9. Test for quinones

A volume of 1 ml of each isolate was allowed to react with 1 ml concentrated sulphuric acid. Appearance of red color indicated the occurrence of quinones (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Harborne, 1973; Trease & Evans, 1989).

3.12.10. Test for Glycoside

Keller Killanis' test: To 1 ml of each fungal extract, 1 ml glacial acetic acid was added and left to cool down. After cooling two drops of $FeCl_3$ were added gently and 2 ml of concentrated H_2SO_4 along the side walls of test tube was dispensed carefully. Development of reddish brown colored ring at the intersection of two layers indicated the presence of glycosides (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Harborne, 1973; Trease & Evans, 1989).

3.12.11. Test for anthraquinones

To 1 ml of each sample, hydrochloric acid diluted to 2% was added. The appearance of red color indicated the presence of anthraquinone presence (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Harborne, 1973; Trease & Evans, 1989).

3.12.12. Test for proteins

Xanthoproteic test: 1 ml of each concentrated methanolic extract was treated with few drops of conc. nitric acid. Presence of proteins in test samples was indicated by the formation of yellow color.

Biuret test: An amount of 0.5 ml of each test solution was taken and equal volume of sodium hydroxide solution (40%) was gradually added to it. After that few drops of 1% $CuSO_4$ solution was added. Appearance of violet color in test samples indicated the presence of protein (Ejikeme et al., 2014; Ezeonu and Ejikeme, 2016; Harborne, 1973; Trease & Evans, 1989).

3.13. Determinant of non-enzymatic antioxidant components

3.13.1. Determination of total phenolic content

Total soluble phenolics in the extracts were assessed using the method described by Singleton and Rossi (1965) with slight modifications. Briefly the fungal extract (0.5ml) was mixed with 0.5 ml of 1000-fold Folin–Ciocalteu phenol reagent and dark incubated for 5 min at room temperature (RT), after that 1 ml of freshly prepared 2% Na_2CO_3 solution was added and mixed well. After 10 min of dark incubation, the absorbance of the blue color that developed was read at 730 nm using UV-Visible spectrophotometer. Gallic acid (5- 20 µg/ml) was used as the phenolic standard and the concentrations of total phenolic compounds were presented as GAE using a standard curve of gallic acid standard curve. All tests were carried out in triplicates.

3.13.2. Determination of total flavonoid content

The total flavonoid content was measured by using colorimetric assay used Zhishen et al., (1999) with slight modifications. Methanolic extract (250µl) was mixed with 1.25 ml deionized water and 75 µl of 5% $NaNO_2$. After 5 min of incubation at room temperature, 0.15 ml of 10% $AlCl_3$ were added and mixed well. The mixture was incubated for 6 min at

room temperature and 0.5 ml of 1 mM NaOH. Finally, the volume was made up to 2.5ml with 275 μl of deionized water and mixed well. The absorbance was measured at 510 nm after 30 minutes of incubation. The flavonoid content was determined from a gallic acid (20-100 μg/ml) standard curve. All tests were carried out in triplets.

3.13.3. Determination of total tannin content

Total tannin content was measured using Folin's Dennis method as described by Nwinuka et al., (2005) with a slight modification. Briefly, 0.25 ml of extract was diluted with 2.25 ml distilled water, 0.50 ml Folin's Dennis reagent was added and allowed to stand for reaction up to 1 min. This mixture was neutralized by addition of 1.0 ml 7 % sodium carbonate (w/v) and kept in a water bath at 25 °C for 20 minutes. The absorbance of resulting blue colour was recorded at 700 nm using UV-VIS spectrophotometer. Tannin estimation was determined from a tannic acid (100-1000 μg/ml) standard curve. All tests were carried out in triplet.

3.13.4. Assay for *β*-Carotene and Lycopene.

The concentration carotenoids lycopene and β-Carotene methanolic extract were estimated spectrophotometrically based on method by Nagata and Yamashita (1992) with a slight modification. Dried fungal mycelial powdered (5 g) was extracted in methanol (100 ml) at room temperature (24 h), filtered (Whatman No #4 filter paper), extraction was repeated thrice and pooled extract was evaporated (42°C) to dryness. Dried methanol extract of pulp and seeds (100 mg) was dissolved by thorough vortexing in mixture of acetone + hexane (4:6 v/v) (10 ml) and filtered (Whatman No #4 filter paper). The absorbance of filtrate was then measured at different wavelengths (453, 505 and 663 nm) to calculate lycopene and β-Carotene contents in 100 ml extract to express their concentration in mg per gram (mg/g). The content of *β*-carotene and lycopene was calculated from the equations given as follows:

$$\text{Lycopene (mg/ml)} = -0.0458A_{663} + 0.372A_{505} - 0.0806A_{453}$$

$$\text{B- Carotene (mg/ml)} = 0.216A_{663} - 0.304A_{505} + 0.452A_{453}$$

3.14. *In Vitro* Antioxidant Assays

3.14.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

Anti-radical activity scavenging activity of the methanolic extract on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method described by Brand-Williams et al., (1995) with some modifications. Briefly, in this assay, a volume of 1.0 mL of various concentrations of fungal extract was mixed with freshly prepared 3.0 mL DPPH solution prepared in 95% methanol and adjusted the absorbance to 1.0 (±0.02) at 517nm. The reaction mixture was vortexed well and then incubated for 30 min at room temperature in dark. After 30 min of dark incubation, the absorbance was measured at 517 nm against 80%-methanol as a blank reference using a UV-VIS spectrophotometer (Evolution 201, Thermo Fisher Scientific). Butylated Hydroxy Toluene (BHT) was used as a positive reference compound and was prepared in similar manner. The percentage free radical scavenging activity of the sample was calculated according to the following equation:

% Scavenging activity = [(Ac-As) / Ac] × 100 where Ac = absorbance of control and As = absorbance of sample. An equal amount of methanol and DPPH without sample served as a control. The IC_{50} value represented the concentration of the methanolic extract that caused 50% inhibition of hydroxyl radical formation was determined through a linear regression analysis.

3.14.2. Scavenging of 2, 2′-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) assay

The long-lived radical cation chromophore 2, 2′-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) was generated through the oxidation of ABTS with potassium persulfate. The scavenging activity of this cation radical was determined by the method of Re et al., 1999. Specifically, 7 mM of ABTS stock solution was mixed with 2.45mM potassium persulfate solutions prepared in deionized water and the mixture was

allowed to stand in the dark at room temperature for sixteen hours to produce a dark-coloured solution containing ABTS radical cation. The radical is stable for up to five days if refrigerated. For the study of methanolic extract radical scavenging activity, the ABTS $^{\bullet+}$ solution was suitably diluted with ethanol to yield an absorbance of 0.70 (± 0.2) at 734nm and equilibrated at 25°C to be used for antioxidant assay. The assay was performed by adding 1ml methanolic extracts to be tested at different amounts to 3ml of ABTS$^{\bullet+}$ radical cation solution and the mixture were shaken gently, incubated for six minutes at 37°C. The reduction of ABTS$^{\bullet+}$ radical cation absorbance by adding compounds that contain antioxidants was measured by the change of absorbance of ABTS$^{\bullet+}$ radical cation at 734nm using deionized water as a blank, on UV- visible spectrophotometer (Thermo Fisher Scientific). A standard solution of ascorbic acid was also prepared and tested at a range of 2 to 10mg/ml in methanol (HPLC grade, HiMedia).

Scavenging effect (%) = [(Ac−As)/(Ac)] × 100.

Where, Ac and As are the absorbance of control and sample, respectively. The result was compared with control which was prepared by adding 1.0ml of methanol in place of the sample.

3.14.3. Evaluation of metal-chelating activity

The Fe^{2+}-chelating ability by the extracts was estimated by the method of Jiang et al., (2005) with a slight modification. The ability of methanolic extract was monitored by measuring the formation of Fe^{2+}-ferrozine complex. A 1-ml aliquot of a methanolic solution of the lyophilized extract (2–10 mg/ml) was reacted with 0.1ml of 2.0 mM aqueous ferrous chloride (Merck) and 3.7ml deionized water. After incubation for 5 min, the reaction was initiated by adding 0.2ml of 5.0 mM ferrozine. The mixture was vortexed and left at room temperature for 10 min to equilibrate. After incubation the absorbance of the resulting solution at 562 nm was determined spectrophotometrically. A lower absorbance indicates a

stronger Fe^{2+}-chelating ability and the percentage chelating capacity were calculated as; Chelating activity (%) = (Ac - As)/Ac × 100, where Ac was the absorbance of the control in the reaction system and As was the absorbance of the sample.

3.14.4. Reducing activity assay

The reductive ability of the extract was determined using the Oyaizu (1986) method with a slight modification. A 1.0-ml aliquot of the lyophilized extract solution (2–10 mg ml^{1} methanol) was mixed with 2.5 ml of 0.2 M phosphate buffer, pH 6.6- and 2.5-ml aqueous solution of 10 g potassium ferricyanide (HiMedia RM1034) in test tubes. The mixture was placed in a water bath at 50°C, for 20 min. Then, 2.5 ml aqueous solution of 100 g trichloroacetic acid (SRL 90544) was added followed by centrifugation at 1200g for 10 min. In the next stage, the upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 %(w/v) ferric chloride (SRL 72287) and allowed to stand for 10 min. The absorbance was measured at 700 nm using a UV-VIS spectrophotometer (Evolution 201, Thermo Fisher Scientific); the higher the absorbance of the reaction mixture, the greater the reducing power. The reference solution was prepared as above, but contained water in place of samples. The reducing power was expressed as ascorbic acid equivalents using a standard curve of ascorbic acid. All these procedures were carried out in triplicates.

3.14.5. Hydroxyl radical scavenging assay

The hydroxyl radicals were generated in an H_2O_2 –$FeSO_4$ system by oxidation of FeSO4 and were assayed by the color change of salicylic acid according to the method of Zhong et al., (2010) with some modification. The hydroxyl radical was produced in a reaction mixture containing 1 ml of sample (2–10 mg/ml), 1 ml of 9 mM $FeSO_4$ and 1 ml of 0.3% H_2O_2 in 0.5 ml of 9 mM salicylic acid–ethanol solutions was mixed well and the mixture was incubated at 37°C for 30 min. The change in absorbance caused by the color change of salicylic acid was

recorded at 510 nm. Gallic Acid was used as the positive control. The hydroxyl radical scavenging activity was calculated as follows:

Scavenging activity (%) = (Ac – As)/ Ac] × 100 where Ac is the absorbance of the control (methanol instead of sample), As is the absorbance of the sample.

3.14.6. Hydrogen peroxide scavenging activity assay

The capability of samples to quench H_2O_2 was determined spectrophotometrically based on the method of Ruch et al., *(*1989) with minor changes. An aliquot of hydrogen peroxide solution 2.0mM prepared in 50mM phosphate buffered saline (pH 7.4) and various concentrations of the extracts (2-10 mg/ml) were mixed (1:0.6 v/v) gently in a test tube covered with aluminium foil which were then capped immediately. Absorbance of H_2O_2 at 230 nm was determined after10 min of dark incubation in a spectrophotometer against a blank solution containing phosphate buffer without hydrogen peroxide. For each sample concentration, a separated blank sample was used for background subtraction.

The percentage inhibition rate of hydrogen peroxide by the methanolic extract was calculated as; Inhibition rate (%) = (Ac - As)/AcX100, where Ac was the absorbance of the control in the H_2O_2 system and As was the absorbance in the presence of the sample.

3.14.7. FRAP Assay

The antioxidant capacity of each methanolic extract was determined by FRAP assay, following the method of Benzie and Strain (1996). Working FRAP reagent was prepared immediately before use by mixing acetate buffer (300 mM, pH 3.6), 2, 4, 6-tri [2-pyridyl]-s-triazine (10 mM in 40 mM HCl) solution, and $FeCl_3.6H_2O$ (20 mmol/L) solution in 10:1:1 ratio, respectively. Three millilitres of FRAP reagent was mixed with the 100 µl of each methanolic extract solution and the content was mixed vigorously. The absorbance was read at 593 nm at the interval of 30s for 4 min. An aqueous solution of known Fe^{2+} concentration

in the range of 1–5 μg/mL was used for calibration. Using the regression equation, the FRAP values (μg Fe (II)/mL) of the each methanolic extract solution were calculated.

3.15. Assay of antimicrobial activity

3.15.1. Test Bacteria

Five reference strains of clinically important pathogenic test bacteria used in this study include; two Gram-positive; *Bacillus subtilis* (MTCC-121) and *Staphylococcus aureus* (MTCC-3160) and three Gram-negative; *Escherichia coli* (MTCC-1698), *Pseudomonas aeruginosa* (MTCC- 4673) and *Salmonella typhimurium* (MTCC-1252). The bacteria were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. These bacterial cultures were maintained by regular subculturing on nutrient agar slants and stored at -20°C using 10% glycerol as preservative.

3.15.2. Preparation of test solutions

The lyophilized methanolic extracts were dissolved in double distilled water to give a working concentration of 01 and 02 mg mL^{-1}. These extracts were further sterilized by filtration through a 0.2-micron syringe filter (Schwalbe et al., 2007).

3.15.3. Antibacterial activity

Antibacterial activity of the synthesized bio silver nanoparticles was carried out according to the agar well diffusion method of Perez et al., (2010), with slight modifications. A loopful of bacterial culture were aseptically inoculated into 10 mL of pre-sterilized Mueller–Hinton broth (HiMedia M391-100G, India) followed by 5-h incubation at 37°C in a shaking condition. These actively growing broth culture suspensions prior to antimicrobial assay were adjusted turbidimetrically to 0.5 McFarland standards with specified pre-sterilized broth to yield a bacterial suspension of 1-2 × 10^8 CFU/ml (Schwalbe et al., 2007).

3.15.4. Agar well diffusion assay

The antimicrobial activities of the fungal extracts were evaluated by means of the agar-well diffusion assay described by Farahmandfar et al., 2019 and Schwalbe et al., 2007. Aseptically, 1000 µl of a bacterial suspension (1-2 × 10^8 CFU/ml) was added to twenty millilitres of the Mueller–Hinton molten agar (cooled 52°C in a water bath). This mixture was poured evenly onto sterile petri dishes (100 x 15 mm). The plate was rotated gently back and forth to ensure even distribution of the inoculated agar culture. Once the agar plates had fully hardened, wells of 9.0 mm diameter were pierced out using a sterile cork-borer. Exactly, 100 µl of the fungal extracts (01 & 02 mg mL^{-1}) suspended in double distilled H_2O was added per well and vortexed until in suspension. The plates were then incubated in an upright position for 24 h at 37°C in an incubator. The antibiotic Streptomycin (100 µg mL^{-1}) (Abbott) served as positive control and EDTA (100 µg mL^{-1}) as a negative control in every experiments. The diameter of the resulting zone of inhibition (ZOI) including the diameter of the well was recorded using a calliper. Growth inhibition was measured as the diameter of clear zone of growth inhibition to the nearest millimetres (0.1 mm) range. Zone of inhibition less than 10.0 mm was not considered. All samples were tested in triplicate and the zone of inhibition results shown are the means ± SD.

3.16. Cell viability assay

The human carcinoma cells, HeLa (cervical cancer), PC3 (prostrate cancer) and HepG2 (hepato carcinoma) were used to investigate the cytotoxic activity evaluation of the methanolic fungal extract. The cells were cultured to reach confluence in RPMI1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO_2 incubator.

Cell viability of the methanolic extract was assessed by the MTT assay as described by Mossman, 1983. MTT [3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide] is a

tetrazolium salt that appear yellow in colour in its oxidized form which is cleaved by mitochondrial and endoplasmic reticulum dehydrogenase of metabolically competent cells, yielding a measurable purple formazan product. This formazan production was quantified spectrophotometrically and it was accepted as proportional to the viable cell number and inversely proportional to the degree of cytotoxicity (Fotakis & Timbrell 2006).

Exponentially growing cells were trypsinized and aseptically collected, counted, and adjusted to a final concentration of 3×10^3 cells/well, to be inoculated on 96-well plates. After 24 hr adherence, the cells were treated with various concentration of fungal extract for 72 hr. Thereafter, the appropriate incubation time, 1:10 volume of MTT solution (5 mg/mL) was added to each well and incubated for 4 hr in dark. Then the medium was carefully removed, and the formazan formed in the wells was dissolved for homogenous measurement in 150 µl of dimethyl sulfoxide, the plates were kept for 5 min on a plate shaker. The absorbance was measured at 570 nm using a microplate reader (company). For the control, SDYB medium (pH 7.4) and Dulbecco's PBS were used in place of the fungal culture filtrate. Cytotoxicity was calculated as the percent reduction in absorbance relative to the control (DMSO). Unless stated otherwise, all experiments were performed in four replicates (n=4).

3.17. Determination of cell apoptosis by PI staining

Determination of cell apoptosis by the propidium iodide (PI) flow cytometric assay is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. The HeLa cells in logarithmic growth phase were suspended at a final concentration of $3x10^4$/mL in a 12-well culture plate. After desired period of incubation, the cells were treated with different concentrations of methanolic extracts for 72 hours. Then, an aliquot of treated cell was harvested, trypsinized, centrifuged at 4^oC, 1500 rpm, supernatant aspirated off and pellet washed with PBS. Pellet was further resuspended on ice cold PBS in which 5µl of PI solution was added briefly mixed

and incubated on ice under dark conditions for 20 minutes. Then the cell apoptosis was analysed by using a flow cytometer (BD FACS Verse) and gate was used to exclude any clumped nuclei. The PI staining was carried out only in HeLa cell lines as this cell exhibited IC_{50}(>50) value within the range of extract concentration tested.

3.18. Annexin-V/PI Staining

Apoptosis was measured by flow cytometry using Annexin-V/PI double staining. Hela cells were seeded in a 6-well plate at a density of 0.50 $x10^6$ cells/well and incubated at 37°C for 24 h. Then, the medium was removed and fresh media with the indicated concentrations of extracts were added. After 48h, cells were collected and washed with ice cold PBS twice and resuspended in 25µl of 1X Annexin-V binding buffer, 1.5µl of Annexin-V staining solution 10µl of PI (stock: 50 µl/ml), (BD Biosciences) staining solution and incubated on ice under dark condition for 20 min in dark. Then, the number of viable, apoptotic and necrotic cells were quantified using flow cytometry.

3.19. Colony Forming Assay

To check the long-term effect of the extracts on HeLa cells, cells were seeded in a 6 well plate at a density of 3000cells/well and incubated at 37˚C for 24h. Then, the media was removed and fresh media with the indicated concentrations of extracts were added. Every, other two days, media was removed and fresh media with desired concentrations of extracts were added for at least 10 days. After ten days, media was removed and each well was washed with PBS, fixed with methanol for 20 min followed by washing with PBS and staining with 0.4% crystal violet for 30 min. After staining, each well was rinsed with tap water to remove the excess stain and the image was analysed using software ImageJ.

3.20. HPLC analysis

Analysis of beauvericin in the extract was done in a HPLC system (Dionex, Ultimate 300) using a reversed-phase HPLC column (Acclaim^{TM} C18, 5µm, 120 Å, 4.6 x 250 mm) with isocratic conditions and a mobile phase of acetonitrile-water (75:25) at a flow rate of 0.5ml/min with UV detection at 210 nm for fifteen minutes. Precise quantities of beauvericin (Sigma, USA) were dissolved in methanol as an internal standard. Beauvericin was determined by comparing peak areas from sample to an internal standard. All reagents used for HPLC analysis were degassed and sterilized using 0.22 µm syringe filter.

3.21. Morphological evaluation of apoptosis using light microscopy

The morphological changes of HeLa cells were determined using an inverted light microscope. HeLa cells were grown in 12 well plates and treated with mycelial methanolic extract at a concentration of 100µg/ml for 72 h. After incubation under optimal conditions, the morphological changes were examined at 4X under inverted light microscope (Nikon TS100).

3.22. FT-IR spectroscopy

The crude extract was subjected to functional groups analysis by FTIR (Perkin Elmer Spectrum 1: FT-IR), having resolution of 1.0 cm^{-1}. Potassium bromide (KBr) pellets was used to obtain the infrared spectra of crude extract powders covering scan range from an entire wavelength region of around 4000- 450 cm^{-1}.

3.23. Statistics

All results are expressed as mean ± standard deviation values of the three sets of observations (Microsoft Corporation, 2018). MTT and Annexin-V/PI assays were analysed using one-way ANOVA followed by Tukey's post hoc test of significance (where different alphabets denote significant difference ($p<0.05$) (RStudio Team, 2020). Multivariate principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) analysis was performed using PAST software (Hammer et al., 2001).

4. RESULTS

4.1 Sampling

Collections were made during extensive fieldwork in Darjeeling Hills between the first week of June to first week of October in 2016 and 2017. The collection data are listed in Table 4.1. All examined collections and raised axenic cultures are deposited in the herbarium of Sikkim University, Sikkim and duplicates of the pure culture materials have been deposited in the herbarium of the St Joseph's College, Darjeeling, India.

4.2 Morphological characterisation

The morphological characters, conidia and phialide from host material and conidia from 14 days old PDA cultures were measured using high power lens of the microscope. All measurements are presented in µm with averages (n=15) in brackets in Table 4.2. The morphological features and the related description of the nine isolates are described below.

Isaria javanica **Friederichs & Bally**

Morphologically conidiogenous structures were phialidic and conidia were formed in chains on verticillate phialides. Phialides are flask shaped 5.2–6.6 x 2.2–2.7 µm in length. The dimensions of conidia were recorded to be 3.1–4.1 x 1.4–2.0 µm on hosts. The conidia formed white or greyish white mass with fusiform to oval, both ends often forming pointed structure. Mean conidial dimensions from cultures grown on 1/3rd PDA supplemented with 0.1% yeast extract were 3.3-4.2 x1.1-1.5 µm. The mycelium was immersed; hyphae branched, septate, hyaline, inter- and intracellular, smooth, and 1.5-2.3 µm thick. Growth was found to be best at 24°C with colonies on PDA growing optimally attaining a diameter of 38±1.41mm after 14 days of dark incubation at pH 7.0. The whitish colony gradually turns pinkish in color with age.

Table 4.1: *Isaria* specimens collected and examined from different ecological regions of Darjeeling hills.

Sample code	Species	Putative Host Order	Host stage	Dominant vegetation	Locality	Latitude (N)	Longitude (E)
IDAR-01	***Isaria javanica* Friederichs & Bally**	Coleoptera	Adult	Hamamelidaceae, Juglandaceae	Upper Labdah, Mungpoo	26º58′26.55″	88º20′22.99″
IDAR-02	***Isaria farinosa* (Holmsk). Fr.**	Lepidoptera	Pupa	Fagaceae, Ericaceae *Sphagnum* sp.	Darjeeling Zoo	27º03′31.00″	88º15′15.86″
IDAR-03	***Isaria tenuipes* Peck**	Lepidoptera	Pupa	Fagaceae, Elaeocarpaceae, Gentianaceae	3rd Mile Forest	27º0′39.61″	88O17′34.6″
IDAR-04	***Isaria tenuipes* Peck**	Lepidoptera	Pupa	Fagaceae, Ericaceae	6th Mile Forest	27º2′9.62″	88º15′45.63″
IDAR-05	***Isaria farinosa* (Holmsk). Fr.**	Hymenoptera	Adult	Ericaceae, Sapindaceae	SJC Medicinal Garden	27º3′34.48″	88º15′3″
IDAR-06	***Isaria tenuipes* Peck**	Lepidoptera	Pupa	Betulaceae, Fagaceae, Juglandaceae	Soom Forest	27º4′27.22″	88º13′53.45″
IDAR-07	***Isaria tenuipes* Peck**	Lepidoptera	Pupa	Betulaceae, Ericaceae, Fagaceae	Lebong Forest	27º3′41.57″	88º16′35.51″
IDAR-08	***Isaria farinosa* (Holmsk). Fr.**	Araneae	Adult	Fagaceae, Ericaceae	Tiger Hill Forest	26º59′48″	88º17′40″
IDAR-09	***Isaria fumosorosea* Wize**	Lepidoptera	Larva	Magnoliaceae, Lauraceae, Rosaceae	Pradhan Medicinal Garden Mongmaya	27º03′36.00″	88º25′48.00″

***Isaria farinosa* (Holmsk). Fr.**

In this specimen also the conidiogenous structures are phialidic and it measured 4.2-6.5 x 2.2-3.1 µm in dimensions and conidia were ellipsoidal to fusiform and measured 2.2-2.7 x 1.2-1.7 µm on the host. The conidia from cultures on PDA after 14 days of dark incubation measured 2.2-3.3 x 1.2-1.8 µm. The mycelium is submerged, hyphae are branched, septate, hyaline, inter- and intracellular, smooth, and 1.4-2.5 µm thick. Growth was recorded best at 24°C with colonies on PDA growing with diameter of 40.75±0.95 mm after 14 days of dark incubation at pH 9.0. Colonies initially appeared whitish in color and gradually it turns light yellow with age.

***Isaria tenuipes* Peck**

Conidiogenous structures are phialidic and it measured 4.1-6.3 x 3.1-4.3 µm in dimensions and conidia were ellipsoidal to cylindrical, slightly curved and measured 3.2-5.3 x 1.3-1.8 µm on the host. While the conidia from cultures on PDA after 14 days of dark incubation measured 4.6-6.8 x 1.9-2.4 µm. The mycelium was submerged, hyphae branched, septate, hyaline, inter- and intracellular, smooth, and 1.3-2.4 µm thick. Growth was best at 24°C with colonies on PDA growing with diameter of 40.75±0.95 mm after 14 days of dark incubation at pH 9.0. Colonies initially appeared whitish in color which gradually it turns creamish yellow in color.

***Isaria fumosorosea* Wize**

Conidiogenous structures are phialidic and it measured 4.6-5.8 x1.8-2.4 µm in dimensions and the conidia were ellipsoidal to cylindrical, slightly curved and measured 3.7-4.7 x1.1-1.9 µm on the host. Conidia from cultures on PDA after 14 days of dark incubation measured 4.1-4.9 x1.2-1.8. The mycelium was submerged, hyphae branched, septate, hyaline, inter- and intracellular, smooth, and 1.5-2.4 µm thick. Growth was found to be the best at 24°C with colonies on PDA growing up to a diameter of 50±0.81 mm after 14 days

of dark incubation at pH 8.0. Colonies appeared whitish in coloration with highly floccose morphology.

4.3. Molecular analysis

Nine isolates of *Isaria* i.e., IDAR-01 to IDAR-09 which have been identified with classical morphological taxonomic approach based upon macroscopic, microscopic and cultural characters were further confirmed their identity with molecular techniques. The nrDNA analysis was carried out by sequencing ITS region of the genomic DNA using specific markers. PCR amplification of ITS region using ITS4 and ITS5 primer generated an amplicon of 543 to 556, specific to entomopathogenic fungus *Isaria* (Table 4.3). The sequences of nine isolates were BLASTN searched against the sequences of *Isaria* species already submitted in the NCBI GenBank indicated that ITS sequence of the present study had a maximal of 98-100 % homology with *Isaria* spp. Thus, sequencing results of all isolates significantly supported the identity of sequences as the members of genus *Isaria.*

4.4. Construction of the phylogenetic tree

Phylogenetic tree was constructed using sequences of nine isolates and voucher sequences of its allies obtained from GenBank. GenBank accession numbers and strain details for global isolates reported earlier are shown in Table 4.4. In order to infer relationship between *Isaria* spp isolates infecting insects in temperate region of Darjeeling Hills based on ITS region, an optimal tree was generated by using maximum likelihood method and evolutionary distances were computed by Neighbour-Joining method (NJM) and Maximum Composite Likelihood method (MCLM). The phylogenetic tree is given in Figure 4.1. It was inferred from phylogenetic analysis that all nine isolates were clustered in a separate subclade. However, ITS region of each isolate could not generate genetic diversity.

Table 4.2: Measurements of morphological characters, conidia and phialide from host material and conidia from 14-day old MEA (Malt Extract Agar) cultures of *Isaria* spp from Darjeeling Hills. All measurements are presented in µm with averages (n=15) and ±SD in brackets.

Species	Putative Host Order	Conidia on MEA length x width	Conidia on host length x width	Phialides on host length x width
***I. javanica* IDAR-01**	Coleoptera	3.77(±0.33) x 1.33 (±0.16)	3.72 (±0.30) x 1.86 (±0.31)	6.0 (±0.49) x 2.8 (±0.14)
***I. farinosa* IDAR-02**	Lepidoptera	2.83 (±0.35) x 1.53 (±0.18)	2.51 (±0.16) x 1.48 (±0.23)	6.17 (±0.62) x 2.65 (±0.28)
***I. tenuipes* IDAR-03**	Lepidoptera	4.75 (±0.67) x 1.83 (±0.34)	3.80 (±0.44) x 1.8(±0.49)	5.27 (0.83) x 2.66 (±0.32)
***I. tenuipes* IDAR-04**	Lepidoptera	5.66 (±0.70) x 2.16 (±0.21)	4.25 (±0.71) x 1.58 (±0.26)	5.25 (±0.86) x 3.71 (±0.38)
***I. farinosa* IDAR-05**	Hymenoptera	2.65 (±0.33) x 1.44 (± 0.24)	2.42 (±0.23) x 1.45 (±0.20)	5.53 (±0.75) x 2.16 (±0.52)
***I. tenuipes* IDAR-06**	Lepidoptera	5.01 (±0.69) x 2.51 (±0.34)	5.12 (±0.74) x 1.58 (±0.27)	5.08 (±0.67) x 2.46 (±0.56)
***I. tenuipes* IDAR-07**	Lepidoptera	6.53 (±1.25) x 2.34 (±0.26)	4.12 (±0.56) x 1.54 (±0.25)	5.30 (±0.53) x 3.78 (±41)
***I. farinosa* IDAR-08**	Araneae	2.65 (±0.33) x 1.4 (±0.33)	2.50 (±0.16) x 1.5 (±0.14)	5.46 (±0.66) x 2.26 (±0.41)
***I. fumosorosea* IDAR-09**	Lepidoptera	4.54 (±0.25) x 1.51(±0.22)	4.25(±0.42) x1.53(±0.27)	5.24 (±0.50) x 2.16(±0.41)

Table 4.3: Molecular identification of nine isolates of *Isaria* spp., collected from different ecological regions of Darjeeling hills based on ITS- rDNA sequencing.

Sl. No.	Sample code	Sequencing code	Closet match	Query coverage	% Similarity	Sequence bp
1	IDAR-01	NL33	*Isaria javanica*	100%	98%	556
2	IDAR-02	NL29	*Isaria farinosa*	100%	99%	555
3	IDAR-03	NL30	*Isaria tenuipes*	100%	100%	555
4	IDAR-04	NL31	*Isaria tenuipes*	100%	99%	551
5	IDAR-05	ST79	*Isaria farinosa*	100%	100%	543
6	IDAR-06	ST80	*Isaria tenuipes*	100%	100%	543
7	IDAR-07	ST82	*Isaria tenuipes*	100%	100%	544
8	IDAR-08	ST83	*Isaria farinosa*	100%	99%	545
9	IDAR-09	ST84	*Isaria fumosorosea*	100%	100%	545

Table 4.4: GenBank sequences included in phylogenetic analysis of *Isaria* species.

Species	Country	ITS	Reference
Isaria amoenerosea	-	AY624168	Luangsa-ard *et al. 2005*
Isaria amoenerosea	Ghana	AY624169	Luangsa-ard *et al. 2005*
Isaria farinosa	Denmark	AY624181	Luangsa-ard *et al. 2005*
Isaria farinosa	UK	AY624179	Luangsa-ard *et al. 2005*
Isaria farinosa	Netherlands	AY624178	Luangsa-ard *et al. 2005*
Isaria fumosorosea	Japan	AY624183	Luangsa-ard *et al. 2005*
Isaria javanica	Indonesia	AY624186	Luangsa-ard *et al. 2005*
Isaria amoenerosea	Brazil	JN998782	D'Alessandro et al., 2013
Isaria fumosorosea	Argentina	JN998801	D'Alessandro et al., 2013
Isaria farinosa	Argentina	JN998784	D'Alessandro et al., 2013
Isaria fumosorosea	Argentina	JN998800	D'Alessandro et al., 2013
Isaria fumosorosea	Mexico	JN998798	D'Alessandro et al., 2013
Isaria fumosorosea	Brazil	JN998795	D'Alessandro et al., 2013
Isaria tenuipes	Brazil	JN998789	D'Alessandro et al., 2013
Isaria tenuipes	Brazil	JN998788	D'Alessandro et al., 2013
Isaria javanica	Argentina	JN998794	D'Alessandro et al., 2013
Isaria farinosa	Argentina	JN998783	D'Alessandro et al., 2013
Isaria fumosorosea	Brazil	JN998796	D'Alessandro et al., 2013
Isaria javanica	Japan	AB539089	Shimazu and Takatsuka, 2010
Isaria javanica	Japan	AB539088	Shimazu and Takatsuka, 2010
Isaria javanica	Japan	AB539087	Shimazu and Takatsuka, 2010
Isaria javanica	Japan	AB539091	Shimazu and Takatsuka, 2010
Cordyceps cardinalis	Japan	AB237660	Yokoyama *et al.* unpublished
C. pseudomilitaris	Thailand	AJ786589	Stensrud *et al. 2005*

4.5. ITS-nrDNA sequences of individual sample

The detailed study on the molecular identification of the fungi was done at National Fungal Culture Collection of India (NFCCI), Biodiversity and Palaeobiology Group, MACS-Agharkar Research Institute, Pune, India.

***Isaria javanica* (IDAR-01)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 556 base pair (bp) sequences are given below.

CCTGATCCGAGGTCACGTTCAGAAGTGGGGTGTTTACGGCGTGGCCGCGTCG
GGTTCCCGGTGCGCGTTGGAGTACTACGCAGAGGTCGCCGCGGACGGGCCGCC
ACTGTATTTCGGGGCCGGCGGTGTGCTGCCGGTCCCCAACGCCGACTCCCCCG
AAGGGGTGTCGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATG
CTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATT
CACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGAT
CCGTTGTTGAAAGTTTTGATTCATTTGTTTTGCCTTGCGGCGGATTCAGAGAGA
CTGATGGAAACAGAGTTTAGGGGTCTCCGGCGGCCGCCTGGGTCCGGGCCGCG
GGCGGCGCAAGGCCGTCCGGACGCCGGGGCGAGTCCGCCGAAGCAACGATAG
GTATGTTCACAAAGGGTTAGGGAGTTGAAAACTCGGTAATGATCCCTCCGCTG
GTTCACCAACGGAGACCTTGTTACG

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 98% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. javanica*.

***Isaria farinosa* (IDAR-02)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 555 base pair (bp) sequences are given below.

CTGATCCGAGGTCACGTTCAGAAGTCGGGGGTTTACGGCGTGGCCACGTCGGGG
TTCCGGTGCGAGTTGGATTACTACGCAGAGGTCGCCGCGGACGGGCCGCCACTTC
ATTTCGGGGCCGGCGGTATACGGCCGGTCCCCAACGCCGATTTCCCCAAAGGGAA
GTCGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGC
GCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA
TCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA
GTTTTGATTCATTTGTTTTGCCTTGCGGCGGATTCAGAAGATACTGAGAATACAGA
GTTTGGGGGTCTCCGGCGGCCGCCTGGATCCAGGCCGCGGCCGGCGCGGGGCCG
GCCGGACGCTGGGGCGAGTCCGCCGAAGCAACGATAGGTATGTTCACAAAAGGG
TTTGGGAGTTGAAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCT
TGTTAC

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 99% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. farinosa*.

***Isaria tenuipes* (IDAR-03)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 555 base pair (bp) sequences are given below.

CCTGATCCGAGGTCACGTTCAGAGGTTGGGGGTTTCACGGCGGGCCGCGTCGGGT
TCCCGGTGCGAGTGCTTGTACTGCGCAGAGGTCGCCGCGGACGGGCCGCCACTCC
ATTTCAGGGCCGGCGGGGTGCTGCCGGTCCCCAAGGCCGACGTCCCGGGGGACG
TCGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCG
CAATGTGCGTTCAAAGATTCGATGATTCACGGAATTCTGCAATTCACATTACGTA
TCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA
GTTTTGATTCGTTTGTGTTGCCTTGCGGCGGATTCAGAGAGGCTGACAGATACAG
GGTTGCGTGGTCCCCGGCGGCCGCCTGGGTCCAGGTCGCGGGCCGGCGCTGGGCC
GTCCGGACGCTGGGGCGGGTCCGCCGAAGCAACTATGGGTAGGTTCACAGAAGG
GTTGGGAGTTGTAAAACTCTGGTAATGATCCCTCCGCTGGTTCACCAACGGAGAC
CTTGTTAC

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 100% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. tenuipes*.

***Isaria tenuipes* (IDAR-04)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 551 base pair (bp) sequences are given below.

GATCGAGGTCACGTTCAGAGGTTGGGGGTTTCACGGCGGGCCGCGTCGGGTTCCC
GGTGCGAGTGCTTGTACTGCGCAGAGGTCGCCGCGGACGGGCCGCCACTCCATTT
CAGGGCCGGCGGGGTGCTGCCGGTCCCCAAGGCCGACGTCCCGGGGGACGTCGA
GGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCAAT
GTGCGTTCAAAGATTCGATGATTCACGGAATTCTGCAATTCACATTACGTATCGC
ATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT
GATTCGTTTGTGTTGCCTTGCGGCGGATTCAGAGAGGCTGACAGATACAGGGTTG
CGTGGTCCCCGGCGGCCGCCTGGGTCCAGGTCGCGGGCCGGCGCTGGGCCGTCCG
GACGCTGGGGCGGGTCCGCCGAAGCAACTATGGGTAGGTTCACAGAAGGGTTGG
GAGTTGTAAAACTCTGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGT
TAC

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 99% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. tenuipes*.

***Isaria farinosa* (IDAR-05)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 543 base pair (bp) sequences are given below.

GGTCACGTTCAGAAGTCGGGGGTTTTACGGCGTGGCCACGTCGGGGTTCCGGTGC
GAGTTGGATTACTACGCAGAGGTCGCCGCGGACGGGCCGCCACTTCATTTCGGGG
CCGGCGGTATACGGCCGGTCCCCAACGCCGATTTCCCCAAAGGGAAGTCGAGGG
TTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCAATGTG

CGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTT
CGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATT
CATTTGTTTTGCCTTGCGGCGGATTCAGAAGATACTGAGAATACAGAGTTTGGGG
GTCTCCGGCGGCCGCCTGGATCCAGGCCGCGGCCGGCGCGGGGCCGGCCGGACG
CTGGGGCGAGTCCGCCGAAGCAACGATAGGTATGTTCACAAAAGGGTTTGGGAG
TTGAAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGT

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 100% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. farinosa*.

***Isaria tenuipes* (IDAR-06)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 543 base pair (bp) sequences are given below.

GTCACGTTCAGAGGTTGGGGGTTTCACGGCGGGCCGCGTCGGGTTCCCGGTGCGA
GTGCTTGTACTGCGCAGAGGTCGCCGCGGACGGGCCGCCACTCCATTTCAGGGCC
GGCGGGGTGCTGCCGGTCCCCAAGGCCGACGTCCCGGGGGACGTCGAGGGTTGA
AATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCAATGTGCGTT
CAAAGATTCGATGATTCACGGAATTCTGCAATTCACATTACGTATCGCATTTCGCT
GCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCGTT
TGTGTTGCCTTGCGGCGGATTCAGAGAGGCTGACAGATACAGGGTTGCGTGGTCC
CCGGCGGCCGCCTGGGTCCAGGTCGCGGGCCGGCGCTGGGCCGTCCGGACGCTG
GGGCGGGTCCGCCGAAGCAACTATGGGTAGGTTCACAGAAGGGTTGGGAGTTGT
AAAACTCTGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTA

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 100% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. tenuipes*.

***Isaria tenuipes* (IDAR-07)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 544 base pair (bp) sequences are given below.

GGTCACGTTCAGAGGTTGGGGGTTTCACGGCGGGCCGCGTCGGGTTCCCGGTG
CGAGTGCTTGTACTGCGCAGAGGTCGCCGCGGACGGGCCGCCACTCCATTTCA
GGGCCGGCGGGGTGCTGCCGGTCCCCAAGGCCGACGTCCCGGGGGACGTCGA
GGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCA
ATGTGCGTTCAAAGATTCGATGATTCACGGAATTCTGCAATTCACATTACGTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA
GTTTTGATTCGTTTGTGTTGCCTTGCGGCGGATTCAGAGAGGCTGACAGATACA
GGGTTGCGTGGTCCCCGGCGGCCGCCTGGGTCCAGGTCGCGGGCCGGCGCTGG
GCCGTCCGGACGCTGGGGCGGGTCCGCCGAAGCAACTATGGGTAGGTTCACAG
AAGGGTTGGGAGTTGTAAAACTCTGGTAATGATCCCTCCGCTGGTTCACCAAC
GGAGACCTTGTTA

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 100% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. tenuipes*.

***Isaria farinosa* (IDAR-08)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 545 base pair (bp) sequences are given below.

GGTCACGTTCAGAAGTCGGGGGTTTTACGGCGTGGCCACGTCGGGGTTCCGGT
GCGAGTTGGATTACTACGCAGAGGTCGCCGCGGACGGGCCGCCACTTCATTTC
GGGGCCGGCGGTATACGGCCGGTCCCCAACGCCGATTTCCCCAAAGGGAAGTC
GAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCG
CAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTT
ATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGA
AAGTTTTGATTCATTTGTTTTGCCTTGCGGCGGATTCAGAAGATACTGAGAATA
CAGAGTTTGGGGGTCTCCGGCGGCCGCCTGGATCCAGGCCGCGGCCGGCGCGG
GGCCGGCCGGACGCTGGGGCGATTCCGCCGAAGCAACGATAGGTATGTTCACA
AAAGGGTTTGGGAGTTGAAAACTCGGTAATGATCCCTCCGCTGGTTCACCAAC
GGAGACCTTGTTA

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 99% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. farinosa*.

Isaria javanica **(IDAR-09)**

The Internal Transcribed Spacer (ITS) sequencing of the isolate IDAR-01 yielded 545 base pair (bp) sequences are given below.

GGTCACGTTCAGAGTTGGGGGTTTCACGGCGGGGCCGCGTCGGGTTTCCGGTG
CGAGCTGTAGTACTTCGCAGAGGTCGCCGCGGACGGGCCGCCACTCCATTTCA
GGGCCGGCGGGGTGCTGCCGGTCCCCAAGGCCGACGTCCCGGGGGACGTCGA
GGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCA
ATGTGCGTTCAAAGATTCGATGATTCACGGAATTCTGCAATTCACATTACGTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA
GTTTTGATTCGTTTGTGTTGCCTTGCGGCGGATTCAGAGAGACTGATGGATGCA
GGGTTGCGTGGTCTCCGGCGGCCGCCTGGGTCCAGGTCGCGGGCCGGCGCGAG
GCCGTCCGGACGCTGGGGCGAGTCCGCCGAAGCAACGATGGGTAGGTTCACA
GGAGGGTTGGGAGTTGTGAAAACTCTGGTAATGATCCCTCCGCTGGTTCACCA
ACGGAGACCTTGTTA

The Basic Local Alignment Search Tool (BLAST) analysis of above nucleotide sequence obtained in the present study showed 100% similarity for 100% query coverage with the sequence available in NCBI database. Hence, molecular results validated the taxonomic results of *I. fumosorosea*.

4.6. Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa

clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 33 nucleotide sequences. There were a total of 3113 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

4.7. Growth parameters of *Isaria* spp.

During the current study, attempts have been made to study critically the effects of some major environmental factors such as temperature, pH and light conditions on the mycelial growth of the nine test isolates, because these factors considerably influence the manifold activities of fungi (Leatham & 1987; Meletiadis et al., 2001; Mahmud & Ohmasa, 2008.). This study was considered necessary for two reasons (1) to ascertain carefully the optimum temperature, pH and light for the best growth of test isolates during the artificial cultivation and (2) to evaluate the growth characteristics of nine isolates for fungal biomass production.

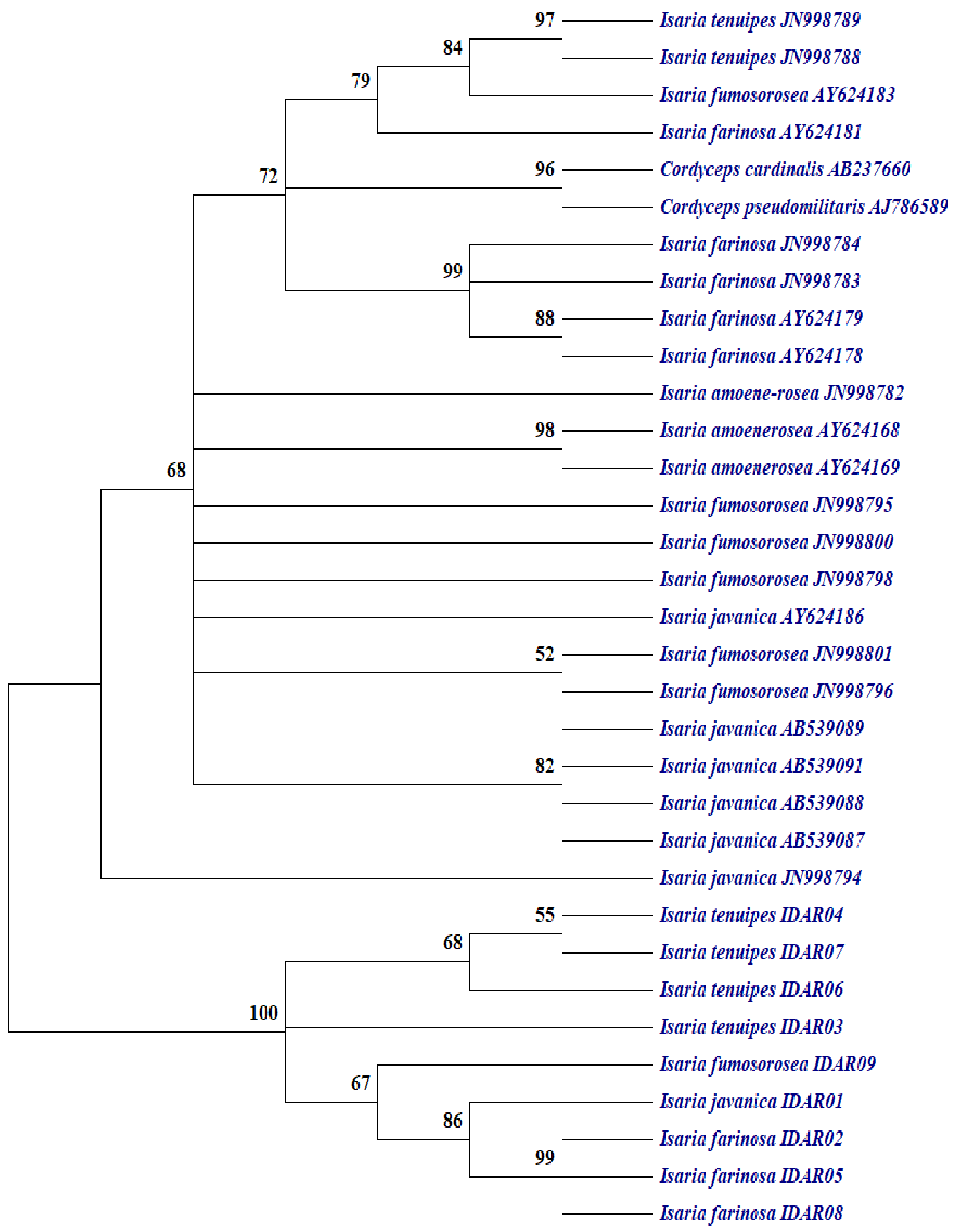


Figure 4.1: Neighbour-joining tree based on analysis of the ITS4-ITS4 rDNA sequences of the isolates IDAR-01, IDAR-02, IDAR-03, IDAR-04, IDAR-05, IDAR-06, IDAR- 07, IDAR-08, and IDAR-09 (From Darjeeling Hills, India) and some related global species. Branch termini are labelled according to isolate numbers, and GenBank accession numbers. Numbers above the nodes indicate bootstrap values (>50%) generated after 500 replications.

4.7.1. Effect of temperature

Temperature is considered to be one of the major environmental factors that influence the growth of mycelium of the fungi. All the nine strains of the genus *Isaria* were grown on Potato Dextrose Agar plate medium with an initial pH of 7.0 and incubated for 14 days under dark condition to study the effect of different temperatures on the mycelial growth of the entomopathogenic fungi. To determine the effect of different temperature on mycelial growth, 5mm agar mycelial seed disc was inoculated on three equidistant points on PDA plates of 90 mm and incubated at each of the following temperatures: 12°, 16°, 20°, 24° and 28°C. The Petri plates were incubated under dark condition for 14 days and thereafter the mean diameter of each radial growth was determined. The results for each treatment are presented in Table 4.5. The Table 4.5., shows that the optimum temperature for maximal radial growth of vegetative colonies lies close to 20°C for eight strains while IDAR-06 exhibited optimal growth at 24°C. While all the strains grew well in a broader range of temperatures (16-28°C). It is also necessary to mention that no mycelial growth in any of the strain was observed at 30°C or beyond. The temperature/growth response is skewed; of the nine strains all favoured comparatively lower mycelial growth at 12°C and a moderate to higher growth at temperature in between 16-24°C. In contrast, an increase or decrease of temperature treatment beyond or below these reduces the radial growth drastically. Thus, the effect of temperature was varied amongst the nine isolates tested. There seemed to be a tendency for a comparatively better growth for all isolates at temperature of 16-24°C. In the present experiment, it was noticed that the temperature treatment of 20°C showed excellent mycelial growth for all the strains with respect to its quality and density. Thus, it was recorded that the trend of mycelium growth with respect to temperature was found to be very similar for all the strains. It was also observed that the strains could survive at temperature as low as 4°C and the mycelial growth was restored

when the temperature was brought back to room temperature (20°C approx.) after being stored at 4°C.

Table 4.5: Effect of temperature (°C) on the mycelial growth and density of different strains of *Isaria* isolates collected from different ecological regions of Darjeeling hills. [a]*Average of three independent colony radial diameters with standard deviation. c: Compact, sc: Moderately compact, st: Moderately thin and t: Thin. Temperature effect was conducted in potato dextrose agar medium (PDA).*

Strain No.	**Mycelial growth (mm)[a] and density**				
	12	**16**	**20**	**24**	**28**
IDAR-01	21.8±0.83c	32.6±1.14c	38±1.22c	35.8±1.64c	31.4±0.54c
IDAR-02	24.8±0.83c	31.8±0.83c	33±1.22c	25.4±1.14c	20.8±0.83c
IDAR-03	16.6±0.54sc	25.8±0.83c	40.6±1.14c	33.2±1.48c	30.8±0.83c
IDAR-04	16.8±0.83sc	23.2±0.83c	34.6±1.67c	33.4±1.51c	31±1.22c
IDAR-05	19.6±1.14c	24.4±1.14c	31.8±1.48c	20.6±1.67c	19.4±0.54sc
IDAR-06	14.8±1.09sc	19.8±0.83sc	34±1.22c	35.8±1.48c	23±1.87c
IDAR-07	21.8±0.83c	35±1.0c	42±1.58c	35.4±1.14c	27±0.70c
IDAR-08	25±0.70c	33.8±0.83c	51.6±1.14c	30±1.22c	25±0.70c
IDAR-09	26.2±0.83c	37.2±1.09c	47.8±0.83c	36.2±0.83c	31±1.58c

4.7.2. Effect of pH

To study the effect of pH on the ability of different strains of genus *Isaria* to grow at different hydrogen ion concentration was studied using the PDA medium. Six different pH conditions were prepared by adjusting the media pH with 1N (HCl/NaOH) and set at an interval of pH 1.0 starting from 4.0 up to 9.0 using Systronics pH meter. Inoculation and incubation techniques were followed as described in the material and method section. Since proper utilization of nutrients provided in the media depends largely on the pH of culture media, hence it is imperative to determine the optimum pH for the growth of an organism. Radial mycelial growth under various tested hydrogen ion concentrations is shown in Table 4.6. All the strains grew relatively well at all pH levels tested, although at lower pH, the growth was comparatively very little. However, out of all the strains IDAR-

01, IDAR-06 and IDAR-08 showed better growth at pH 6, 7 and 8 while IDAR-02, IDAR-03, IDAR-04, IDAR-05, and IDAR-09 exhibited better growth at pH: 7, 8 and 9 respectively. It is interesting to note that the mycelial growth of any strain of the genius *Isaria* did not show an abrupt decline above or below pH 7. Furthermore, the mycelial density of all the nine isolates were always observed to be extremely good which in all cases developed abundant mycelial density (+++) at neutral pH and was recorded stable at neutral pH for each replication. It indicated that the different strains of *Isaria* were less sensitive to change in pH. Results as enumerated in Table 4.6., indicate that among all the strains, overall mycelial growth is highest for IDAR-09 (50±0.81mm) at pH 8 followed by IDAR-08 (48.75±0.95mm), at pH 7.

Table 4.6: Effect of pH on the mycelial growth and density of different strains of *Isaria* isolates collected from different ecological regions of Darjeeling hills. [a]*Average of three independent colony radial diameters with standard deviation. c: Compact, sc: Moderately compact, st: Moderately thin and t: Thin. pH effect was conducted on potato dextrose agar medium (PDA).*

Strain No.	Mycelial growth (mm) and density					
	pH 4	**pH 5**	**pH 6**	**pH 7**	**pH 8**	**pH 9**
IDAR-01	37±0.81c	37.25±0.5c	37±1.63c	38±1.41c	37.5±0.57c	37.5±1.73c
IDAR-02	36.25±0.95c	34±1.41c	34.25±0.95c	32.5±1.29c	38.5±0.57c	40.75±0.95c
IDAR-03	29±0.81sc	36.75±0.50sc	34±2.61sc	40.25±0.95sc	41.75±0.95sc	41.75±1.25sc
IDAR-04	26±0.81sc	32.25±0.50sc	31.75±2.16sc	30±1.41sc	31.5±1.29sc	32±0.81sc
IDAR-05	29.25±0.95c	29±0.81c	30.25±0.95c	31±0.81c	30.25±1.50c	33±0.81c
IDAR-06	25±0.81sc	31±0.81sc	30±0.81sc	34±1.41sc	33.25±2.36sc	32.5±1.29sc
IDAR-07	29.75±1.70c	39.25±0.95c	42±0.81c	43.5±0.57c	45.25±0.50c	46.5±0.57c
IDAR-08	34.25±1.25c	42.5±1.29c	46.25±0.95c	48.75±0.95c	46±1.41c	47.5±0.57c
IDAR-09	43±0.81c	45.75±0.95c	45.25±0.95c	47.25±0.95c	50±0.81c	43.75±1.25c

4.7.3. Effect of light

To determine the effect of light on the mycelial growth, PDA medium on a Petri plate was inoculated and exposed to different period of light at 20°C. Of the two sets of plates, one set was incubated under 12 hours continuous light (400 lumen) from 12 cm afar. The second set was incubated under complete darkness for a period of 14 days. In both cases the total incubation period was 14 days. The results are given in Figure 4.2. There was no significant effect of light on the mycelial growth of any of the strains. But it is interesting to note that IDAR-08 showed relatively better growth (51.6±1.14mm) under complete darkness. Similarly, in case of IDAR-03 (47±1mm) and IDAR-04 (46.33±2.08mm), stimulation of luxuriant mycelial growth was observed when the culture was exposed to 12 hours darkness/light for a period of 14 days. Since, the strains are non-phototrophic light intensity is expected not to influence the growth of mycelia. Furthermore, no pronounced colony pigmentation was produced by any of the nine strains irrespective of light/dark conditions.

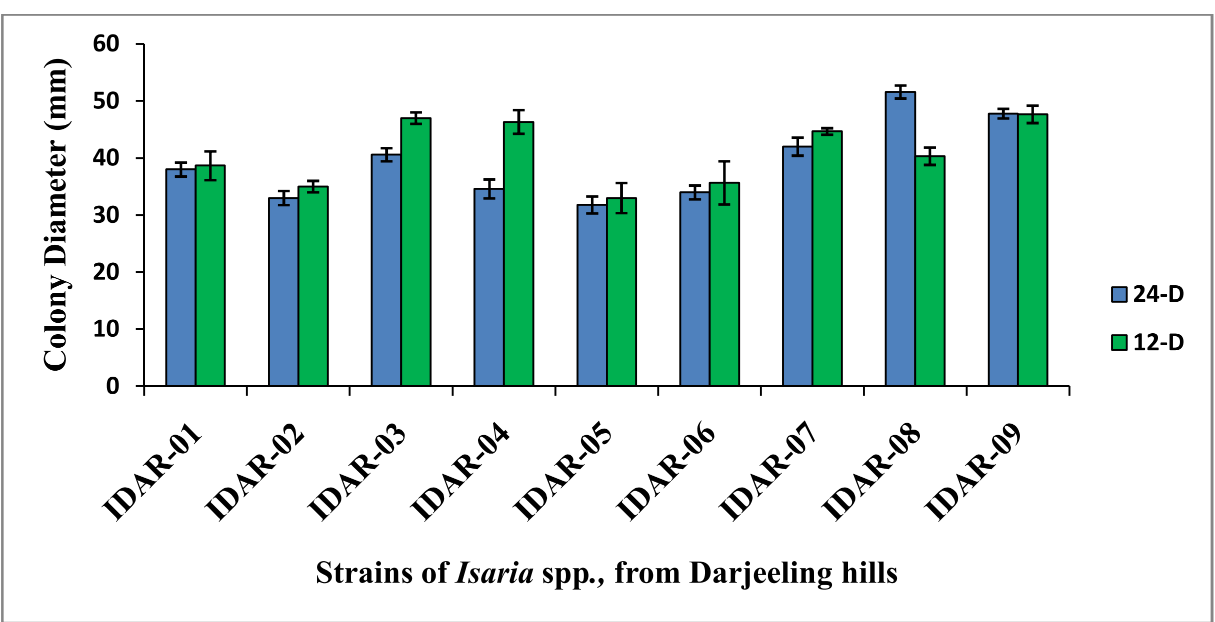


Figure 4.2: Growth properties of nine strains of Isaria isolates from Darjeeling hills on different light-dark settings. Mycelial growth was measured as the diameter of colony (mm) on the culture plates (n=3 plates ± SD of nine readings).

4.8. Effect of different culture media

Fifteen different culture media were used to screen the optimal mycelial growth of nine isolates of the genus *Isaria.* Of the fifteen-culture media tested, MCM medium was the most suitable for favourable growth especially in case of IDAR-01, IDAR-02, IDAR-06, IDAR-07, IDAR-08 and IDAR-09, CDA medium for IDAR-05 and GP medium for the growth of IDAR-04 respectively. The results are presented in Table 4.7. In these media, the colony diameter and density were greater in comparison to other media tested.

However, in general, semi synthetic media like PDA and SDA were also found equally good for the mycelial growth when all the isolates were taken into consideration. It was also recorded that the synthetic media such as glucose peptone and Czapek's agar medium was found to be overall satisfactory in growing all isolates. Regarding the mycelial growth of isolates on salt stress media such as MSA, the trend of growth was dissimilar to the rest of the media tested. It may be inferred that high salt stress of 7.5% (w/v NaCl) resulted in very poor mycelial growth in IDAR-01, IDAR-02, IDAR-05, IDAR-08 and IDAR-09. In the present study 7.5 % NaCl concentration has been found to be inhibitory for the growth of IDAR-03, IDAR-04, IDAR-06 and IDAR-07.

4.9. Effect of carbon source

After making out the role of physical factors in the growth of all isolates an attempt was made to see how far the nature of carbon effects mycelial growth. In order to understand the effect of carbon source in basal medium lacking carbon an experiment was set. The effect of different carbon source on growth by nine strains was investigated in the basal medium supplemented with 1% separately autoclaved individual carbon sources with an initial pH of 7.0 and dark incubated at 20°C for 14 days. Since the carbon source is known

to influence the radial colony growth of almost all fungi, twelve different carbon sources (at 1%, w/v) were tested to determine the most suitable one for mycelial growth.

The result shown in table 4.8 reveals the suitability of various carbon sources for the growth of nine strains. It appears from the results that the supplementation with 1% (w/v) influenced the mycelial growth of all isolates significantly. The growth of all isolates with all carbon sources was evident from the table with varying mycelial growth and density. The result indicated that almost all the nine strains could utilize a wide range of carbon sources for mycelial growth. Among twelve carbon sources tested, some of the isolates such as IDAR-04, IDAR-05, IADR-06, IDAR-07 and IDAR-08, exhibited pronounced vegetative growth on media where the carbon source was disaccharides like maltose and sucrose. The monosaccharides such as dulcitol excreted positive growth influence on respective isolates (IDAR-02, IDAR-08 and IDAR-09), (Table 4.8). It appeared that the disaccharides were most preferably useful for the mycelial growth. However, very weak growth was recorded in the media supplemented with xylose or sodium acetate as the carbon source. Little mycelium density was found on control plate lacking any carbon source.

4.10. Effect of nitrogen source

An experiment was conducted to work out the effects of supplementation of amino acids, inorganic nitrogen and complex organic nitrogen substance in basal media on mycelial growth. The effect of complex organic, amino acids and inorganic nitrogen substance on growth was tested in basal medium supplemented with inorganic and organic nitrogenous substances at 0.5% (w/v) level with pH adjusted to 7.0. However, the nitrogen source was autoclaved separately and aseptically combined afterwards. The experiment was conducted for the period of fourteen days (dark incubation) at 20°C and the results of colony diameter and density so obtained are recorded in Table 4.9.

Table 4.7: Effect of different culture media on mycelial growth of nine isolates of *Isaria* spp., collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD).

	Isolates								
Medium	**IDAR-01**	**IDAR-02**	**IDAR-03**	**IDAR-04**	**IDAR-05**	**IDAR-06**	**IDAR-07**	**IDAR-08**	**IDAR-09**
CDA	44.00±1	37.67±1.52	40.67±2	47.00±2	44.00±1	31.67±1.52	50.33±1.52	49.67±2.51	60.67±1.52
CMA	21.67±0.57	16.00±1	31.00±1	27.67±1.52	22.67±2.08	25.67±1.52	31.00±1	27.33±1.52	40.33±1.52
GP	39.33±0.57	28.67±2.08	48.00±1	52.00±1	33.33±1.52	48.00±2	29.67±1.52	33.33±2.51	48.33±2.08
HB	15.00±1	26.33±1.52	22.33±0.57	30.00±1	35.33±3.05	13.33±1.52	37.33±0.57	43.00±1	51.00±1
HO	41.62±1.52	42.00±1	29.67±1.52	39.00±1	24.67±0.57	25.00±1	40±1	39.00±1	49.00±1
LILLY	43.33±2.08	40.00±1	28.67±1.52	35.33±1.15	29.00±1	33.33±0.57	39.33±1.52	51.33±1.52	53.33±0.57
ME	26.66±1.52	26.66±2.08	37.66±1.52	32.33±1.15	19.00±2.64	35.66±0.57	36.33±1.52	31.33±1.52	31.33±4.16
MCM	47.67±0.57	47.67±1.15	50.00±1	51.00±1	37.33±0.57	50.67±1.52	56±1	59.00±1	58.00±1
MPDA	39.00±0.57	37.00±1	37.00±1	37.00±1	34.00±1	33.67±1.52	42±1	42.67±0.57	45.67±1.52
MSA	21.67±1.15	17.00±0	-	-	20.67±0.57	-	-	18.67±1.15	18.33±0.57
NA	27.67±1.15	19.33±0.57	38.00±2	42.67±2.08	26.00±2	28.00±2	26.67±2.08	15.00±2	34.67±2.51
OMA	25.00±2.64	17.00±1	29.67±1.52	28.67±0.57	24.33±1.52	30.00±1	25.33±0.57	25.33±1.15	35.33±1.15
PDA	40.67±1.15	31.33±1.15	43.67±2.51	44.67±1.15	42.67±1.15	39±2.64	45.33±1.52	39.00±1	47.67±1.52
SDA	34.00±1.73	29.00±1	43.00±1	41.67±0.57	21.67±2.08	43.33±2.08	33.67±1.52	37.67±2.08	42.67±0.57
TA	27.33±2.51	16.67±1.52	25.67±2.08	28.33±0.57	21.33±2.51	21.67±1.15	20.67±0.57	19.00±1	37.00±1

— : *No growth was recorded*

CDA: Czapek Dox Agar., CMA: Corn Meal Agar, GP: Glucose Peptone Agar, HB: Hennerberg Agar., HO: Hopkins Agar., ME: Malt Extract Agar, MCM: Mushroom Complete Medium, MPDA: Martin Peptone Dextrose Agar Medium, MSA; Malt Salt Agar, NA: Nutrient Agar, OMA: Oat Meal Agar, PDA: Potato Dextrose Agar, SDA: Sabouraud Dextrose Agar, TA: Tryptone Agar.

Table 4.8: Effect of different carbon source on the mycelial growth of various strains of *Isaria* spp., collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD). *Effect of carbon source was conducted on basal salt medium with each carbon source being added at 1.0% (w/v) separately.*

Carbon source	**Isolates**								
	IDAR-01	**IDAR-02**	**IDAR-03**	**IDAR-04**	**IDAR-05**	**IDAR-06**	**IDAR-07**	**IDAR-08**	**IDAR-09**
	Mycelial growth (mm) and density								
Dextrose	30.66±0.57	33±1	33±1	29.33±2.5	28.33±1.52	29.66±1.52	35.33v2.08	39.66±1.52	39.66±0.57
Dulcitol	29.33±0.57	38.66±0.57	27±1	25.66±1.52	33±1.73	30.33±1.52	34.33±1.52	44.33±0.57	45.33±0.57
Fructose	29.66±0.57	33.66±0.57	29.6±0.57	31±1	34.33±0.57	23±1	40.66±0.57	39.33±0.57	40±1
Galactose	30±1	36.33±0.57	18.66±2.08	20±1.73	32.33±0.57	18.33±1.52	17±1	39.33±0.57	40.66±1.52
Mannose	30.33±0.57	34.66±1.15	31.66±1.52	30±1	24.33±0.57	29.33±0.57	38.66±0.57	39.33±0.57	38.66±0.57
Trehalose	29.33±1.52	35.33±0.57	32.66±1.15	33±1	38.66±0.57	24.33±0.57	38±1	38±1	39.33±0.57
Xylose	24.66±0.57	37±1	17.66±1.15	17±1	24.33±0.57	18±1	21±2	20.66±2.08	36.66±0.57
Maltose	32±1	22±1	36±1	34.66±1.52	31.66±1.52	31.66±2.08	46±1	46.33±0.57	47.33±0.57
Sucrose	33.33±0.57	36±1	28.33±0.57	28.66±1.52	36±1.73	27.66±1.52	40.33±1.52	39.33±0.57	39.33±1.15
Raffinose	30.33±0.57	38±1	32.3±0.57	28.66±1.52	30.33±1.52	25.33±0.57	38.66±0.57	34±1	43±1
Starch	30.66±0.94	37.33±0.23	31.66±0.47	32±2.12	31.66±0.23	25.66±0.23	40±0.70	42.33±0.47	40.66±0.23
Na-Acetate	31±1	28±1	20.33±2.08	25±1	19.33±0.57	30±1	36±1	41±1	47±1
Control	31±1	22.33±0.57	36±1	25.33±1.15	22±2	23±1	28.66±1.15	40.33±1.52	45.33±0.57

It is clear from the Table 4.9 that mycelial growth occurred in all nitrogen sources. Supplementation of basal growth medium lacking nitrogen with organic and inorganic nitrogenous sources in general showed a stimulatory effect on mycelial growth and density in the selected isolates (Table 4.9), but the growth responses were variable with different nitrogenous substances. Data clearly indicates that all the amino acids tested (asparagine, glycine, cysteine and glutamic acid) supported the growth of all isolates.

Glutamic acid as nitrogen source among amino acids was superior in accelerating the mycelial growth of all isolates producing larger colony diameter followed by asparagine, cysteine and glycine. Yeast extracts amongst organic nitrogenous compounds positively supported mycelial growth. However, among the nitrate nitrogen, sodium nitrate was found to be superior to calcium nitrate and potassium nitrate with respect to mycelial growth and density. All the tested nitrates were better than ammonium sulphate for mycelium growth, although it fulfils the requirement of sulphur. Urea as nitrogen was found to exhibit lesser colony growth of all isolates and very feeble mycelial density was recorded in all the isolates.

Thus, all the nine isolates grew approximately equally well with amino acids, nitrate and complex nitrogen substance. Finally, in the absence of nitrogen source, in the control plates, there is predominance of very thin mycelial growth.

Table 4.9: Effect of different nitrogen source on the mycelial growth of various strains of *Isaria* spp., collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD). *Effect of nitrogen source was conducted on basal salt medium with each nitrogen source being added at 0.5% (w/v) separately.*

Nitrogen source	Isolates								
	IDAR-01	IDAR-02	IDAR-03	IDAR-04	IDAR-05	IDAR-06	IDAR-07	IDAR-08	IDAR-09
	Mycelial growth (mm) and density)								
Asparagine	29.66±2.08	32.66±0.57	22.66±1.15	23.33±1.15	29.66±1.15	26.66±0.57	29.33±1.15	37.66±0.57	39±1
Glycine	25±1	33.66±1.15	22.33±1.52	26.33±0.57	28.661.15	23.33±0.57	31.66±2.08	40.33±2.08	36.33±1.15
Cysteine	25±1	22.66±1.52	23.66±0.57	20.66±2.51	19.66±1.52	16.33±0.57	23.33±0.57	20.33±0.57	34.33±2.08
Glutamic acids	44.33±1.52	34±1	40.66±1.52	41.66±1.52	41.33±0.57	39.661.52	40±1	41±1	33±1
$CaNO_3$	36.33±0.57	15.33±1.15	22.66±0.57	18.66±0.57	19±1	20.66±1.15	30±1.73	32.66±0.57	19.33±3.21
KNO_3	38.66±0.57	37±1	23.33±2.08	24.33±1.52	28.66±1.15	25±1	35±1	37.33±1.52	44.66±1.15
$NaNO_3$	42.66±0.57	28.33±1.52	28.66±1.52	45.66±1.52	44.66±1.52	32±1	36±1	44±1	46.33±0.57
NH_4SO_4	25.66±0.57	21.33±1.52	16.33±0.57	16.66±0.57	21.33±0.57	16.33±0.57	19.66±1.52	23.33±0.57	36.33±0.57
Casein	40.66±0.57	34.33±1.52	38.33±1.52	36.66±1.52	29.66±0.57	40.33±1.52	40.33±0.57	33±1	37±3
Peptone	35.33±0.57	29.33±1.52	40±1	33±2	24.66±0.57	36.66±1.52	46±1	32±1	39±1
Yeast Extract	40±1	41.33±2.08	39.66±0.57	34.33±1.52	28.66±1.52	40.33±1.52	47.33±0.57	48.66±0.57	40.33±1.15
Urea	32±1.15	24.5±2.08	32±2.08	32.5±3.51	16.5±1	13±1.15	13.5±1.52	28±2.51	21±1.15
Control	15±1	19.33±1.52	21±1	20±1	20.33±0.57	19±2	20.66±2.08	23±1	23.33±0.57

4.12. Effect of carbon to nitrogen ratio

The radial mycelial growth of all nine strains of *Isaria* spp., with different C/N ratios (adjusted with different concentration of D-glucose and proteose peptone) for a period of fourteen days is presented in Table 4.10. An optimum C/N ratio of 50: 1 (12.5 g D-Glucose or 5% C and 3.84 g Proteose Peptone) was observed for IDAR-01, IDAR-03 and IDAR-09 while 60:1(15 g D-Glucose or 6% C and 4.61 g Proteose Peptone) for IDAR-04, IDAR-06 and IDAR-08, respectively and a very low C/N ratio of 5:1(1.25 g D-Glucose or 0.5% C and 0.769 g Proteose Peptone) for IDAR-05. The negative control i.e., media containing agar only, showed a mycelial growth with extremely poor level of mycelial density for all nine strains, revealing it could not sustain rich mycelial growth.

4.13. Effect of Vitamins

To study the vitamin requirements of all nine sample strains of *Isaria* spp., an experiment was designed. The basal medium was boiled with activated charcoal (5g/l) and filtered to eliminate any trace of vitamin present in the basal medium (Lilly and Barnett, 1951). Both the basal medium and the stock solution of vitamins (thiamine, biotin, pyridoxine, nicotinic acid, and riboflavin) were sterilized separately and mixed at the rate of 0.5 mg vitamin/1 before inoculation. The results are given in table 4.11. The radial mycelial growth of the medium supplemented with thiamine-HCl was the highest among the tested vitamins for IDAR-09 (41±1mm), even higher than the control medium with all vitamins (control 1) and without vitamin (control 2), although there is no significant difference among these treatments and the media containing biotin (VH), ascorbic acid (VC) nicotinic acid and thiamine-HCl (Table 4.11). It was significant to note that the basal medium (without vitamin) was found to be a poor medium for mycelial growth in all cases excepting IDAR-08 and IDAR-09 which showed comparatively better growth in basal medium than that of a medium containing either ascorbic acid or pyridoxine.

Table 4.10: Effect of different C/N ratios on the mycelial growth and density of various strains of *Isaria* spp collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD). [a]*Average of three independent colony radial diameters with standard error. c: Compact, sc: Moderately compact, st: Moderately thin and t: Thin. Effects of C/N ratio was conducted on basal salt medium.*

Strain No.	Mycelial growth (mm) and density							
	01:01	**05:01**	**10:01**	**20:01**	**30:01**	**40:01**	**50:01**	**60:01**
IDAR-01	29.8±2.77t	32.8±1.30sc	31±1.87c	30.4±1.51c	36±1c	37.8±1.09c	40.4±1.14c	39.4±.89c
IDAR-02	32.8±1.92sc	30.8±1.48sc	42.6±1.67sc	40.8±.83sc	41.2±.83sc	39.4±1.14c	39.8±1.30c	40.8±.83c
IDAR-03	23.8±.83t	24.6±1.14sc	34±3.56sc	36±1sc	37.6±.89c	39.6±1.14c	41.6±1.14c	40.4±1.34c
IDAR-04	38.6±1.14t	37±1.30sc	35.4±1.14sc	36±1sc	37.8±0.83sc	35.6±1.14sc	37.8±0.83c	39.4±1.58c
IDAR-05	41.2±2.58t	36.4±1.14sc	32.2±0.83sc	30.4±1.14sc	30.4±1.14sc	31.8±1.30c	33±0.70c	33.6±1.14c
IDAR-06	23.2±1.48t	26±1.58sc	31.6±1.14sc	29.8±0.83sc	34.6±0.89sc	34±1sc	34±1c	35±1.58c
IDAR-07	33.6±1.14t	33.61.51sc	38.2±0.83sc	40.8±0.83sc	37.2±1.64sc	47.6±2.07c	45.6±1.51c	45.8±0.83c
IDAR-08	30.6±1.51t	40±2.91sc	29.8±0.83sc	45±0.70sc	42.2±1.30c	46.4±1.34c	47.2±1.09c	48.6±1.14c
IDAR-09	34.6±1.94t	40.4±1.14sc	36.8±0.83sc	44.6±0.89c	45.4±1.14c	40.6±1.14c	48.2±0.83c	45.6±0.89c

Table 4.11: Effect of different vitamin source on the mycelial growth of various strains of *Isaria* spp., collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD). *Effect of vitamin source was conducted on basal salt medium with each micro-syringe filter sterilized vitamin source being added at 0.5µg/ml separately. T-HCl= Thiamine HCl.*

Strain No.	**Mycelial growth (mm)**							
	Ascorbic Acid	**Biotin (VH)**	**Inositol**	**Nicotinic Acid**	**Pyridoxine (VB6)**	**Riboflavin**	**T-HCl**	**Control**
IDAR-01	28.66±0.57	27.66±1.52	27.66±0.57	26.33±1.15	28.66±0.57	26.33±1.15	25.33±0.57	22.33±0.57
IDAR-02	25.33±0.57	21±1	25.66±1.52	23.33±1.52	23.66±1.52	24±1	26±1	21.33±0.57
IDAR-03	24.33±0.57	20±1	18.33±0.57	24.33±0.57	22.33±0.57	22.66±0.57	22.66±0.57	20.66±0.57
IDAR-04	18.66±0.57	22±2.64	20±1	18±1	17.33±0.57	18.66±0.57	18.66±0.57	15.66±0.57
IDAR-05	21.33±0.57	28±1.73	20.33±1.52	21.66±0.57	20.33±0.57	18.66±	20.66±0.57	17.33±0.57
IDAR-06	20.66±0.57	25.33±0.57	23±1	19±1	20.33±0.57	19.33±0.57	19.66±0.57	17±0
IDAR-07	22±1	22.66±0.57	23.66±0.57	21.66±1.52	21±1	21.66±0.57	20.66±1.15	18±1
IDAR-08	33±1	34.66±0.57	34.33±1.52	29.66±0.52	30.33±0.57	30.66±0.57	30.66±0.57	26.33±0.57
IDAR-09	36.66±1.52	33.33±0.57	34±1	37.33±0.57	38±1	36.66±0.57	41±1	30.33±0.57

4.14. Effect of Organic acids:

When various organic acids (citric acids, fumaric acid, malic acid, oxalic acid and tartaric acid) were added to the basal medium at a concentration of 0.01% (w/w), oxalic acid was found to be excellent for the mycelial growth of IDAR-03, IDAR-04, IDAR-05, IDAR-07, IDAR-08 and IDAR-09 respectively and citric acid for IDAR-02 and IDAR-06 followed by maleic acid for IDAR-01 (Table 4.12). After 14 days of dark incubation, the mean colony diameters of strains IDAR-08 and IDAR-09 grown in the presence of oxalic acid were 35.66 mm and 35.0 mm, respectively. It was also evident from the results that visual ratings of the mycelial density were extremely poor (-), poor (+) to moderate (++) in few strains. Furthermore, the mycelial growth was found to be scanty and thus it could be assumed from the results that supplementation of organic acids in the basal medium had minimal effect on the growth of mycelia.

Table 4.12: Effect of different organic acids source on the mycelial growth of various strains of *Isaria* spp collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD). *Effect of organic acid source was conducted on basal salt medium with each organic acid source being added at 0.01% (w/v) separately.*

Strain No.	**Mycelial growth (mm)**				
	Citric acid	**Fumaric acid**	**Malic acid**	**Oxalic acid**	**Tartaric acid**
IDAR-01	22±1	12.66±1.15	27±3.05	24.33±1.52	21.33±0.57
IDAR-02	29.33±1.52	18±1	23±2.51	29±1	15.33±1.52
IDAR-03	28±2.64	23±1	20.5±2.64	30.66±2.08	24.33±1.52
IDAR-04	30±1	19±2	29.5±1	32±1	18±1
IDAR-05	24.33±0.57	19±2.64	33.5±1	38±1	26.33±1.15
IDAR-06	31.33±1.52	21±1	21.5±1	31±1	20±1
IDAR-07	30±1	20±2	24.5±1	28.33±1.15	28±1
IDAR-08	29±1	23±1	21.5±1	35.66±1.15	32±1
IDAR-09	34.66±1.52	23.66±2.51	36.5±1	35±2	35±1

4.15. Effects of Mineral elements

Eight mineral macro and micro elements in its salt form were used to study their effects on the process of mycelial growth elongation. The mycelial growth is sensitive towards the presence of mineral elements and therefore an attempt was made to find out its effects on the mycelial growth. All the trace elements were added separately to MPDA medium at a concentration of 0.01% (w/v) and the macro elements at a concentration of 0.05% (w/v). The mycelial growth of almost all the strains was significantly increased in the MPDA medium with all the macro-elements tested than the medium (control) without any mineral element. The lack of any of the macro-elements from the complete medium did not have any significant effect on mycelial growth, although the highest yield of colony diameter occurred in the complete medium supplemented with calcium. There was no significant difference of the colony diameters in the medium supplemented K^+, Mg^{2+}, Ca^{2+} and Na^+ and between the medium without any macro-element.

It is clear from the results that the mycelial growth of IDAR-01, IDAR-05, IDAR-08 and IDAR-09 was significantly enhanced in the medium containing Fe as the trace-elements, compared with that in the control medium (Table 4.13). The colony diameter in the basal medium without mineral nutrient's (control) was significantly less than that in the medium supplemented with mineral nutrients. The medium supplemented with copper had the toxic effect on growth in case of IDAR-01 and IDAR-09. The growth of IDAR-01 (12.33±1.52 mm) and IDAR-09 (15±1mm) was found to be adversely affected with supplementation of copper in the basal media and its presence significantly decreased the growth of these strains. Thus, all the strains exhibited excellent mycelial density of visual ratings (+++) on supplementation of mineral elements.

Table 4.13: Effect of different mineral elements source on the mycelial growth of various strains of *Isaria* spp., collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD). *Effect of mineral elements source was conducted on Martin's peptone dextrose agar with each macro and micro element source being added at 0.05% and 0.01% (w/v) separately.*

Strain No.	**Mycelial growth (mm)**								
	Fe	**Zn**	**Mn**	**Cu**	**K**	**Mg**	**Ca**	**Na**	**Control**
IDAR-01	38±1	33.66±1.52	32.66±0.57	12.33±1.52	38±1	37.33±1.52	39±1	39±1	32.66±1.52
IDAR-02	26.66±1.52	40±1	32.66±2.08	36.66±1.52	34.66±0.57	36.66±1.52	44.66±1.15	40±1	32±2
IDAR-03	37.66±1.52	27.66±2.08	36±1	46±1	39±1	38±1	42.66±0.57	36.66±1.52	30±1
IDAR-04	39.33±2.08	40.33±2.51	38.66±1.52	38.33±1.52	32±1	35±1	33.66±0.57	36±1	35±1
IDAR-05	35±1	16±1	33.33±0.57	29.66±0.57	44.33±1.52	41±1	45±1	35±1	32±2
IDAR-06	32±1	31.33±1.52	32.66±2.51	35±1	40±1	36±1	33.66±0.57	35±1	29±1
IDAR-07	38±1	46±1	48.66±0.58	49.33±0.57	42±1	44.33±1.52	38.33±1.52	41.33±1.52	36±2
IDAR-08	50±1	52±1	50.33±1.52	42.33±0.57	36.33±0.57	39±1	38.66±1.15	41±3.60	38±2
IDAR-09	50±1	48±1	44.66±1.52	15±1	46±1	40±1	48±1	47.66±0.57	33.66±1.52

4.16. Effect of Medium Composition on Radial Growth Rate.

Radial growth was measured in terms of mycelial elongation, diameter of the radial mycelial colony on the surface of agar media. The mycelial growth rate was calculated as described previously by Nguyen *et al.,* (2020) using the formula: V = D / T, where V is the mycelial growth rate (mm/day), D is the diameter growth (mm), and T is the incubation time (days).

In this experiment, three types of fungal nutrient media were used to determine the growth rate in mm per day. Mycelial growth was measured as the mean of three diameters taken during the incubation period of fourteen days. As recorded in the Figure 4.3, the growth rate was found to be varied from 1.5-3.5 mm on SDA and PDA agar plates whereas 3.4-4.2 mm with MCM agar plate. Growth rate of all the isolates was also effected by varied composition of medium.

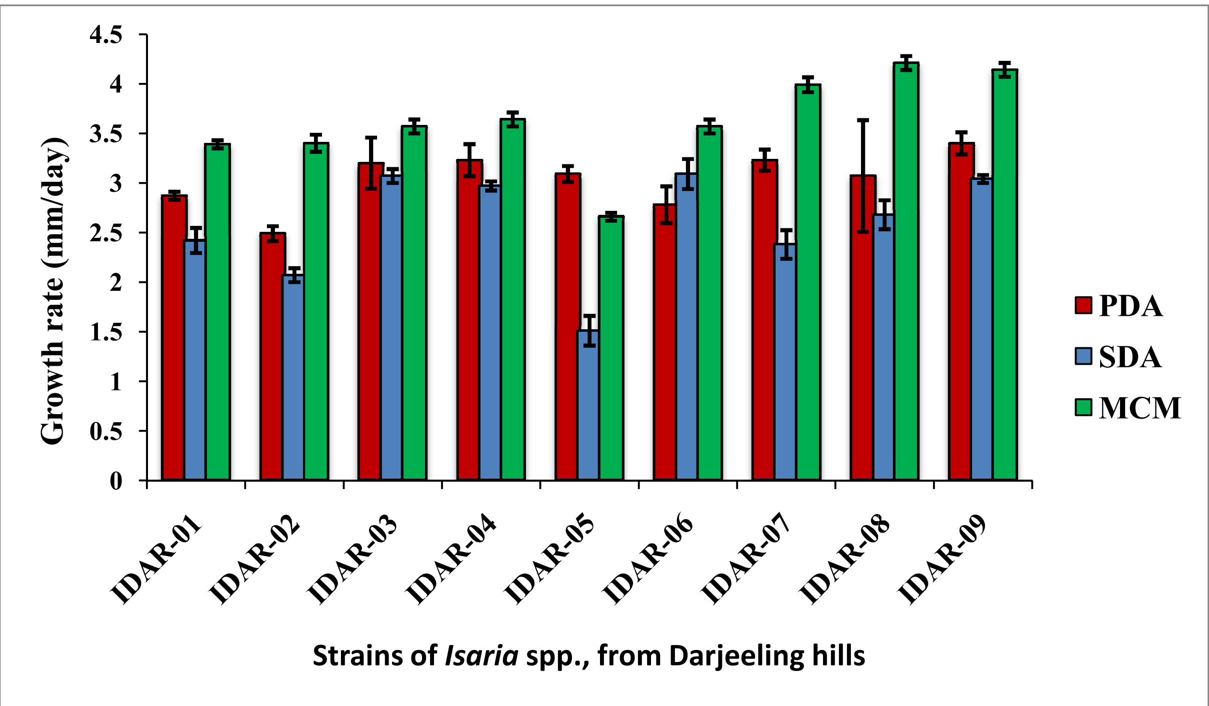


Figure 4.3: Effect of different media source on the mycelial growth of various strains of *Isaria* spp., collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the diameter of the colony (mm) on the culture plates (n=3 plates ± SD of nine readings) at 20°C, PDA= Potato Dextrose Agar, SDA= Sabouraud Dextrose Agar and MCM= Mushroom complete Medium.

4.17. Static fermentation and inoculation density

An experiment was conducted for selection of optimum inoculum size and its effect on mycelial dry weight of all nine isolates. To select the optimum inoculum size, three to eight mycelial discs (5 mm) were inoculated in 30 mL of potato dextrose broth in a 300ml culture vessel and incubated at 20°C for 14 days. It was evident from the figure that the optimal mycelial growth in liquid static culture increased as the number of mycelial discs increased up to five to six, but more than six discs did not increase the dry weight (Figure 4.4). Thus, three to five mycelial discs were found to be optimum for 30 mL of liquid culture. However, three discs gave a comparable mycelial yield in terms of dry weight and thus it was used as a standard size of inoculum for all the experiment carried out in liquid static media.

4.18 Effect of different media and incubation time on the growth of fungal biomass and media pH.

The effect of incubation period on the growth of nine isolates was studied *in vitro* using CDB, MCM and PDB broth medium. To determine the optimum culture period, three mycelial discs (5 mm) were inoculated in 30 mL of each broth medium and incubated at 20°C (dark) for 5 to 15 days under liquid static condition. Mycelial dry weight was measured as mentioned above for all experiments. The results of mean mycelial dry weight and pH of nine isolates as affected by three culture broth media are incorporated in Table 4.14. The pH of the growth medium was initially adjusted at 7.0 which became acidic between day five and day fifteen of growth. The pH of the medium remained low for all the isolates with exception to IDAR-01.

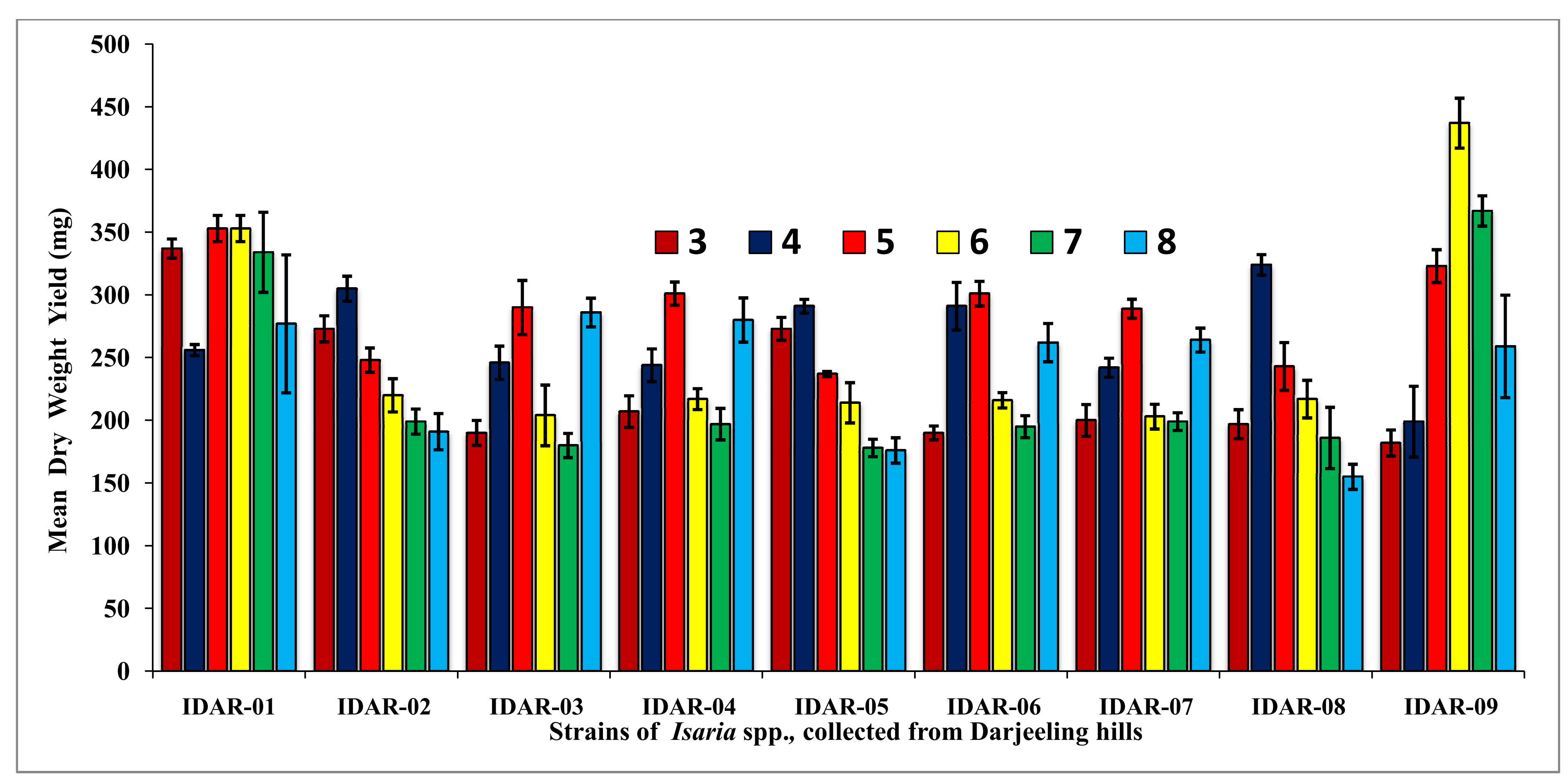


Figure 4.4: Effect of inoculum size on mycelial dry weight of nine isolates of genus *Isaria* spp., collected from different ecological regions of Darjeeling hills on potato dextrose agar broth incubated at 20°C for 14 days liquid under static condition.

The pH of IDAR-01 grown on CDB medium increased slightly towards alkaline pH following fifteen days of continual growth. The results clearly showed that the mycelial biomass content of all nine isolates increased with time up to ten days and then declined while IDAR-01 exhibited maximum growth up to fifteen days of incubation in CDB and MCM, IDAR-03, IDAR-04, IDAR-06, IDAR-07, IDAR-08 and IDAR-09 gave similar results in CDB. In this experiment it was also noted that the rate of growth was maximum between five to ten days based on the mycelial dry weight.

Of the three different liquid culture media used per strain at static condition, PDB and MCM produced the best results for the majority of strains analysed. Generally, these media significantly improved the overall mycelia biomass production over time of each of the selected species. However, for large scale production of fungal biomass, PDB is selected as preference to other two tested liquid medium owing to ready availability. Thus, PDB liquid static culture for ten days at 20°C is recommended for further research.

Table 4.14: Effect of growth media, incubation period on fungal biomass and pH over time on the mycelial growth and dry weight of various strains of *Isaria* spp., collected from different ecological regions of Darjeeling hills. Mycelial growth was measured as the dry weight of the mycelium in a liquid static culture (n=3 flask ± SD of three readings).

		5-Days		10-Days		15 Days	
Fungi	**Medium**	**DMW (g)**	**Final PH**	**DMW (g)**	**Final PH**	**DMW (g)**	**Final PH**
IDAR-01	**CDB**	0.27±0.04	7.49±0.37	0.27±0.04	7.29±0.23	0.37±0.03	8.22±0.14
	MCM	0.21±0.03	3.9±0.02	0.25±0.04	3.63±0.26	0.42±0.01	4.53±0.16
	PDB	0.13±0.03	5.27±0.24	0.39±0.13	5.78±0.16	0.33±0.11	3.83±0.22
IDAR-02	**CDB**	0.28±0.06	4.67±0.54	0.45±0.08	6.07±1.18	0.40±0.1	6.01±0.57
	MCM	0.31±0.06	3.94±0.09	0.47±0.06	4.71±0.32	0.43±0.32	4.88±0.69
	PDB	0.22±0.04	5.2±0.14	0.47±0.05	3.92±0.03	0.36±0.02	3.28±0.15
	CDB	0.407±0.012	6.44±0.303	0.373±0.033	5.87±0.05	0.38±0.04	6.01±0.15
IDAR-03	**MCM**	0.22±0.02	4.46±0.03	0.340.04	4.48±0.25	0.22±0.05	4.30±0.01
	PDB	0.39±0.47	4.83±0.07	0.40±0.01	4.59±0.19	0.25±0.02	3.62±0.04
	CDB	0.27±0.03	6.30±0.12	0.31±0.008	6.07±0.17	0.32±0.01	6.38±0.22
IDAR-04	**MCM**	0.22±0.02	4.46±0.03	0.34±0.04	4.48±0.25	0.22±0.05	4.30±0.01
	PDB	0.39±0.47	4.83±0.07	0.40±0.01	4.59±0.19	0.25±0.02	3.62±0.04
	CDB	0.25±0.03	5.17±0.12	0.39±0.14	5.98±0.16	0.32±0.03	3.69±0.29
IDAR-05	**MCM**	0.21±0.03	3.92±0.10	0.29±0.004	3.64±0.06	0.18±0.03	4.66±0.66
	PDB	0.22±0.05	5.53±0.35	0.31±0.10	4.04±0.129	0.27±0.02	3.62±0.11
	CDB	0.28±0.02	6.33±0.13	0.25±0.13	6.17±0.11	0.30±0.01	6.70±0.11
IDAR-06	**MCM**	0.18±0.01	3.73±0.31	0.37±0.01	6.02±0.05	0.34±0.24	3.64±0.0+6
	PDB	0.14±0.005	5.86±0.54	0.45±0.05	4.22±0.15	0.24±0.01	3.83±±0.06
	CDB	0.23±0.04	6.85±0.22	0.20±0.07	6.37±0.28	0.27±0.05	6.54±0.32
IDAR-07	**MCM**	0.18±0.01	5.75±0.03	0.29±0.01	4.60±0.58	0.23±0.06	5.40±0.09
	PDB	0.162±0.01	5.51±0.03	0.36±0.006	4.82±0.06	0.20±0.06	5.14±0.10
	CDB	0.31±0.05	7.03±0.49	0.26±0.06	6.36±0.29	0.37±0.03	7.49±0.24
IDAR-08	**MCM**	0.22±0.03	5.75±0.09	0.42±0.03	4.60±0.58	0.24±0.20	5.76±0.43
	PDB	0.206±0.007	5.62±0.21	0.37±0.01	5.83±0.23	0.38±0.01	4.48±0.25
	CDB	0.29±0.04	6.72±0.16	0.30±0.03	6.54±0.09	0.39±0.07	6.81±0.26
IDAR-09	**MCM**	0.15±0.009	4.97±0.21	0.44±0.21	5.08±0.75	0.25±0.08	4.61±0.7
	PDB	0.37±0.39	5.31±0.08	0.30±0.01	3.56±0.08	0.24±0.06	3.07±0.12

4.19. Percent (%) yield of crude extract of various strains of *Isaria* spp., collected from different ecological regions of Darjeeling hills.

5 g finely ground pulverised mycelium was extracted by maceration three times with 70% methanol (aqueous), evaporated to dryness and weighed to obtain the yield. The same procedure was repeated for all investigated fungal isolates and the results of percent yield of mycelial extract of nine isolates are given as a percentage of the original weight of crude sample (Table 4.15). The wet weight values of all nine extracts under study was found to range from 8.17±5.20g for mycelium of *I. farinosa* IDAR-05 to 2.79±8.74g in *I. farinosa* IDAR-08. The dry weight of nine isolates was found ranging maximum from 0.634±0.06g in *I. tenuipes* IDAR-06 to a minimum of 0.308±0.12g in *I. fumosorosea* IDAR-09. Among the nine isolates the percent yield of cultured mycelial methanol extract was the highest for *I. fumosorosea* IDAR-09 (14.02%) while extract of *I. farinosa* IDAR-05 had the smallest yield (10.55%). The extraction yield percentage of all the extract was found to be in the following relative decreasing order of: IDAR-09(14.02%)> IDAR-07(13.19%)> IDAR-01(12.97%)> IDAR-08 (11.95%)> IDAR-06 (11.69%)> IDAR-03 (11.50%)> IDAR-02 (11.31%)> IDAR-04 (10.65%)> IDAR-05 (10.55%). The data herein reveals that the extraction yield of all isolates was found similar when compared amongst isolates with aqueous alcohol as an extraction solvent. The probable explanation may be attributed to the higher solubility of carbohydrates and proteins in protic solvent water present in the extraction solvent (7:3 v/v Me-OH-H_2O).

Table 4.15: Extraction yield efficiency of various strains of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for mycelial extraction. (n=3, ± SD).

Isolates	Wet weight (g)	Dry weight (g)	Yield (%)[3]
IDAR-01	4.40±4.63	0.385±1.30	12.97±0.07
IDAR-02	5.52±7.94	0.469±0.49	11.31±0.11
IDAR-03	5.92±3.45	0.399±0.16	11.50±0.47
IDAR-04	5.95±5.86	0.410±0.11	10.65±0.02
IDAR-05	8.17±5.20	0.366±0.30	10.55±0.12
IDAR-06	4.78±5.25	0.457±0.50	11.69±0.12
IDAR-07	4.16±1.06	0.634±0.06	13.19±0.22
IDAR-08	2.79±8.74	0.373±0.11	11.95±0.18
IDAR-09	5.53±4.60	0.308±0.12	14.02±1.48

4.20. Qualitative phytochemical analysis

Qualitative analysis for the detection of various phytochemicals viz. alkaloids, anthraquinones, flavonoids, saponins, tannins, terpenoids, glycosides, phenols, steroids, triterpenoids and proteins, was carried out from cultured mycelial methanol extract of nine isolates. Results summarised in Table 4.16., indicated that various classes of phytochemical did not obey the polarity of solvents for compound resolution. The preliminary chemical screening results confirmed the presence of flavonoids, phenolic compound, phlobatannin, tannins, saponins, proteins and glycosides in all nine isolates and steroid were present in only in IDAR-04 and IDAR-06. The presence of these phytochemicals in the concentrated methanolic extracts based on visual inspection varied quantitatively from mild positive to highly positive in all extracts of different isolates. Anthraquinones, alkaloid, terpenoid and quinines were absent in all nine isolates. Further, cultured mycelial extracts contained the maximum phytochemical classes.

Table 4.16: Preliminary biochemical analysis for the detection of phytochemicals in concentrated methanolic extract from mycelia of *Isaria* isolates from Darjeeling hills. (n=3).

Test of Compound class	IDAR-01	IDAR-02	IDAR-03	IDAR-04	IDAR-05	IDAR-06	IDAR-07	IDAR-08	IDAR-09
Alkaloids	−	−	−	−	−	−	−	−	−
Phenolic compounds	++	+++	+++	+++	++	++	++	++	+++
Flavonoids	−	+	+	+	−	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+
Phlobatannin	−	+	−	+	+	+	+	−	+
Glycoside	++	+++	++	+++	++	++	++	++	+++
Proteins	+	+	+	+	+	+	+	+	+
Saponins	+	++	+	+++	+	+	++	+	+
Anthraquinones	−	−	−	−	−	−	−	−	−
Quinones	−	−	−	−	−	−	−	−	−
Steroid	−	−	−	+	−	+	−	−	−
Terpenoids	−	−	−	−	−	−	−	−	−

Note: +: Mild positive; ++: Positive; +++: Highly positive; −: Negative

4.21. Comparison of IR spectra from four different species of genus *Isaria*

In order to examine the probable chemical compositions of the crude methanolic extract, FTIR (Fourier Transform Infrared) spectroscopy was used. The spectrum analysis (Fig. 4.5/A) of the lyophilized extract of *Isaria javanica* IDAR-01 shows the characteristic absorption peaks at 3302 cm^{-1} (R_2-N-H stretch), 2924 cm^{-1} (aliphatic C-H stretching, asymmetric) 2853 cm^{-1} (C-H stretching, symmetric) respectively. The vibration at 1746 cm^{-1} indicates γ C=O stretching and γ C-(C=O)-C stretching at 1638 cm^{-1} whereas peak at 1468 and 1378 cm^{-1} indicate γ C-H bending followed by spectrum at 1240 cm^{-1} and 1165 cm^{-1} indicates γ C=O stretching and minor peak at 722 cm^{-1} reflects γ C-C bending out of plane.

The FTIR spectrum analysis (Fig 4.5/B) of the lyophilized extract of *Isaria tenuipes* IDAR-04 shows the characteristic absorption peaks at 3292 cm^{-1} (γ -N-H stretch), 2931 cm^{-1} (aliphatic C-H stretching, asymmetric) and 2855 cm^{-1} (C-H stretching, symmetric) respectively. The vibration at 1622 cm^{-1} indicates γ C=N stretching and γ N-O (cis) stretching at 1405 cm^{-1} whereas peak at 931 indicate γ N-O stretch.

The FTIR spectrum analysis (Fig 4.5/C) of the lyophilized extract of *Isaria farinosa* IDAR-02 shows the similar characteristic absorption peaks at 3343 cm^{-1} (γ -O-H stretch) indicating the functional group of a carbohydrates, 2929 cm^{-1} (aliphatic C-H stretching, asymmetric) and 2855 cm^{-1} (C-H stretching, symmetric) respectively indicating lipids. The vibration at 1653 cm^{-1} indicates γ C=O stretching and δ ($C-H_2$) stretching at 1454 cm^{-1} whereas peak at 1404 indicate γ C-N stretch. There was a strong absorptive peak for the methanol extract in the vicinity of 890 cm^{-1}, which indicated higher sugar composition compared with intensities of peak at 1259 cm^{-1} and at 1080 cm^{-1} indicating the presence of ethers.

The FTIR spectrum analysis (Fig 4.5/D) of the lyophilized extract of *Isaria fumosorosea* IDAR-09 shows the characteristic absorption peaks at 3300 cm^{-1} (R_2-N-H stretch), 2927 cm^{-1} (aliphatic β ($C-H_2$) stretching, asymmetric), 2853 cm^{-1} (γ ($C-H_2$) stretching, symmetric) 1643

cm^{-1} (γ (C-O stretching) respectively indicating the probable presence of higher lipid content. The vibration at 1460 cm^{-1} indicates δ (C-H_2) suggesting the existence of nucleosides. There was a strong absorptive peak for the methanol extract in the vicinity of 1258 cm^{-1}, 1081 cm^{-1} and 630 cm^{-1} indicating the presence of ethers.

The overall results showed the presence of N–H stretching vibration, C-H stretching, C=N stretching and N-O stretching vibration which could possibly indicate the presence of some alkene, amine and nitro compounds in methanolic extract.

In the present study, FTIR data was evaluated only for four isolates representing four different species. This limitation was based on prospective observational studies of nucleophile functional groups from FTIR chromatogram. We did not include other isolates based on anticipation that these nucleophiles that could participate in oxidation reduction reactions are also present in the replicate collection from various ecological regions.

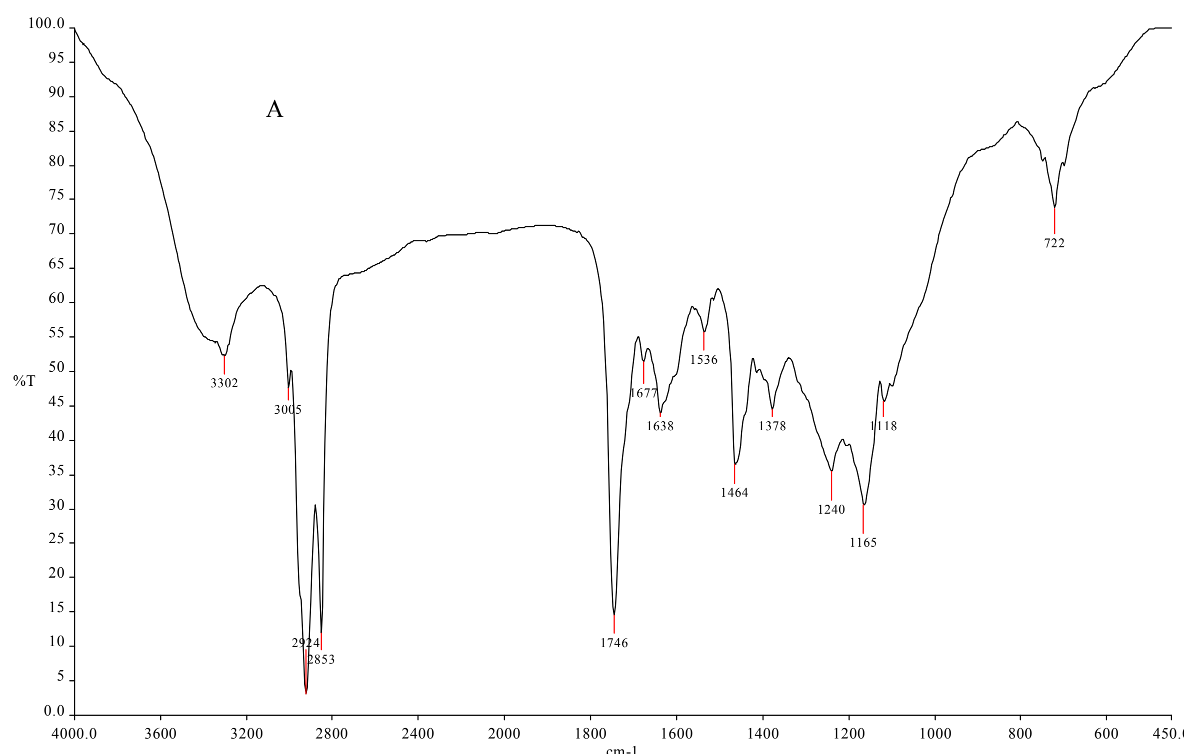


Figure 4.5: Fourier transform infrared spectra of lyophilized extract of **A.** *Isaria javanica* IDAR-01 prepared as KBr pellet and scanned in the range 4000 to 450 cm^{-1}.

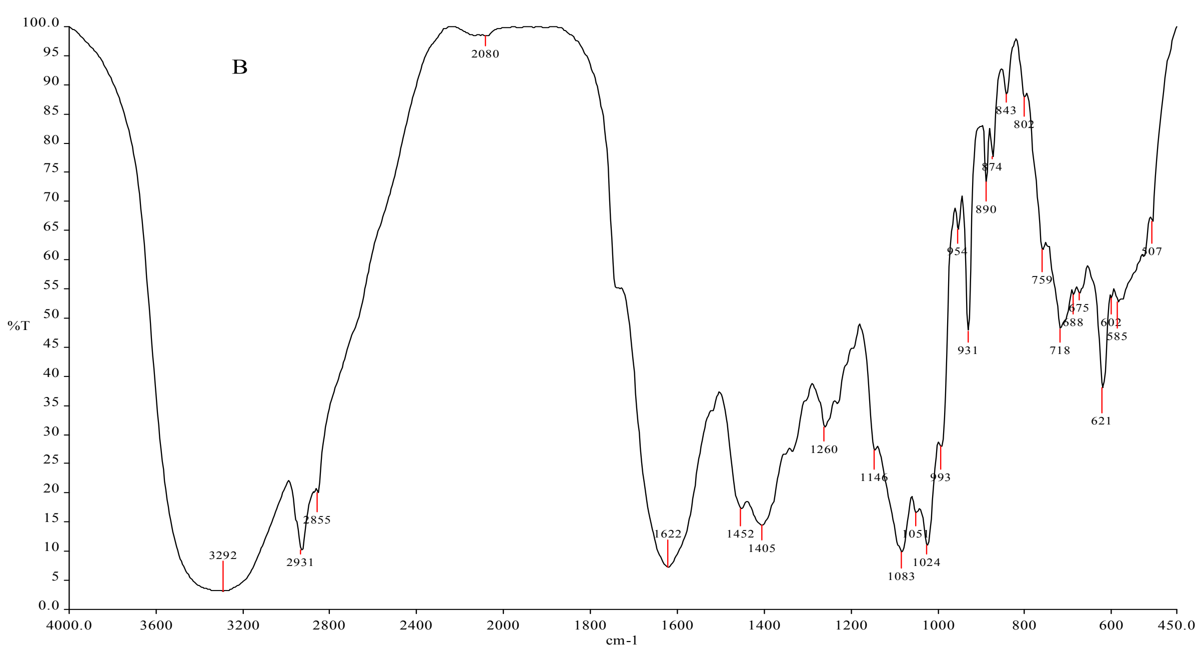


Figure 4.6: Fourier transform infrared spectra of lyophilized extract of **B**. *Isaria tenuipes* IDAR-04 prepared as KBr pellet and scanned in the range 4000 to 450 cm^{-1}.

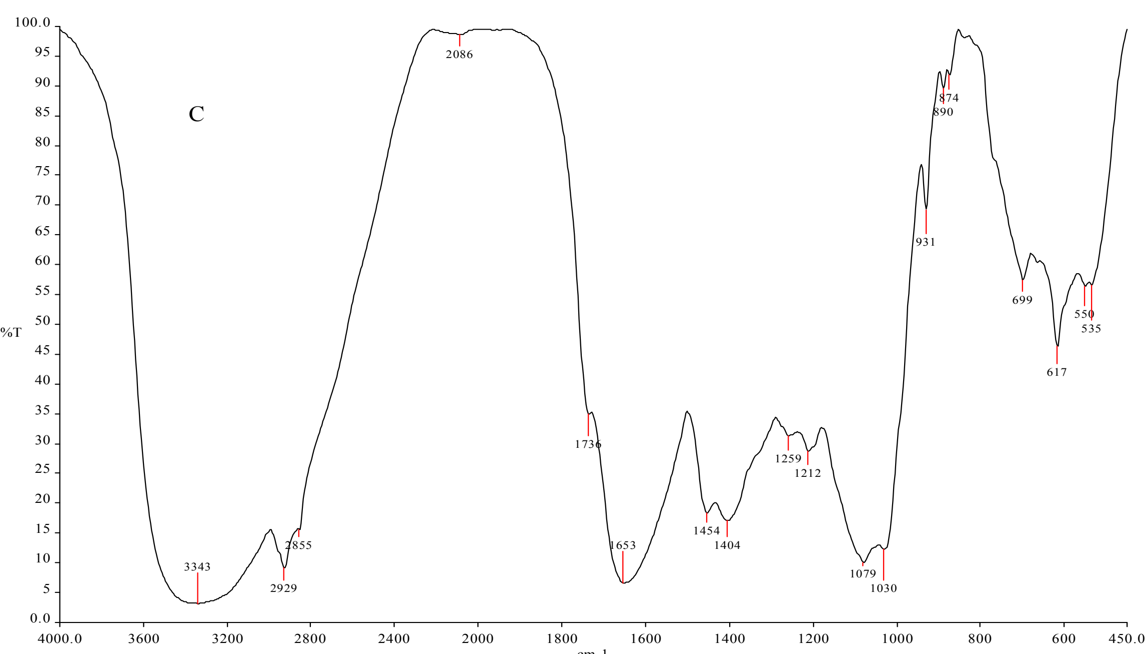


Figure 4.7: Fourier transform infrared spectra of lyophilized extract of **C.** *Isaria farinosa* IDAR-02 prepared as KBr pellet and scanned in the range 4000 to 450 cm^{-1}.

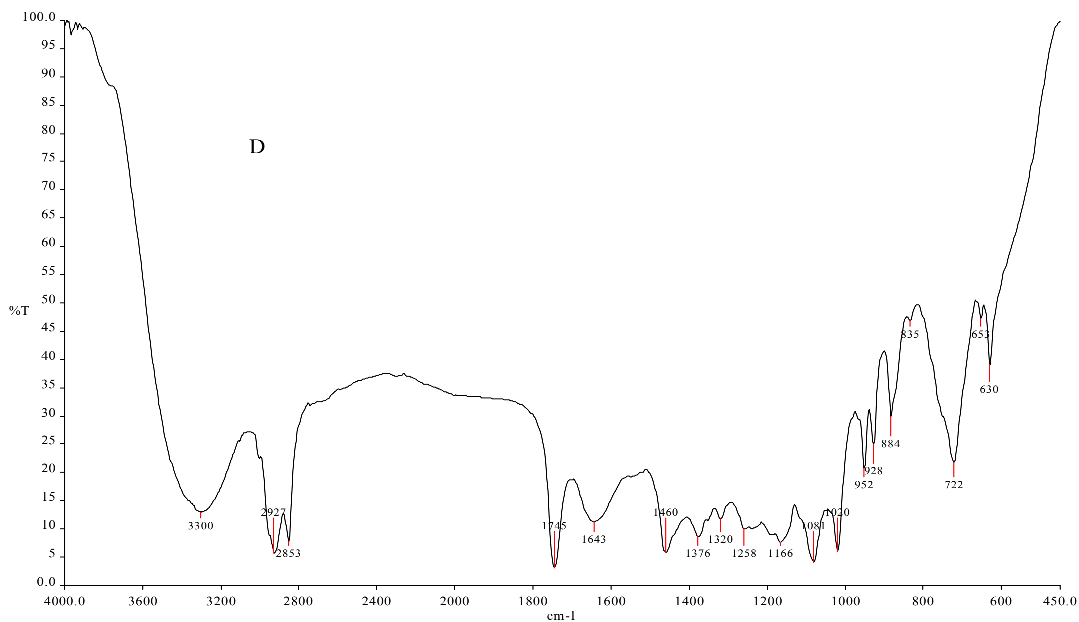


Figure 4.8: Fourier transform infrared spectra of lyophilized extract of **D.** *Isaria fumosorosea* IDAR-09 prepared as KBr pellet and scanned in the range 4000 to 450 cm^{-1}.

4.22 Total phenolic content (TPC)

Figure 4.9., shows the TPC of the extracts measured using the Folin-Ciocalteu method. TPC values were obtained from the calibration curve y =0.0023x+0.0657 with R^2 = 0.9936, where x is the absorbance and y is the concentration of gallic acid solution (µg/mL) expressed as µg GAEs/g. The TPC values of all nine extracts under study was found in ranging from 115.58 µg GAE/g for methanol extract of IDAR-01 to 304.47µg GAE/g for IDAR-02 methanol extract (Figure 4.9) and they decrease in the following relative order: IDAR-02 > IDAR-08 > IDAR-06> IDAR-05 > IDAR-07 > IDAR-03 > IDAR-09> IDAR-04 > IDAR-04 > methanol.

4.23 Total flavonoid content

As presented in figure 4.10., the TPC of the extracts were measured using the $AlCl_3$ method. TFC values were obtained from the calibration curve y =0.0345x+with R^2 = 0.9629, where x is the absorbance and y is the concentration of quercetin solution (µg/mL) expressed as µg QEs/g. The TFC values of the extracts range from 7.39 µg QEs/g for

methanol extract of IDAR-06 to 16.22 µg GAE/g for IDAR-03 methanol extract (Figure 4.10) and they increase in the following relative order: IDAR-06 >IDAR-07 > IDAR-05 >IDAR-04 >IDAR-02 < IDAR-08 >IDAR-09 >IDAR-01 >IDAR-03.

4.24 Total tannin content (TTC)

As presented in figure 4.11., the TTC of the extracts was measured using the $AlCl_3$ method. TTC concentration values were obtained from the calibration curve y =0.0047x-0.0026 with R^2 = 0.992, where x is the absorbance and y is the concentration of tannic acid solution (µg/mL) expressed as mg TAEs/g. The TTC values of the extracts range from 0.257 mg TAEs/g (Tannic Acid Equivalent) for methanol extract of IDAR-08 to 0.483 µg TAEs/g for IDAR-05 methanol extract (Figure 4.11) and they increase in the following relative order: IDAR-08 >IDAR-09 >IDAR-01 >IDAR-03 >IDAR-04 >IDAR-02 >IDAR-06 >IDAR-07 >IDAR-05.

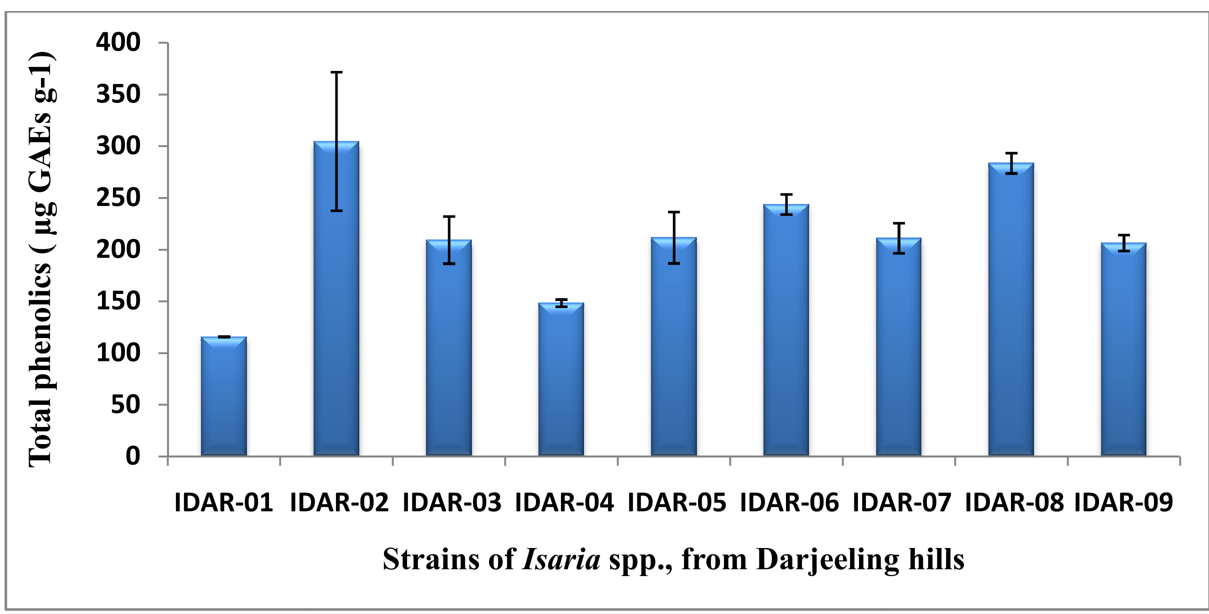


Figure 4.9: Total phenolic content of nine isolates of *Isaria* collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for total phenolics extraction from dried mycelium. (n=3, ± SD).

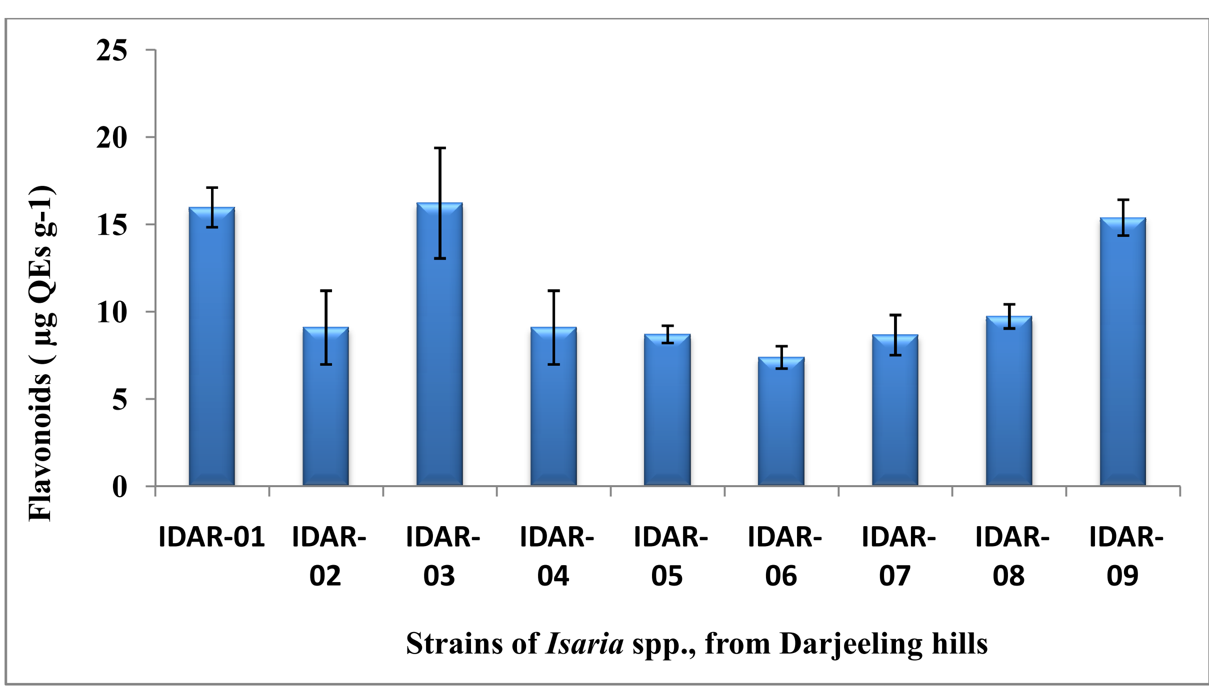


Figure 4.10: Total flavonoid content of nine isolates of *Isaria* collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for total flavonoid extraction from dried mycelium. (n=3, ± SD).

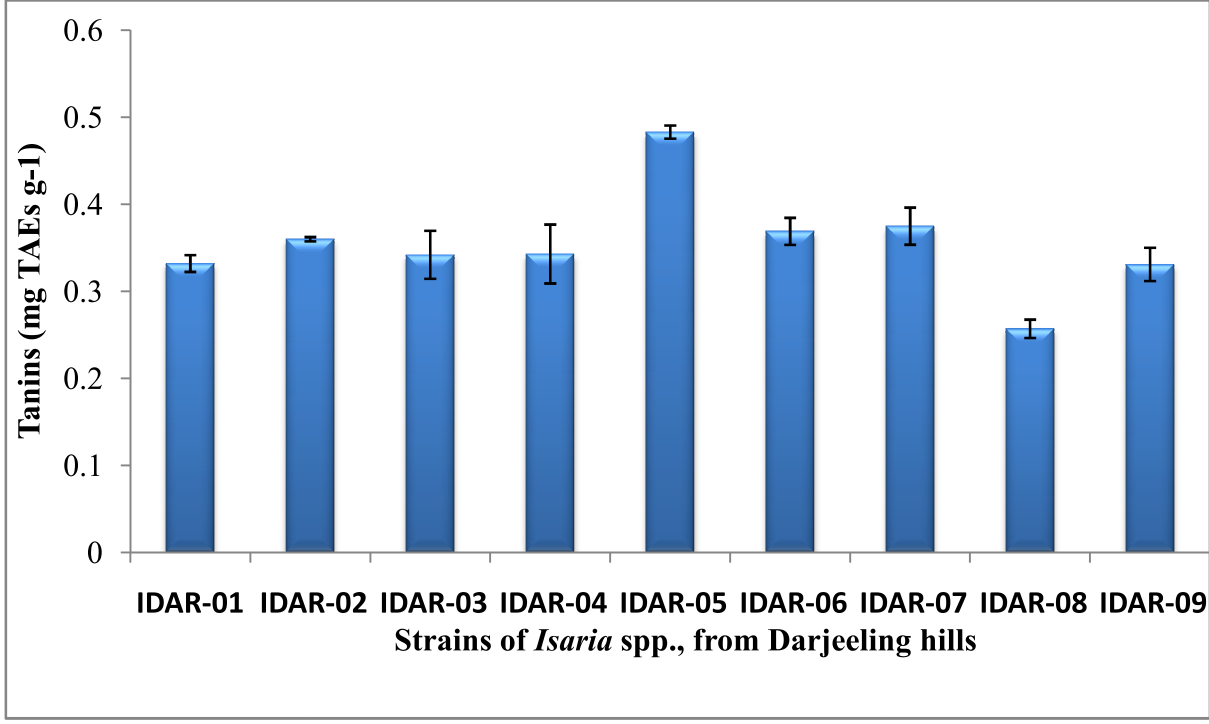


Figure 4.11: Total tannin content of nine isolates of *Isaria* collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for total tannin extraction from dried mycelium. (n=3, ± SD).

4.25 Total β-carotene content

The β-carotene content values of all nine extracts under study was found ranging from 0.055mg/100g extract for methanol extract of IDAR-01 to 0.255mg/100g for IDAR-02 methanol extract (Figure 4.12) and they increase in the following relative order: IDAR-01 > IDAR-06 > IDAR-04> IDAR-07 > IDAR-03 > IDAR-05 > IDAR-08> IDAR-09 > IDAR-02.

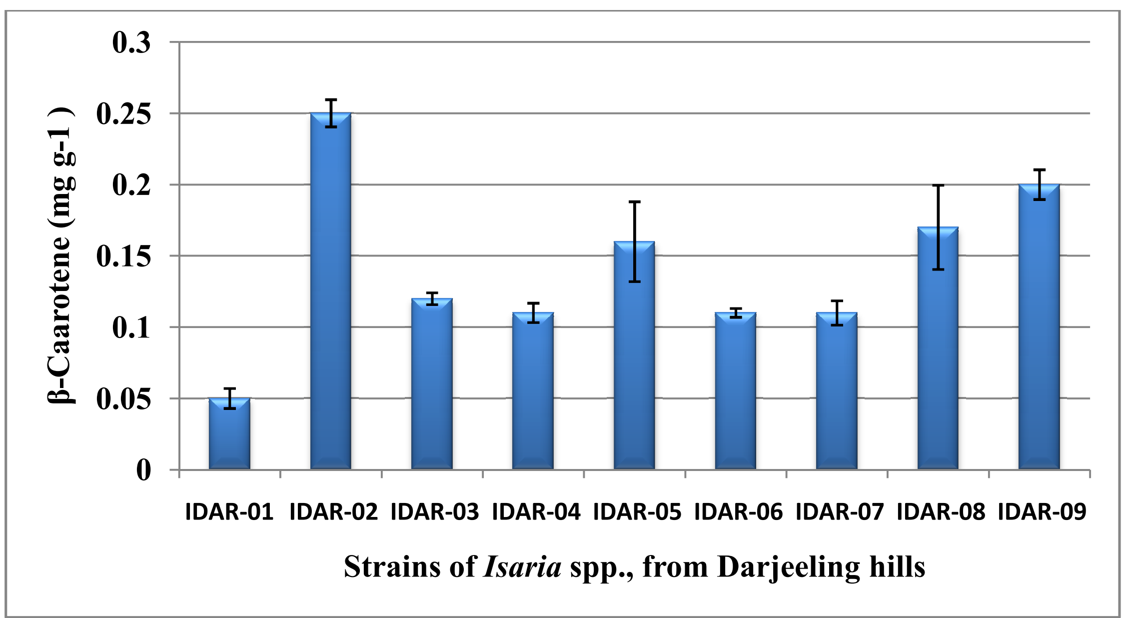


Figure 4.12: Quantitative estimation of β-carotene of nine isolates of *Isaria* collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for β-carotene extraction from dried mycelium. (n=3, ± SD).

4.26 Total lycopene content

The lycopene content of all nine extracts under study was found in ranging from 0.00207mg/100g of extract for methanol extract of IDAR-01 to 0.041mg/100g for IDAR-04 methanol extract (Figure 4.13) and they gradually increase in the following relative order: IDAR-01 > IDAR-08 > IDAR-02> IDAR-05 > IDAR-03 > IDAR-06 > IDAR-09> IDAR-07 > IDAR-04. Amongst the nine isolates maximum amount of lycopene was

evaluated in *I. tenuipes* (IDAR-04), while minimum amount of was there in *I. javanica* (IDAR-01).

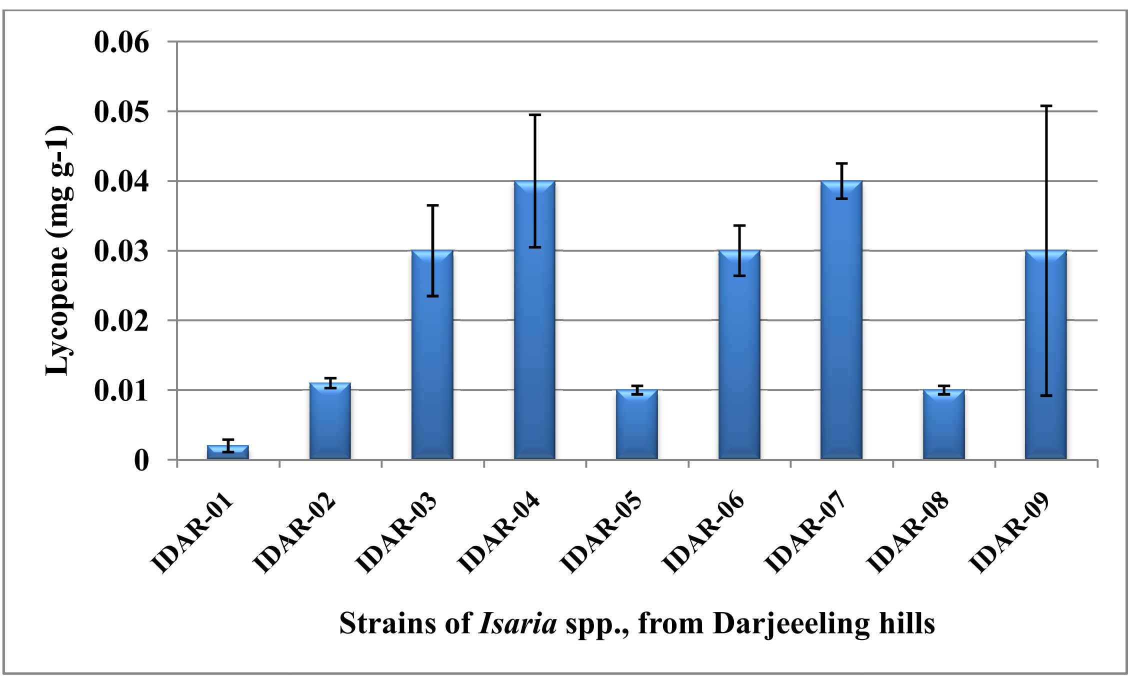


Figure 4.13 Quantitative estimation of lycopene in various extracts of *Isaria* collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for lycopene extraction from dried mycelium. (n=3, ± SD).

The overall extraction yield of various bioactive potential fractions of aqueous methanol mycelial extract is depicted in Table 4.17.

Table 4.17: Estimation of phenolic compounds, carotenoid, and lycopene content of nine isolates of *Isaria* spp collected from different ecological regions of Darjeeling hills. (n=3, ± SD).

Isolates	TPC (µg GAEs/g)	TFC (µg QEs/g)	TTC (mg TAEs/g)	β-Carotene (mg/100g Extract)	Lycopene (mg/100g Extract)
IDAR-01	115.58±0.12	15.98±1.13	0.332±0.009	0.055±0.007	0.002±0.009
IDAR-02	304.47±6.96	9.1±2.11	0.360±0.002	0.255±0.009	0.011±0.007
IDAR-03	209.11±2.28	16.22±3.16	0.342±0.02	0.123±0.004	0.035±0.006
IDAR-04	148.09±3.51	9.1±2.11	0.343±0.03	0.119±0.006	0.041±0.009
IDAR-05	211.43±2.48	8.71±0.5	0.483±0.007	0.162±0.02	0.0115±0.006
IDAR-06	243.45±9.77	7.39±0.64	0.369±0.01	0.114±0.003	0.035±0.003
IDAR-07	210.85±14.56	8.67±1.14	0.375±0.02	0.116±0.008	0.040±0.002
IDAR-08	283.31±9.81	9.74±0.69	0.257±0.01	0.178±0.02	0.0108±0.006
IDAR-09	206.21±7.69	15.39±1.02	0.331±0.01	0.208±0.01	0.0355±0.02

TPC= Total Phenol Content; GAE= Gallic Acid Equivalent; TFC= Total Flavonoid Content; QE= Quercetin Equivalent; TTC=Total Tannin Content; TAE= Tannic Acid Equivalent

4.27 DPPH Assay

All the nine isolates were screened for their antiradical activities. The extracts from all isolates were evaluated for their DPPH radical scavenging abilities at various increasing concentrations ranging from 2-10 mg/ml. The aqueous methanolic extract (70%v/v) from nine isolates of *Isaria* spp showed moderate to high degree of DPPH radical scavenging activity. The variation in the percent inhibition of DPPH radical became more prominent at 4-10 mg/ml concentrations. The extract concentration at 4mg/ml and in the increasing ones beyond that, all the extracts maintained a good percentage of inhibition with exception in isolates IDAR-02 and IDAR-05. However, in comparison, the aqueous methanolic extract showed less DPPH activity than that of synthetic antioxidant standard BHT (Butylated Hydroxytoluene). As can be seen in the Figure 4.14., the various isolates of the genus *Isaria* spp. were shown to scavenge the stable DPPH radical to different degrees. The methanolic extracts of IDAR-09 scavenged the highest percentage of DPPH radicals (77.63±0.64%) compared to other isolates where the ability to scavenge was found to be rather moderate. The lowest scavenging ability was recorded with IDAR-01 (3.33±0.25%). Analysis of the IC_{50} values for the extracts from nine isolates revealed the values as 2.54 ± 0.39/mg/ml, for the IDAR-09, it was 2.91 ± 0.17 mg/ml for IDAR-04 and that for the IDAR-03 the IC_{50} was 3.11± 0.20 mg/ml (Table 4.18). This assay shows second line of defence to scavenge free radicals by suppressing chain initiation and/or by breaking the chain propagation reaction. Thus, the percent inhibition of DPPH radical differed among the different concentrations of mycelial extracts. The IC_{50} value for BHT was found to be 7.2 ± 0.90 µg/ml.

4.28 ABTS radical scavenging activity

The aqueous methanol extract from different isolates of *Isaria* spp was found to efficiently scavenge ABTS radicals generated by the reaction between 2, 2'- azinobis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS) and ammonium persulphate. Result as

presented in Figure 4.12 revealed that the activity was found to be in a dose dependent manner. Amongst the nine isolates tested, IDAR-01 was the most effective with high scavenging activity (83.65±0.37), recorded at 10mg/ml and the lowest was recorded with IDAR-07 (35.92±0.96) at the same concentration tested. The scavenging activity of the 70% methanol extract was comparable among the IDAR-02 and IDAR-03 where the activities ranged from 23.03±0.45% to 60.47±2.47% and 17.83±1.12% to 65.23±0.80% respectively. Thus, IDAR-01 had the greatest activity to quench ABTS radical followed by IDAR-03, IDAR-02, IDAR-09, IDAR-05, IADR-08, IDAR-04, IDAR-06 and the lowest scavenging ability was recorded for IDAR-07 (Figure 4.12). However, as can be seen from the figure 4.15., it was noticeable that percent inhibition of ABTS radical differed among the different isolates of the genus *Isaria*. Considering the higher concentrations, in 10mg/mL tested, the percent inhibition of IDAR-05, IDAR-08 and IDAR-09 appear to be similar to each other. It is worth mentioning that even higher concentration of extracts of IDAR-06 and IDAR-07 could not scavenge 50% of ABTS radical exhibiting IC_{50} >10mg/ml. In the results on the IC_{50} it was recorded that the variance of its values with different isolates was relatively larger amongst one another. The lowest IC_{50} values were recorded for IDAR-01 with 2.9±0.27mg/ml followed by IDAR-02 with 3.36mg/ml. The IC_{50} value for BHT was found to be 2.77 ± 0.55 μg/ml.

4.29 Hydroxyl radical scavenging activity

The •OH scavenging activity of mycelial methanolic extracts of nine isolates was assessed by its ability to compete with salicylic acid for •OH radicals in the •OH generating/detecting system. As shown in Figure 4.16., as concentrations of the mycelial methanolic extracts increased, their •OH scavenging activity also increased and the enhanced activity was maximally discernable at highest concentration tested (10mg/mL) in all the isolates. Further, the scavenging effects of methanolic extracts from IDAR-07, IDAR-08 and IDAR-09 on

hydroxyl free radicals were observed to be the highest (>50%) at 10 mg/ml. At 10 mg/ml, scavenging effects for the methanolic extracts from IDAR-07, IDAR-08 and IDAR-09 were 55.0%, 50.47% and 55.78%, respectively. In addition, at 2-10mg/ml, the scavenging effect of methanolic extracts from the isolates of IDAR-01, IDAR-02, IDAR-03, IDAR-04, IDAR-05 and IDAR-06 on hydroxyl radicals was below 50.0%. In general, all the mycelial extracts showed relatively weak ability to scavenge OH radical and this apparent inhibition of hydroxy radical was noted over the control. However, the scavenging effect of mannitol at 200μg/ml was 25.17%. The IC_{50} values of extracts from IDAR-07, IDAR-08 and IDAR-09 were 3.95±0.32, 5.60±0.45 and 3.44±0.06 mg/ml respectively. The IC_{50} values of other extracts were found to be more than 10mg/ml.

4.30 Hydrogen peroxide Scavenging Activity

To ascertain whether the mycelial extract will exhibit the scavenging effect on hydrogen peroxide in a concentration dependent manner, the hydrogen peroxide scavenging assay was performed. In the present study, different concentrations of mycelial extract ranging from 2mg/ml to 10mg/ml were used in hydrogen peroxide scavenging assay. As revealed in the Figure 4.17., it was observed that there was a prominent difference in the scavenging ability between the standard and the test extracts. In addition, moderate level of hydrogen peroxide scavenging activity was observed for all the extracts with >50% scavenging activity recorded for IDAR-04 and IDAR-06 in presence of methanolic extracts at a concentration of 10 mg/ml. According to the present results, <50% scavenging activity were recorded for the rest of the isolates IDAR-01, IDAR-02, IDAR-03, IDAR-05, IDAR-07, IDAR-08 and IDAR-09 even at a highest tested concentration. In general, the scavenging activity of all the isolates under investigation was close to each other and the pattern of inhibition was more or less similar. However, a positive standard sodium pyruvate at a concentration 2mg/ml was found to be far more effective in exhibiting hydrogen peroxide scavenging activity

(56.29%). IC_{50} values could not be obtained for hydrogen peroxide scavenging activity for seven isolates with exception to *I. tenuipes* (IDAR-04) with 3.85±0.08mg/ml, and *I. tenuipes* (IDAR-06) with 3.60±0.50mg/ml. However, IC_{50} value of the remaining methanol extract from cultured mycelium was higher than 10 mg/ml, which was the highest concentration tested.

4.31 Determination of reducing power

The respective reducing power values (Figure 4.18) of the aqueous methanolic extracts revealed that IDAR-01 had the highest value followed by IDAR-09, IDAR-05, IDAR-07, IDAR-02, IDAR-08, IDAR-04, IDAR-03 and IDAR-06 that also showed good reducing power activity. The ferrous reducing antioxidant power varied from 1.329 for IDAR-01 to 0.32 for IDAR03.

4.32 Chelating effect on ferrous ions

The chelating activity at various concentrations of aqueous methanolic extracts was examined and compared to a chelating standard, EDTA. Figure 4.19., displays the effective concentrations (mg ml^{-1}) at which ferrous ions were chelated. The figure 4.16 shows that the chelating ability of all aqueous methanolic extracts was excellent showing >80 chelating power at highest tested concentration. According to their IC_{50} value (mg ml^{-1}), the extract of IDAR-02 had the most chelating power of the isolates analysed (Table 4.18).

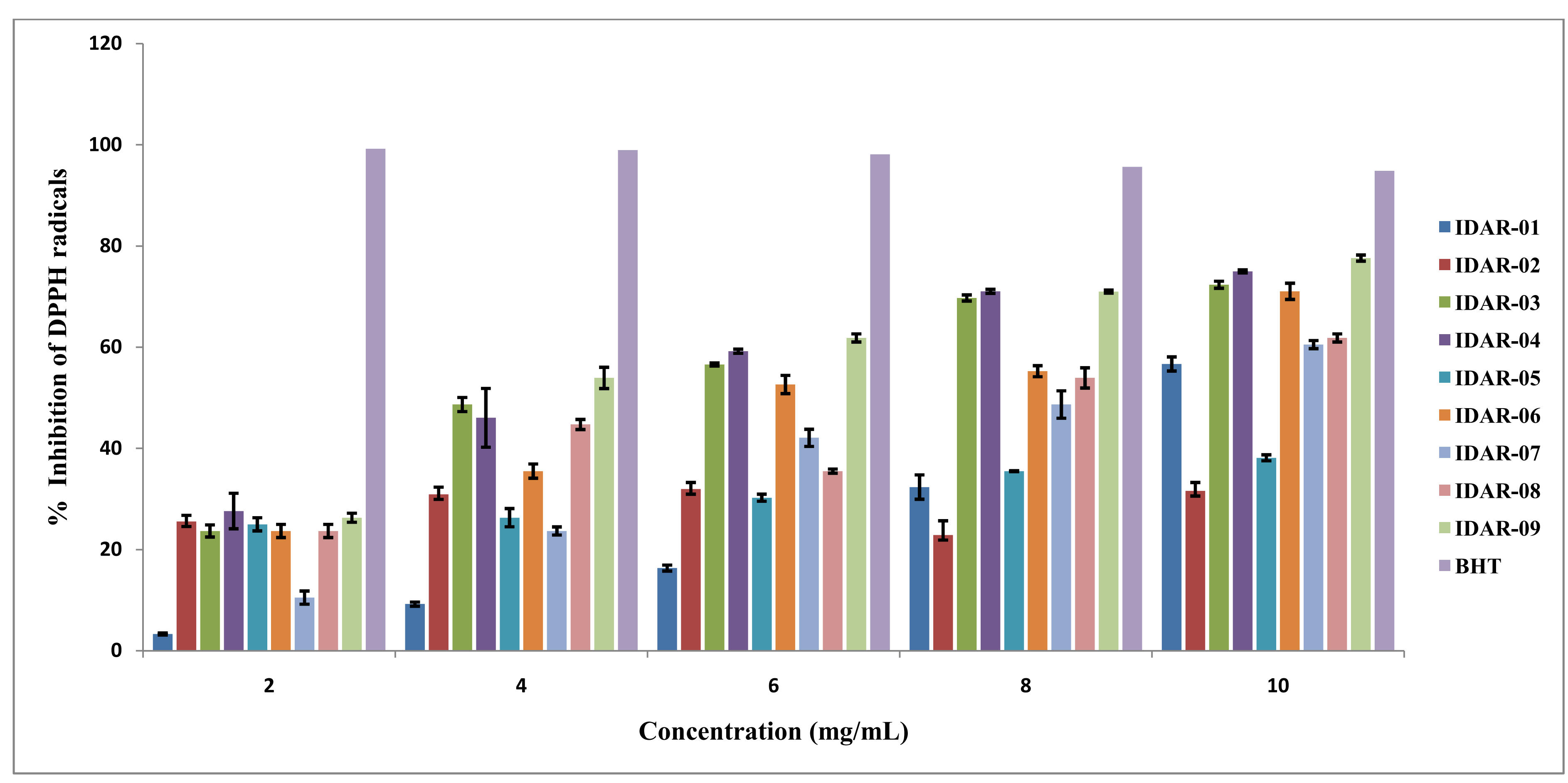


Figure 4.14: DPPH radical scavenging activities (%) of extracts (2-10 mg ml^{-1}) of nine isolates of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for extraction of antioxidant components from dried mycelium. (n=3, ± SD).

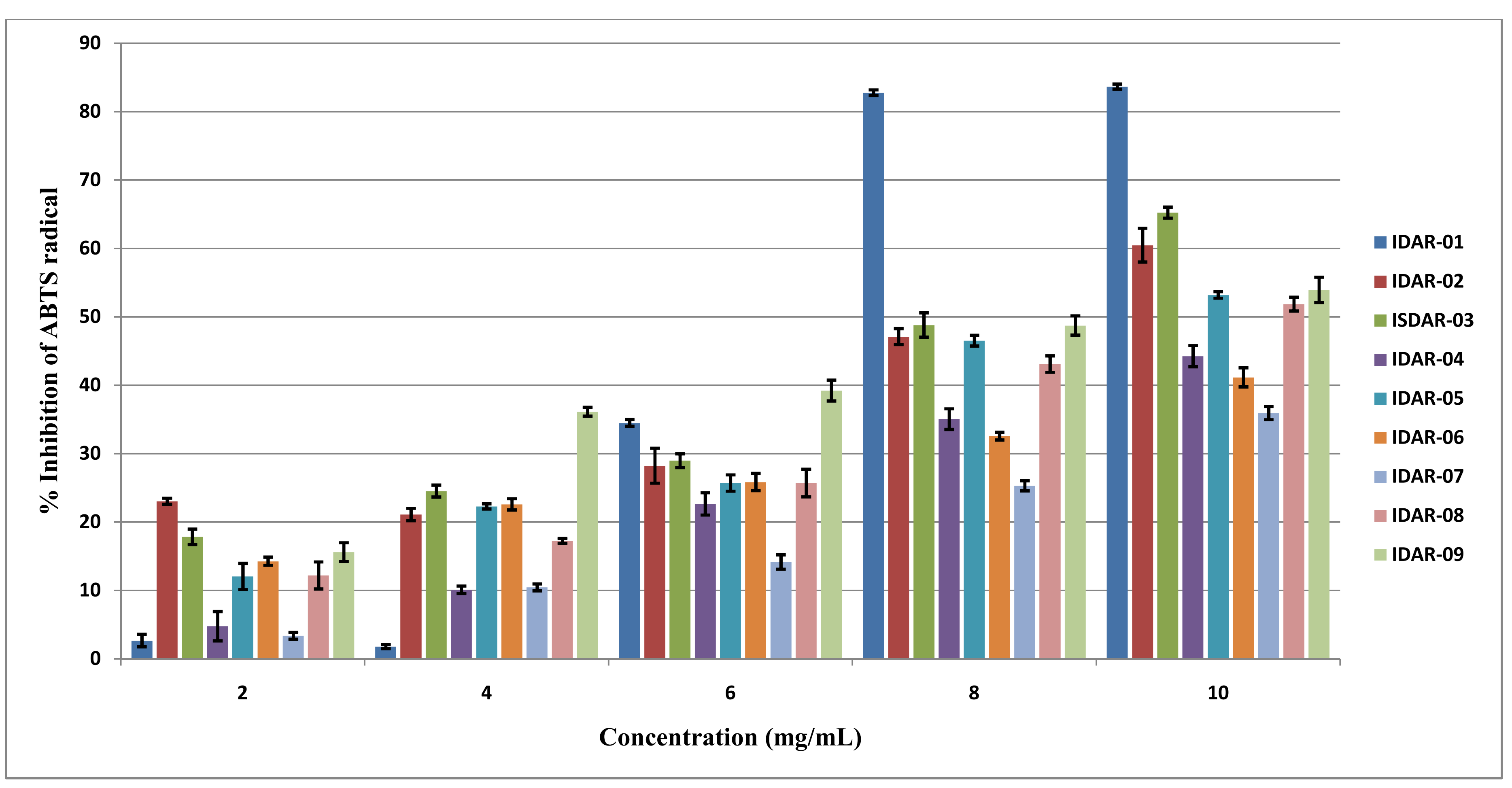


Figure 4.15: ABTS radical scavenging activities (%) of extracts (2-10 mg ml^{-1}) of nine isolates of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for extraction of antioxidant components from dried mycelium. (n=3, ± SD).

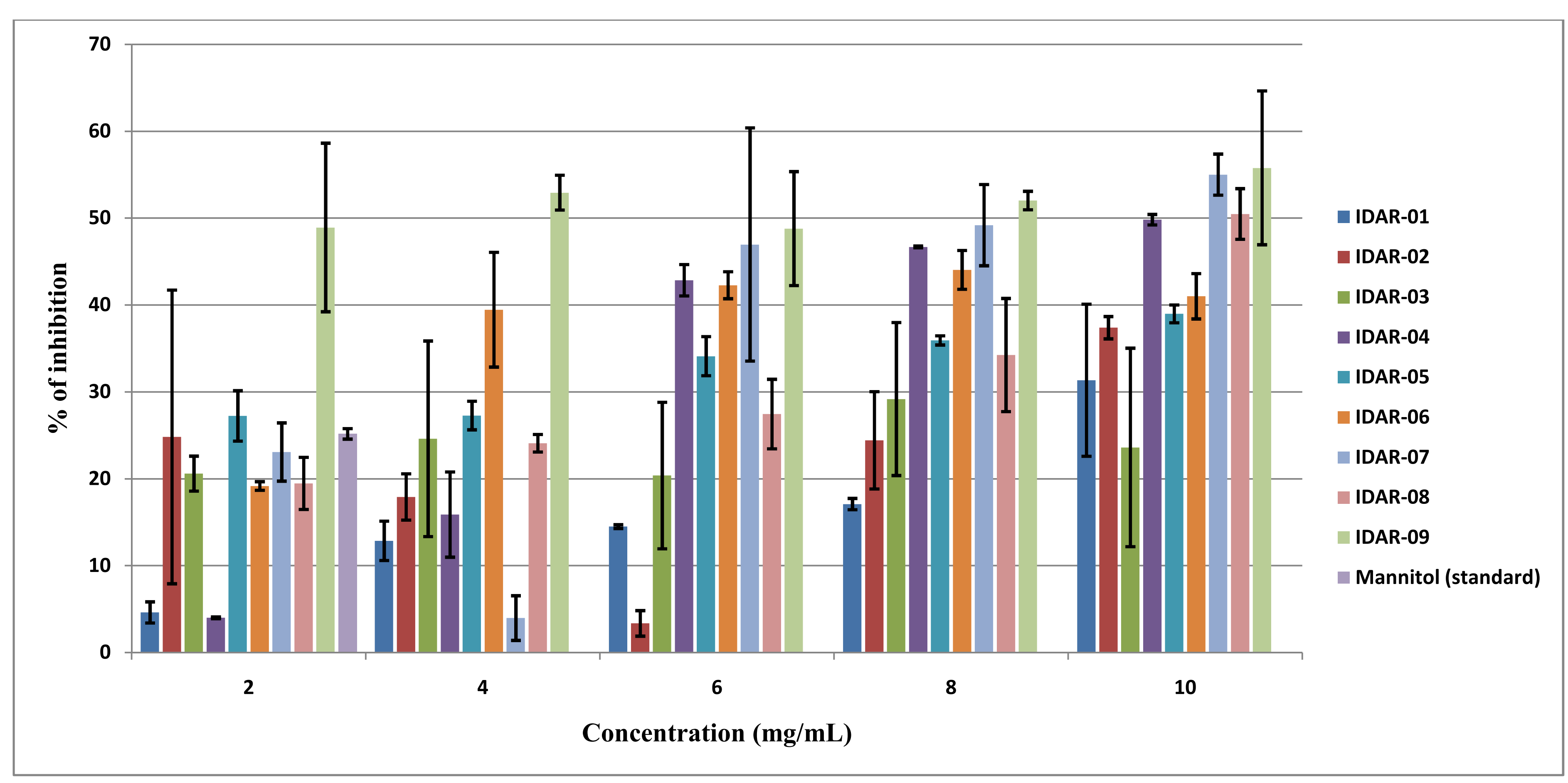


Figure 4.16: Hydroxyl radical scavenging activities (%) of extracts (2-10 mg ml^{-1}) of nine isolates of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for extraction of antioxidant components from dried mycelium. (n=3, ± SD).

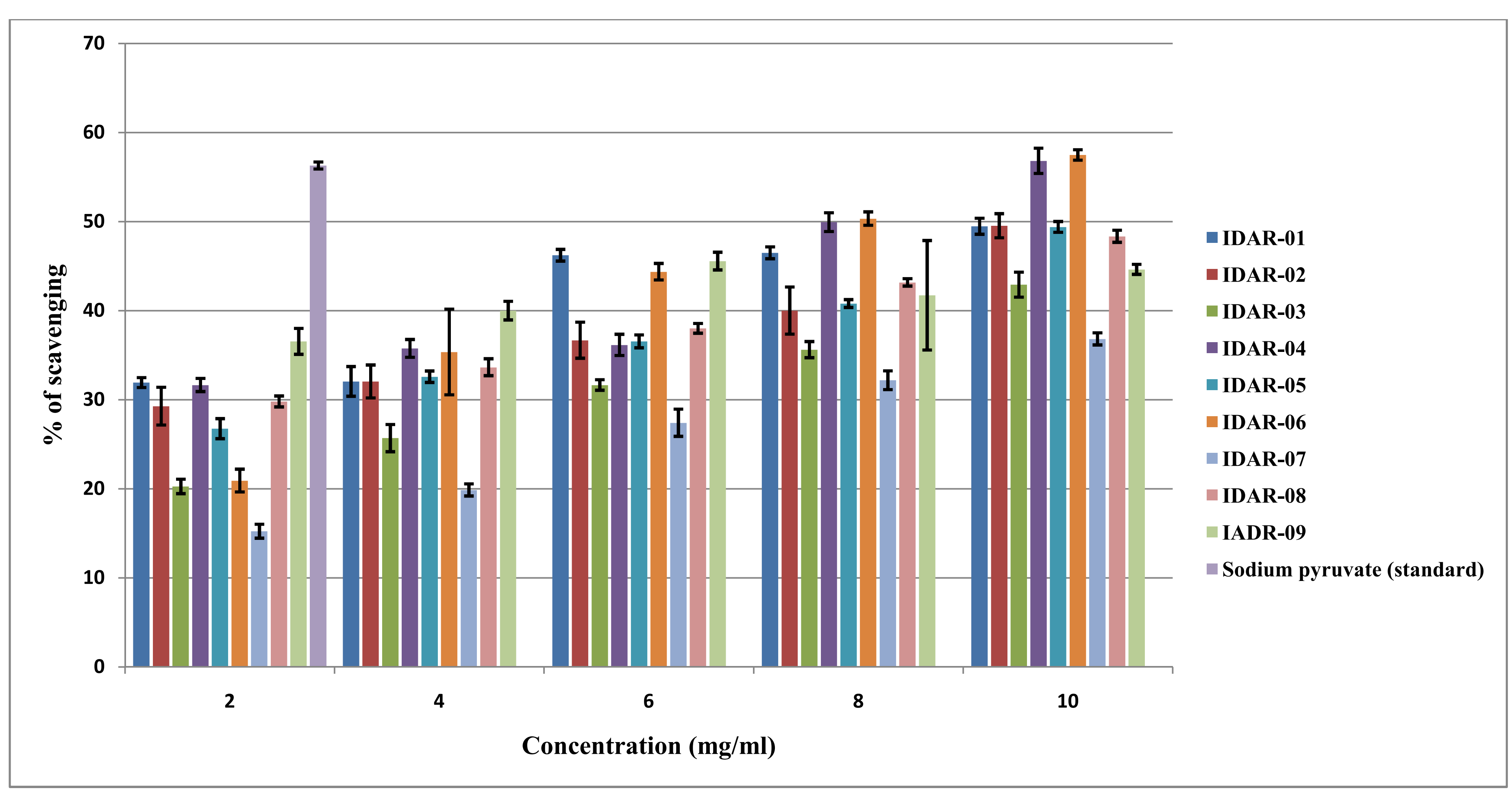


Figure 4.17: Hydrogen peroxide scavenging activities (%) of extracts (2-10 mg ml^{-1}) of nine isolates of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for extraction of antioxidant components from dried mycelium. (n=3, ± SD).

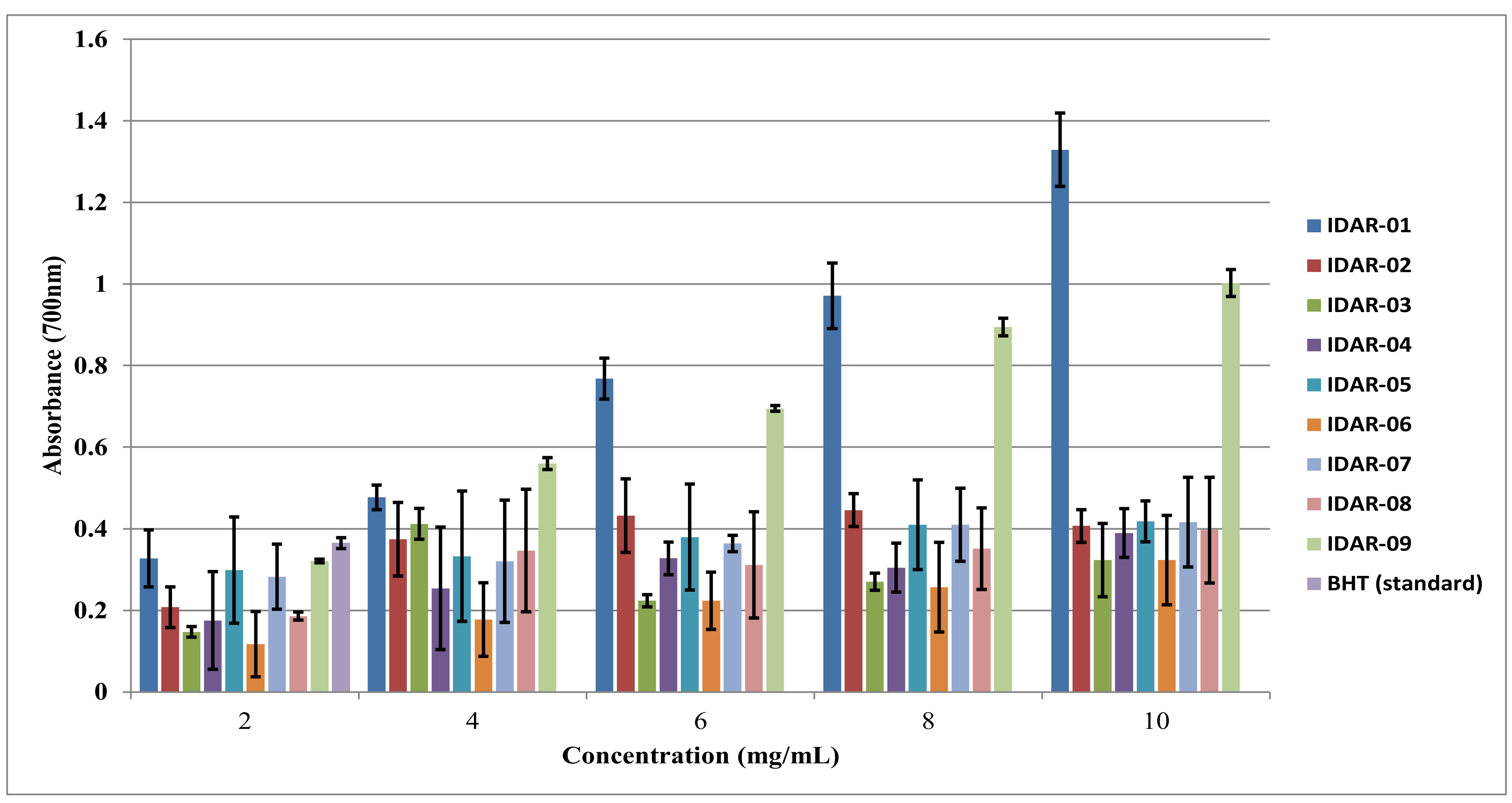


Figure 4.18: Evaluation of ferric reducing power of extracts (2-10 mg ml^{-1}) of nine isolates of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for extraction of antioxidant components from dried mycelium. (n=3, ± SD).

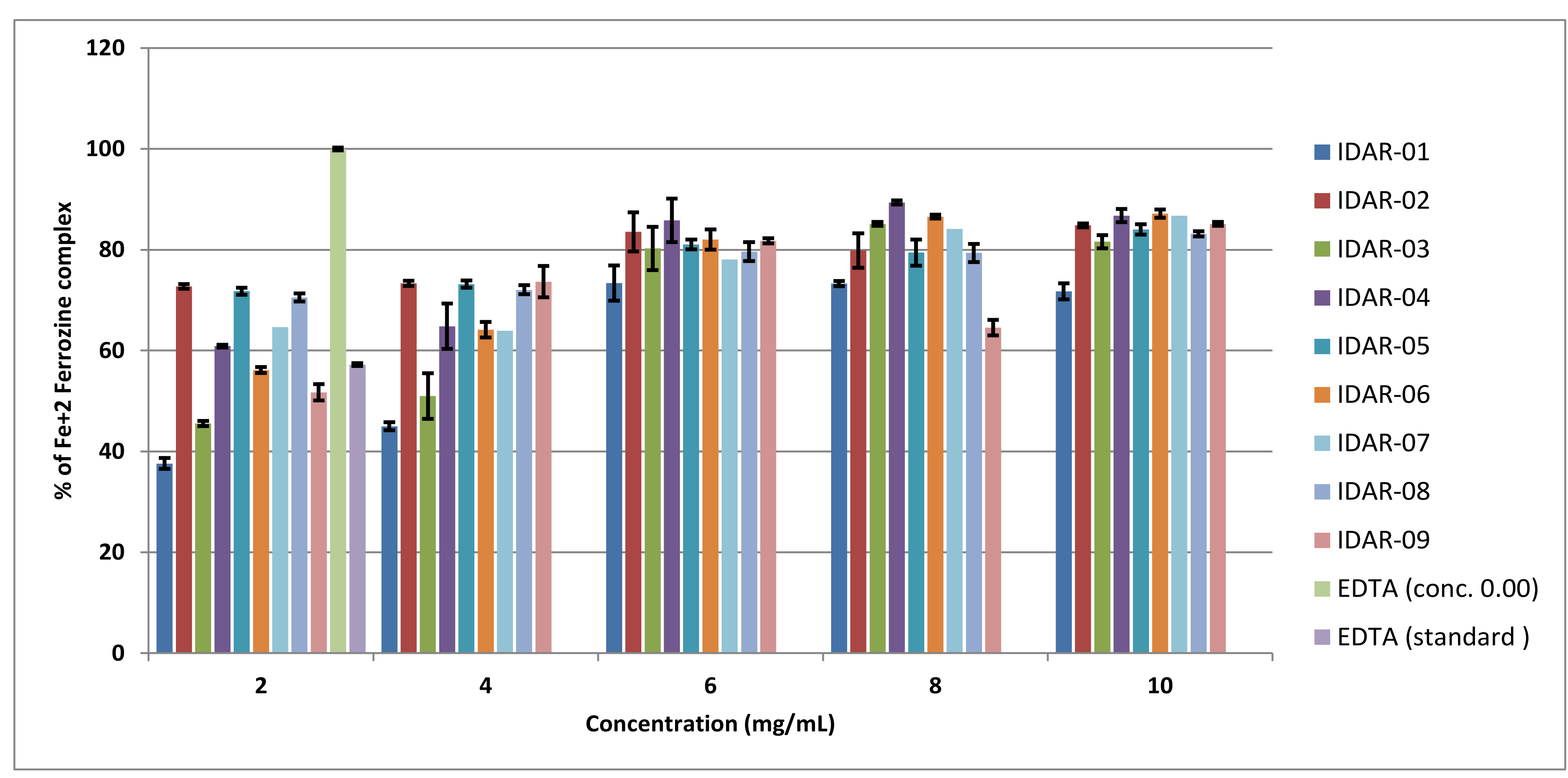


Figure 4.19: Chelating effect on ferrous ions (%) of extracts (2-10 mg ml^{-1}) of nine isolates of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for extraction of antioxidant components from dried mycelium. (n=3, ± SD).

4.33 IC_{50} in Antioxidant Properties

The IC_{50} of aqueous methanolic extracts is inversely related to its antioxidant capacity, as it expresses the amount of antioxidant required to decrease the test reagent concentration by 50%. It is mostly obtained by interpolation from a linear regression analysis. A lower IC_{50} indicates a higher antioxidant potential of a test extracts. Table 4.18 shows the IC_{50} values in the antioxidant activity assay of the extracts.

Table 4.18: Overview of the antioxidant activities of extracts (IC_{50}=mg/mL, n=03±SD) of nine isolates of *Isaria* spp., collected from Darjeeling hills. Aqueous methanol (70%) was used as a solvent for extraction of antioxidant components from dried mycelium. (n=3, ± SD).

Isolates	DPPH activity	ABTS activity	Hydroxyl activity	H_2O_2 activity	Chelating effect
IDAR-01	>10	2.9±0.27	>10	>10	2.64±0.48
IDAR-02	>10	3.36±0.83	>10	>10	2.05±0.17
IDAR-03	3.11±0.20	4.04±0.09	>10	>10	2.42±0.15
IDAR-04	2.91±0.03	6.14±0.07	>10	3.85±0.08	2.21±0.02
IDAR-05	>10	4.65±0.17	>10	>10	2.29±0.01
IDAR-06	3.42±0.22	>10	>10	3.60±0.50	2.25±0.03
IDAR-07	4.12±0.21	>10	3.95±0.32	>10	2.24±0.08
IDAR-08	3.74±0.13	4.91±0.21	5.60±0.45	>10	2.33±0.03
IDAR-09	2.54±0.39	4.17±0.06	3.44±0.06	>10	2.44±0.1
BHT	<2.0	—	—	—	—
AA	—	<2.0	—	—	—
Mannitol	—	—	<2.0	—	—
EDTA	—	—	—	—	<2.0
Na-Pyruvate	—	—	—	<2.0	—

4.34 Principal Component Analysis (PCA) of antioxidant parameters of nine *Isaria* spp., isolates

The results of the phytochemical content and antioxidant activity of methanol mycelial extracts from nine isolates of *Isaria* spp., are considered variables. However, it cannot be stated that the 70% methanol mycelial extracts as an independent variable but it does influence grouping of the data and clustering. On these variables, PCA was performed to understand the interrelationships based on the content of total phenol, total flavonoid, total tannin, total carotene, total lycopene, and antioxidant assay components viz, DPPH (1,1-diphenyl-2- picrylhydrazyl radicals), 2, 2′-azinobis-(3—ethylbenzothiazoline-6-sulphonate radical cation scavenging assay (ABTS), hydroxyl radical scavenging assay (OH), hydrogen peroxide scavenging assay (HPSA) and chelating activity (CE) of methanol extracts from nine isolates of *Isaria*. In the present study, Figure 4.20., depicting the rotated score PCA plot provides a clear picture of the correlation of the principal component with the phytochemicals and between antioxidant activity parameters. Two factor loadings were obtained that accounted for the PC1 of 59.26 % and PC2 of 25.53% of the total variability (Variance-covariance = 84.79%) of the methanol extracts, chosen on the basis of their matrix eigen values (>1) using Varimax factor rotation. DPPH·-scavenging ability and OH scavenging activity were shown to be highly loaded on PC1 with loadings 0.669, and 0.466, respectively. Thus, OH and DPPH·-scavenging activity were loaded closely, which indicated the two properties are in close relationship with respect to their antioxidant activity. However, the high loading (-5.4) of lycopene on PC3 that accounts for 7.98% variance where other parameters were recorded to have low loading implies its independence from the other variables. On the other hand, the ABTS cation scavenging ability of the methanol extract shows a loading of -0.573 on PC1, Fe (II)-chelating ability shows a loading of 0.010 on PC2, while total flavonoid content has a

loading of 0.201 on PC2, which showed low loading of the other phytochemical parameters.

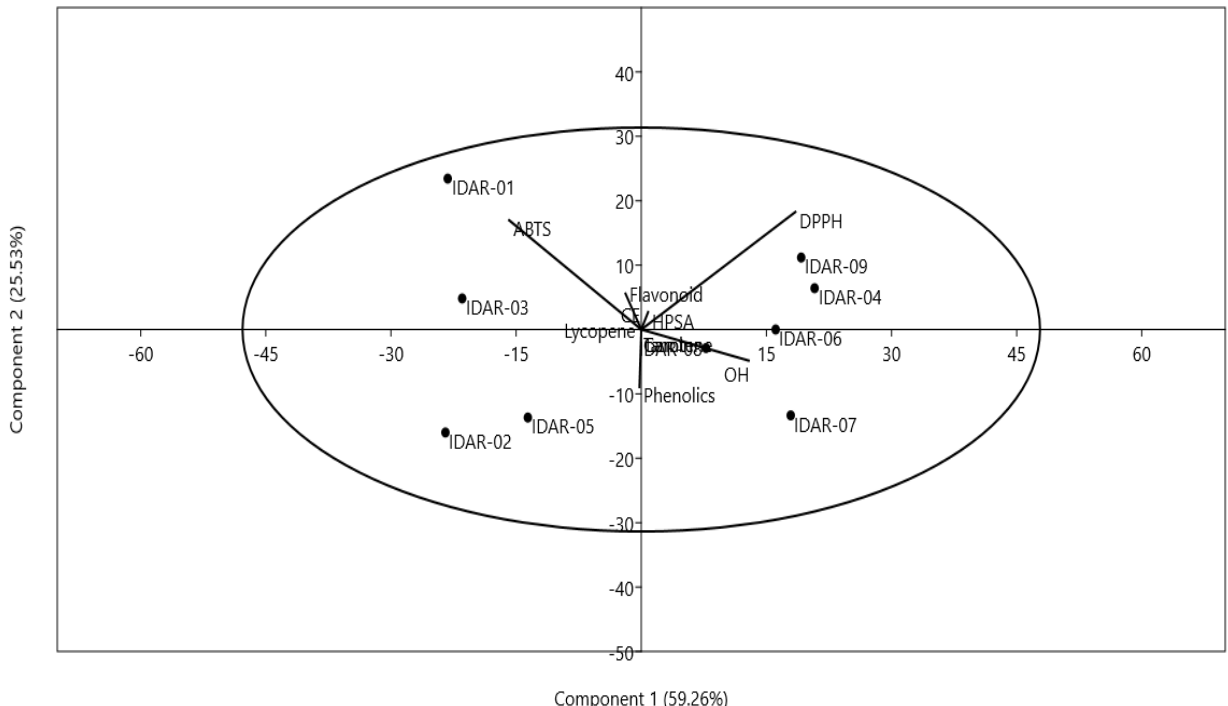


Figure 4.20: Principal component analysis biplot of nine *Isaria* isolates collected from Darjeeling hills based on the content of total phenol, total flavonoid, total tannin total carotene, total lycopene, and antioxidant assay components DPPH (1,1-diphenyl-2-picrylhydrazyl radicals), ABTS (2, 2′-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation), OH (Hydroxyl radical scavenging assay) HPSA (hydrogen peroxide scavenging assay) and CE (chelating activity).

Eigen values provide a comprehensive summary of the influence of the original variables on the principal components. Thus, it is widely used as a statistical platform for data interpretation. Figure 4.21., shows the percentage of Eigen values and variance explained by the principal components. From the figure it is found that two principal components carry over 84.97 % of the information contained in primary variables. The scree plot also shows that there are three values on its slope, while the other five are off the slope. In the present study, two principal components were selected for representation of statistical

behaviour which accounts to 84.97% of their variance for a set of analysed variables in the methanol extracts of nine isolates of *Isaria*.

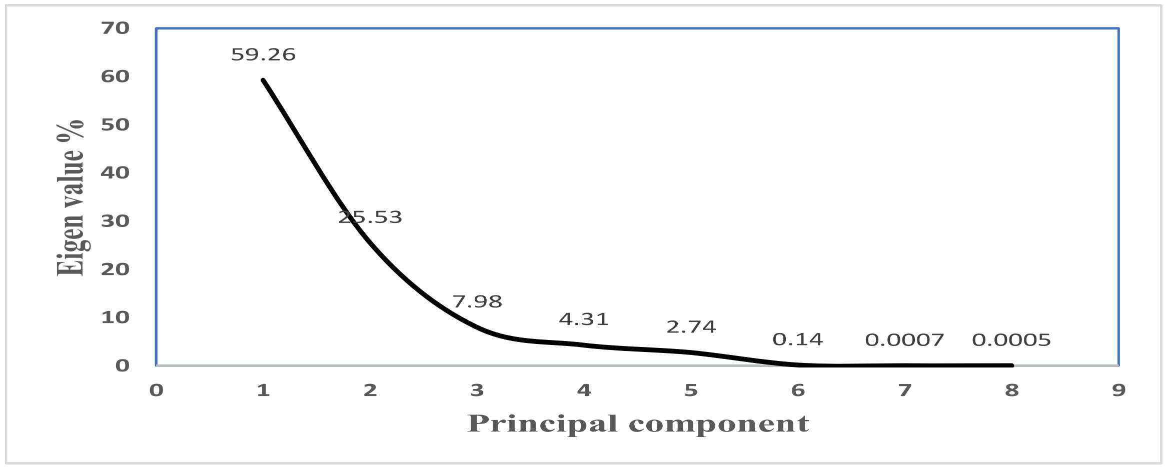


Figure 4.21: The scree plot of methanol extracts of nine *Isaria* isolates collected from Darjeeling hills based on the content of total phenol, total flavonoid, total tannin total carotene, total lycopene, and antioxidant assay components DPPH (1,1-diphenyl-2-picrylhydrazyl radicals), ABTS (2, 2´-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation), OH (Hydroxyl radical scavenging assay) HPSA (hydrogen peroxide scavenging assay) and CE (Chelating activity).

4.35 Classification of the nine isolates based on agglomerative hierarchical clustering (AHC)

To simplify the chemical pattern recognition and to visualize the relationships between nine isolates of *Isaria* collected from different ecological region of Darjeeling hills, agglomerative hierarchical clustering (AHC) using paired group UPGMA (unweighted pair group method with arithmetic mean) was used. The dataset viz., the phytochemicals and antioxidant parameters related to the 70% methanol extracts was joined into clusters, thus increasing their in-group homogeneity (Figure 4.22). The nine isolates of the *Isaria* spp., have been classified into five significant clusters. The first and second cluster comprises of IDAR-01, IDAR-02, IDAR-03 and IDAR-05, which had high content of total phenolics, total tannins, and carotene. The third and fourth cluster was composed of the isolates IDAR-04, IDAR-06,

IDAR-08 and IDAR-09 with the characteristics of high content of total phenolics, and low content of lycopene and β-carotene while the IDAR-07 was clustered separately with high content of lycopene and tannin. The result obtained in the present study is in conformity with those of the PCA in which the distribution of phytochemical and antioxidant activity of methanol extracts from nine isolates of the *Isaria* spp., on the score plot shows a similar tendency. Furthermore, the comparison between the agglomerative hierarchical clustering using paired group UPGMA and PCA is significant, which showed that the PCA score plot data were in line with single linkage Euclidean distance scores.

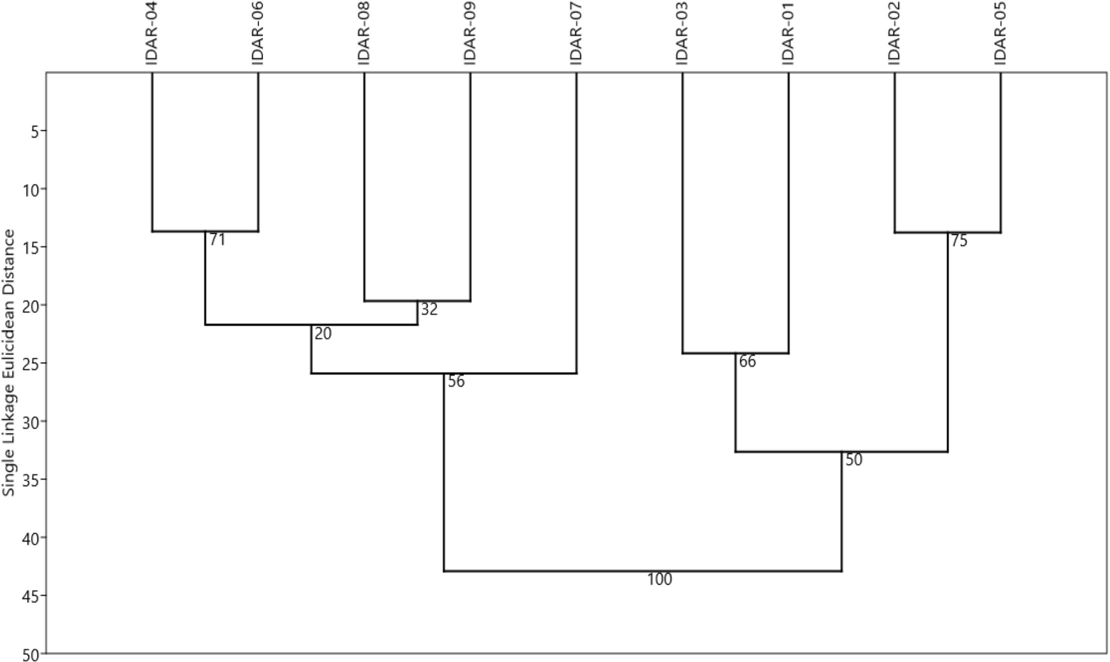


Figure 4.22: Dendrogram of cluster analysis with single linkage Euclidean distance of methanol extracts of nine *Isaria* isolates collected from Darjeeling hills based on the content of total phenol, total flavonoid, total tannin total carotene, total lycopene, and antioxidant assay components DPPH (1,1-diphenyl-2- picrylhydrazyl radicals), ABTS (2, 2´-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation), OH (Hydroxyl radical scavenging assay) HPSA (hydrogen peroxide scavenging assay) and CE (Chelating activity).

4.36 Antibacterial activity

Results obtained by agar diffusion assay on bacterial strains that had zones of inhibition of more than 11 mm in diameter were considered active for the assay of antibacterial activity. The data were defined as the diameters of zone of inhibition (ZOI) in nearest millimetre scale. Antibacterial effect was examined for different cultured mycelial methanol extracts against all cited bacteria. The variable results of two groups of bacteria are presented in Table 4.19 and 4.20. The bactericidal properties of methanol extract were observed and recorded after 24 hours of incubation. At a tested concentration (10.00 mg/ml) extract particularly *I. tenuipes* IDAR-04 and *I. tenuipes* IDAR-03 showed strong antibacterial activity against *E. coli* with an inhibition zone of 15.08±0.87 and 14.33±1.20mm respectively. In case *P. aeruginosa* the highest zone of inhibition was recorded with *I. fumosorosea* IDAR-09 (14.83±0.76mm) followed by *I. tenuipes* IDAR-04 (14.66±0.57mm) and with *K. pneumoniae*, the highest zone of inhibition was found with *I. tenuipes* IDAR-04 (15.33±1.15), followed by *I. tenuipes* IDAR-03 (15±1.00mm). Methanol extracts from all isolates showed similar results as observed in case of Gram-negative bacteria with some variations. The cultured mycelial methanolic extracts displayed relatively better inhibitions zones with three Gram positive bacteria ranging from 11.0-16.0mm (Table 4.20). It was found in the present study that the methanolic extract at a concentration of 10mg/mL from *I. tenuipes* IDAR-06 demonstrated highest activity against *B. subtilis* with zone of inhibition of 16.33±0.57mm followed by *I. farinosa* IDAR-06 with ZOI of 15.66±0.57mmm. While the isolates *I. fumosorosea* IDAR-09 exhibited highest activity towards *S. aureus* with ZOI of 16±1.00 followed by *I. tenuipes* IDAR-07 where ZOI was recorded to be 15.66±1.15 mm. Isolates of *I. farinosa* IDAR-02 demonstrated highest activity against *S. pneumoniae* with ZOI of 14±1.00mm followed by *I. tenuipes* IDAR-04, which demonstrated the ZOI value of 13.75±0.86mm respectively.

The methanol extracts exhibited antibacterial activity of differing magnitudes in which the zone of inhibition of different bacteria in 24 h was recorded in the following ascending order of ZOI: *S. pneumoniae* (14±1.00mm) > *P. aeruginosa* (14.83±0.76mm) > *E. coli* (15.08±0.87 mm) > *K. pneumoniae* (15.33±1.15mm) > *Staphylococcus aureus* (16±1.00mm) >*Bacillus subtilis* (16.33±0.57mm). The result also indicated that the methanol extracts exhibited excellent antibacterial properties in two test bacteria *Staphylococcus aureus* (50%) and *Bacillus subtilis* (65.32%) in comparison to streptomycin used as a positive control at a concentration of 100.00µg/ml. However, no antibacterial activity as expected was recorded in a negative control sample containing EDTA at a concentration of 100.00µg/ml. Thus, in general it seems that all methanolic extracts demonstrated significantly better antibacterial activity against Gram positive bacteria when compared with Gram negative bacteria. Overall, a broad spectrum of antibacterial activity was exhibited.

Table 4.19: Antibacterial activity of dried mycelial extracts of nine isolates of *Isaria* spp collected from Darjeeling hills against Gram-negative bacteria in terms of zone of inhibition (mm). Aqueous methanol (70%) was used as a solvent for extraction of antibacterial components from dried mycelium. (n=3, ± SD).

Isolates	Conc. (mg mL^{-1})	Growth inhibition Zone Diameter (mm)[1] Antibacterial activities					
		E. coli	%[2]	*P. aeruginosa*	%[2]	*K. pneumoniae*	%[2]
IDAR-01	5	N.D.	—	N.D.	—	N.D.	—
	10	12±1.0	42.35	11.33±0.57	—	11.66±0.57	53
IDAR -02	5	11.07±0.68	39.07	12.16±0.28	—	11±1.0	50
	10	13.61±0.97	48.04	12.5±0.5	—	14±1.0	63.63
IDAR -03	5	12.5±0.5	44.12	N.D.	—	11±1.0	50
	10	14.33±1.20	50.58	13±1.0	—	15±1.0	68.18
IDAR -04	5	12.5±0.5	44.12	13.33±0.57	—	12.66±0.57	57.54
	10	15.08±0.87	53.22	14.66±0.57	—	15.33±1.15	69.68
IDAR -05	5	11.66±1.15	41.15	11.66±0.57	—	N.D.	—
	10	12.66±1.15	44.68	12.66±0.33	—	12.66±0.57	57.54
IDAR -06	5	N.D.	—	N.D.	—	11.33±0.57	51.5
	10	12.66±1.15	44.68	13±1.0	—	13.33±0.57	60.59
IDAR -07	5	N.D.	—	11±1.0	—	11.33±0.57	51.5
	10	12.66±0.57	44.68	14±1.0	—	12.33±1.52	56.04
IDAR -08	5	11.83±0.76	41.75	12.5±0.5	—	12.33±0.57	56.04
	10	13.5±0.5	47.65	14.33±0.57	—	14.66±0.57	66.63
IDAR -09	5	12.66±0.57	44.68	12.5±0.5	—	11.5±0.50	52.27
	10	14±1.0	49.41	14.83±0.76	—	13.66±1.15	62.09
STANDARDS							
Negative control[3]	0.1 mg mL^{-1}	N.D.	—	N.D.	—	N.D.	—
Positive control[4]	0.1 mg mL^{-1}	28.33±0.57		–	—	22±1.00	

N.D./— means that no antibacterial activity was detected. A zone of inhibition (mm) > 11 mm was considered active. [1] Values are means ± SD of triplicate experiments. %[2]; Percentage inhibition based on corresponding positive control; Control[3]; EDTA (0.1 mg mL^{-1}), Control[4]; Streptomycin (0.1 mg mL^{-1}),–; no growth recorded.

Table 4.20: Antibacterial activity of dried mycelial extracts of nine isolates of *Isaria* spp collected from Darjeeling hills against Gram-positive bacteria in terms of zone of inhibition (mm). Aqueous methanol (70%) was used as a solvent for extraction of antibacterial components from dried mycelium. (n=3, ± SD).

Isolates	Conc. (mg/mL)	Growth inhibition Zone Diameter (mm)[1] Antibacterial activities					
		B. subtilis	%[2]	*S. aureus*	%[2]	*S. pneumoniae*	%[2]
IDAR -01	**5**	12.66±0.57	50.64	12.66±0.28	39.56	11±0.57	40.74
	10	14±1.00	56	13.66±0.57	42.68	13.75±0.50	50.92
IDAR -02	**5**	12.66±0.57	50.64	11±1.0	34.37	12±0.57	44.44
	10	15±1.0	60	13.33±1.15	41.65	14±1.00	51.85
IDAR -03	**5**	11.66±0.57	46.64	12.33±1.15	38.53	11.25±0.86	41.66
	10	13±1.00	52	15±1.00	46.87	14.5±1.00	53.70
IDAR -04	**5**	12.33±0.57	49.32	13±1.00	40.62	N.D.	—
	10	13.66±0.57	54.64	16±1.00	50	13.75±0.86	50.92
IDAR -05	**5**	13.33±0.57	54.64	12.33±0.57	38.53	12±1.52	44.44
	10	15.66±0.57	62.64	15.33±0.57	47.90	15±1.00	55.55
IDAR -06	**5**	12.66±0.57	50.64	12.83±0.28	40.09	N.D.	—
	10	16.33±0.57	65.32	15.66±1.52	48.93	14.25±1.04	52.77
IDAR -07	**5**	12.66±1.15	50.64	12.33±0.57	38.53	N.D.	—
	10	15±1.00	60	15.66±1.15	48.93	13.5±0.57	50
IDAR -08	**5**	12±0.50	48	12.66±0.57	39.56	12.5±0.57	46.29
	10	14±1.00	56	14.33±1.52	44.78	13.75±0.28	50.92
IDAR -09	**5**	12.66±0.57	50.64	13.16±0.76	41.12	12.5±0.57	46.29
	10	15.33±0.57	61.32	16±1.0	50	13.5±1.0	50
STANDARDS							
Negative control[3]	**0.1 mg mL^{-1}**	N.D.		N.D.		N.D.	
Positive control[4]	**0.1 mg mL^{-1}**	25±1.00		32±1.00		27±1.00	

N.D. means that no antibacterial activity was detected. A zone of inhibition (mm) > 11 mm was considered active. [1] Values are means ± SD of triplicate experiments. %[2]; Percentage inhibition based on corresponding positive control; Control[3]; EDTA (0.1 mg mL^{-1}), Control[4]; Streptomycin (0.1 mg mL^{-1}), − ; no growth recorded.

4.37 Evaluation of dried mycelial extracts of nine isolates of *Isaria* spp collected from Darjeeling hills for cytotoxicity

To evaluate the antiproliferative activity of methanol extracts on HeLa, HepG2, and PC3 cells MTT assay was performed. It was observed that methanol extracts decreased the number of cells in the monolayer in the concentration dependent manner. The exposure of methanolic extracts of all nine isolates in an increasing concentration resulted in significant dose-dependent inhibition of the growth of HeLa, HepG2, and PC3, and greater inhibition for all isolates was more pronounced in case of HeLa cells particularly. As in figure 4.23 and 4.25, it was observed that percent survival was distinct only at relatively high extract concentration in the case of HepG2 and PC3 cells (>100μg/ml). Concentrations of dried mycelial extracts which decreased the absorbance by > 10% when compared with the control were considered to be cytotoxic. According to the cytotoxicity assay, the dosages of each mushroom extract for cellular antioxidant assay were: 1.0, 3.0, 10.0, 30.0 and 100.0μg/ml respectively. After HeLa cells had been incubated with methanol extracts for 72 hours, among the methanol extracts from nine isolates studied in the present research, IDAR-03, IDAR-04, IDAR-07, IDAR-08 and IDAR-09 exhibited significant cytotoxicity against HeLa cells in a dose dependent manner (Figure 4.23). After prolonging exposure to methanol extracts for 72 hours, all the isolates extracts decreased the HeLa cell proliferation at all tested concentration compared to control cells. Cytotoxicity of IDAR-04 and IDAR-09 was found to be more, because it inhibited the HeLa cells by 94.48 and 93.09% respectively. Similarly, methanol extracts showed dose dependent antiproliferation effects on HepG2 cells with a similar trend of percent survival of HeLa cells, but, the rate of cell death with increasing concentrations (1-100μg/ml) of the extracts was significantly less than that observed for HeLa cells (Figure 4.24). The cytotoxic potential of seven isolates was found to exhibit less than 50 % inhibition even at 100μg/mL concentration. It

was found that only IDAR-06 and IDAR-07 could bring about percent inhibition of 64.35 and 64.53% respectively at 100µg/ml. Whereas the cytotoxicity profile of PC3 cells, treated with the methanol extracts showed a comparable profile with HepG2 cells. More than 50% cytotoxicity was observed with IDAR-03 (55.93%), IDAR-04(55.93%) and IDAR-09 (59.35%) respectively at highest tested concentration (100µg/ml). Except these isolates, other isolates could not result in 50% cytotoxicity. The effect of extracts was greater at higher concentration between 30 and 100µg/ml showing a significant decrease in cell proliferation in the monolayer and decreasing the number of cells in these concentrations ($p<0.05$). However, this effect was more evident with HeLa cells where methanol extracts from all isolates decreased the percentage of viable cells by more than 50% with 1-100µg/ml ($p<0.05$). In general, greater antiproliferative activity was demonstrated greater in HeLa cells followed by HepG2 and PC3 cell lines. The standard deviation of cell viability assay was calculated for all three cell lines as shown in Table 4.21, 4.22 and 4.23.

In order to simplify the analysis on the results of effects of methanol extracts on three cancer cell lines and to determine the relative positions of test isolates in the standard toxicity activity scale, half maximal inhibitory concentration (IC_{50}) values were derived from the dose response curve of nine isolates. The half maximal inhibitory concentration is a measure of the effectiveness of the compound in inhibiting biological or biochemical function. This quantitative measure indicate how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process by half maximal. It is commonly used to measure of antagonist drug potency in pharmacological research. Cytotoxicity was also analysed by calculating IC_{50} values. Dose dependent cytotoxic activity of nine isolates over HeLa, HepG2 and PC3 cells were analysed and the IC_{50} values of test samples derived from dose response cytotoxic activity graph were calculated

and presented in Table 4.24. Among the nine isolates the highest cytotoxicity was exerted by IDAR-01 followed by IDAR-07 for HeLa cells, IDAR-07 and IDAR-06 for HepG2 cells and IDAR-03 and IDAR-05 for PC3 cells. In HeLa cells the cytotoxic activity of methanol extract was manifold compare to HepG2 and PC3 cells. Thus, HeLa cells were found to be most sensitive to the methanol extract from dried mycelium of *Isaria* spp. However, a complete cell death was not recorded in the present study. Even at highest tested concentration, it was found to be around 94.48% with IDAR-04 at 100µg/ml. However, it was also noted that IC_{50} value of extracts from mycelium was higher than 100µg/ml, which was the highest concentration tested.

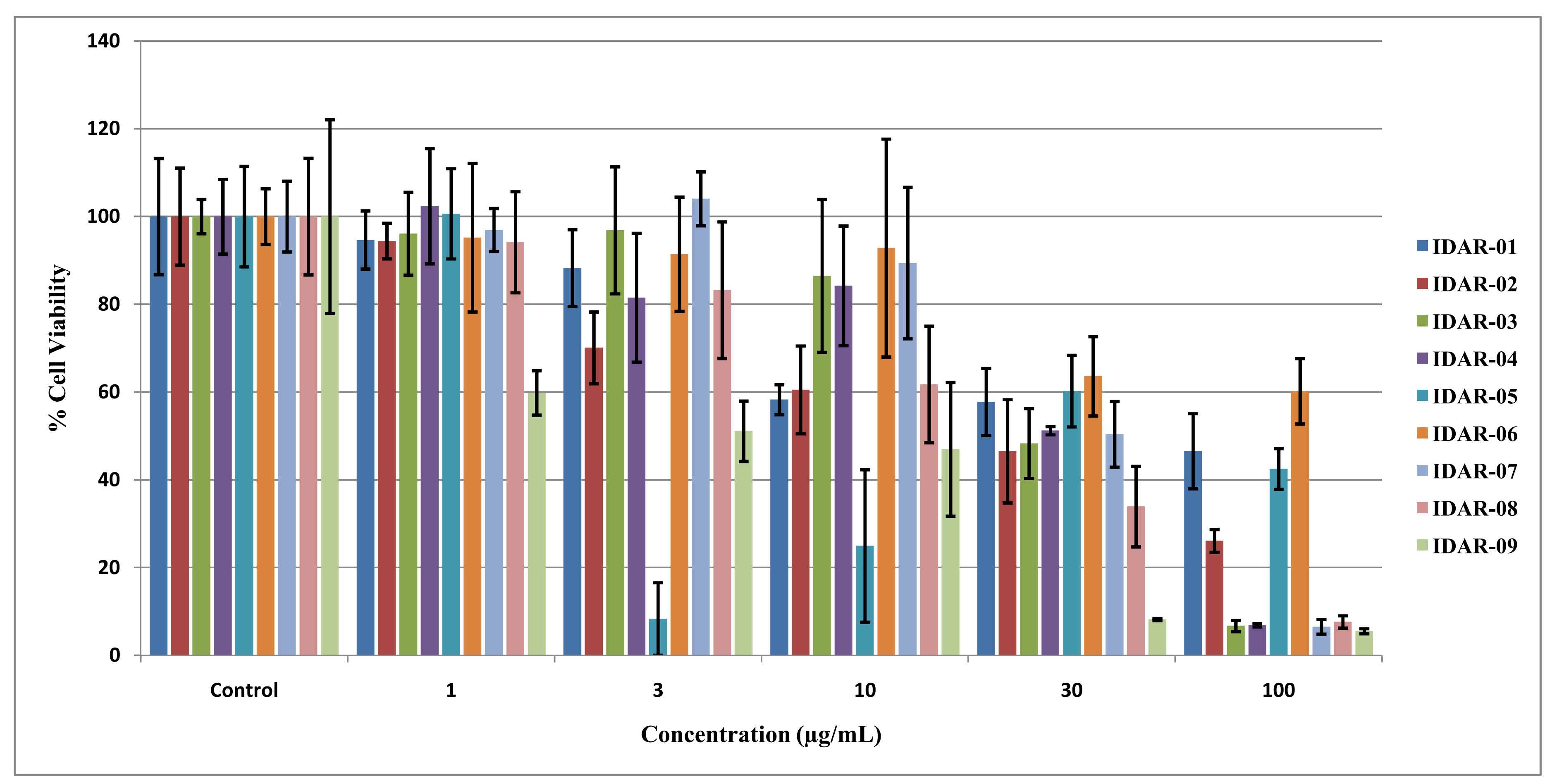


Figure 4.23: Cell survivals of HeLa cells treated with various concentrations of methanol extract of nine isolates of *Isaria* spp., collected from different ecological region of Darjeeling hills. Each value is expressed as mean ± standard deviation (n=4).

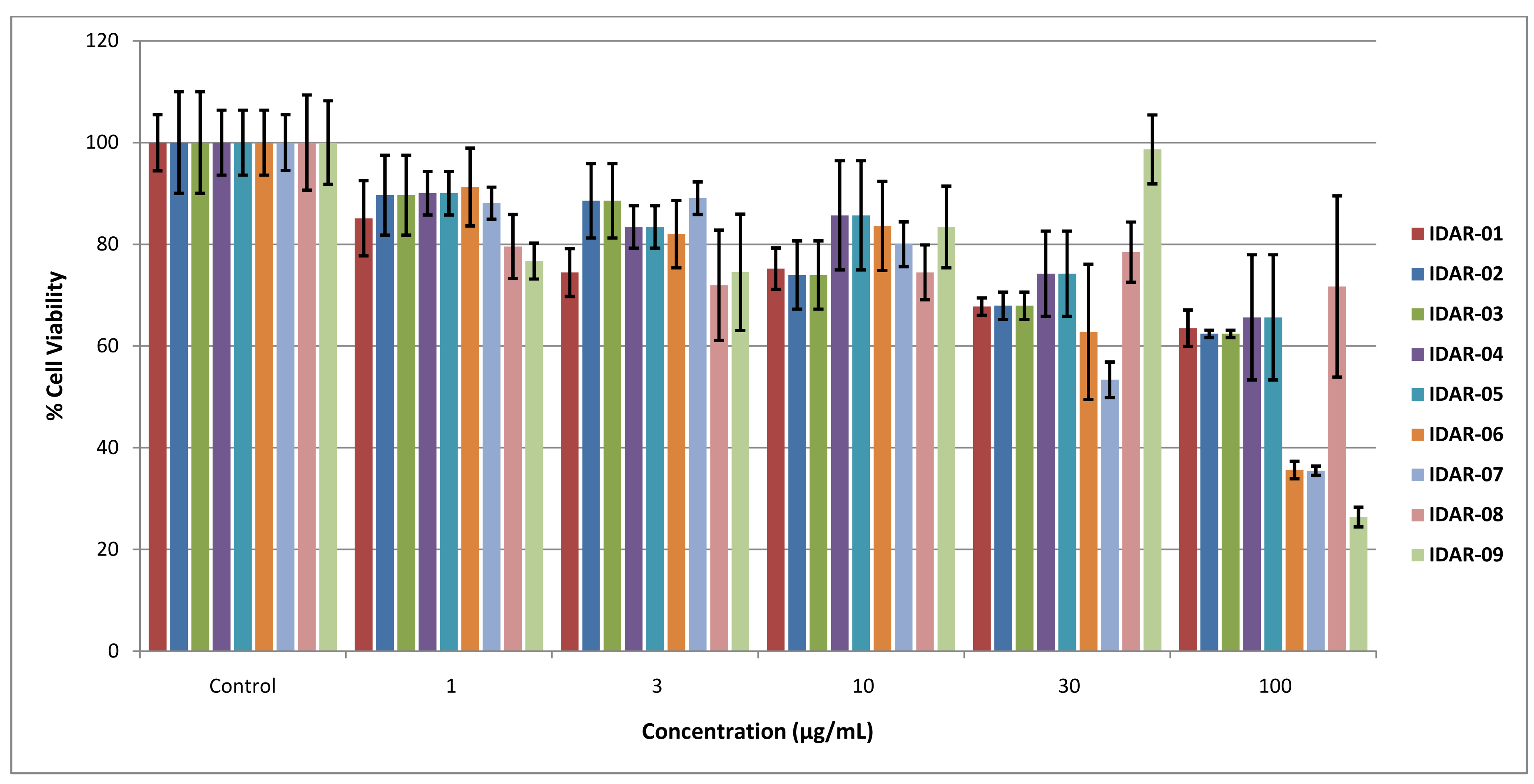


Figure 4.24: Cell survivals of HepG2 cells treated with various concentrations of methanol extract of nine isolates of *Isaria* spp., collected from different ecological region of Darjeeling hills. Each value is expressed as mean ± standard deviation (n=4).

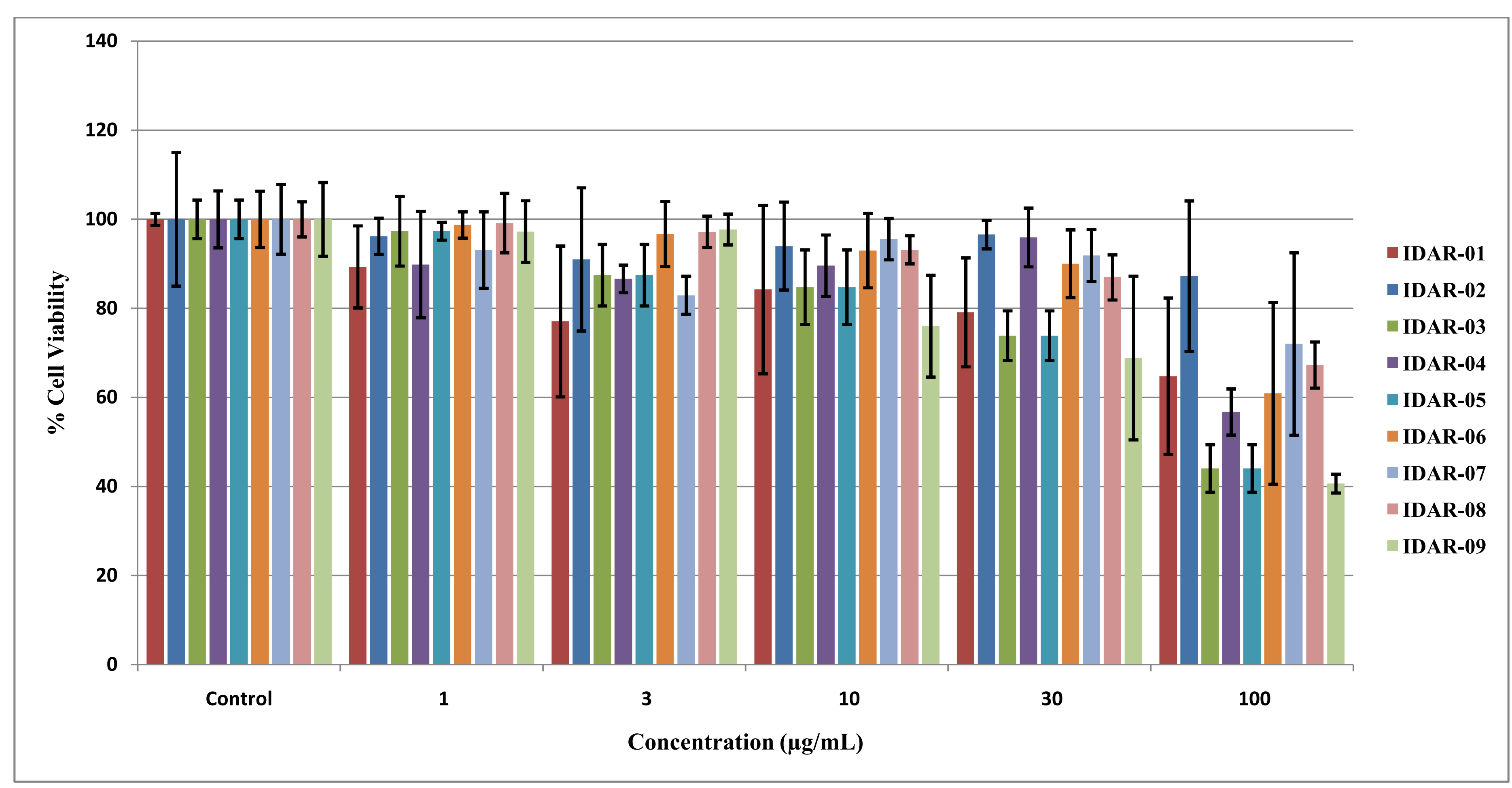


Figure 4.25: Cell survivals of PC3 cells treated with various concentrations of methanol extract of nine isolates of *Isaria* spp., collected from different ecological region of Darjeeling hills. Each value is expressed as mean ± standard deviation (n=4).

Table 4.21: Cell survivals of HeLa cells treated with various concentrations of methanol extract of nine isolates of *Isaria* spp., collected from different ecological region of Darjeeling hills. Data are representative as a mean ±SD of four independent experiments indicates *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to control using two paired t-test.

Isolate	Cell viability on HeLa Cell Line					
	Control	**1µg**	**3µg**	**10µg**	**30µg**	**100µg**
IDAR-01	100±13.23	94.67±6.61^{ns}	88.255±8.75ns	57.76±3.42***	58.28±7.65***	46.55±8.55***
IDAR-02	100±11.05	94.42±4.03^{ns}	70.12±8.16***	60.53±10.01***	46.53±11.77***	26.12±2.61^{ns}
IDAR-03	100±3.89	96.09±9.44^{ns}	96.88±14.47^{ns}	86.47±17.41^{ns}	48.28±7.95^{ns}	6.74±1.27^{ns}
IDAR-04	100±8.51	102.37±13.13*	81.51±14.66*	84.22±13.64^{ns}	51.22±0.94*	6.91±0.39^{ns}
IDAR-05	100±11.43	100.64±10.27^{ns}	8.31±8.27*	24.96±17.37^{ns}	60.24±8.14***	42.52±4.65^{ns}
IDAR-06	100±6.36	95.20±16.91^{ns}	91.42±13.01^{ns}	92.84±24.81^{ns}	63.64±9.03***	60.21±7.42^{ns}
IDAR-07	100±8.04	96.94±4.90^{ns}	104.06±6.14^{ns}	89.06±17.25^{ns}	50.40±7.46^{ns}	6.53±1.67^{ns}
IDAR-08	100±13.28	94.17±11.51^{ns}	83.24±15.55^{ns}	61.24±13.27***	33.91±9.16***	7.64±1.40^{ns}
IDAR-09	100±22.06	59.82±5.05***	51.10±6.87***	46.99±15.23***	8.21±0.23^{ns}	5.52±0.58^{ns}

Table 4.22: Cell survivals of HepG2 cells treated with various concentrations of methanol extract of nine isolates of *Isaria* spp., collected from different ecological region of Darjeeling hills. Data are representative as a mean ±SD of four independent experiments indicates *p <0.05, **p<0.01, ***p<0.001 as compared to control using two paired t-test.

Isolate	**Cell viability on HepG2 Cell Line**					
	Control	**1µg**	**3µg**	**10µg**	**30µg**	**100µg**
IDAR-01	100±5.52	85.13±7.38***	74.46±4.71***	75.20±4.06***	67.75±1.72ns	63.50±3.57^{ns}
IDAR-02	100±17.97	89.65±12.10^{ns}	88.56±6.47^{ns}	73.97±17.13**	67.90±5.61**	62.41±1.62**
IDAR-03	100±9.99	89.65±7.84^{ns}	88.56±7.32^{ns}	73.97±6.71*	67.90±2.69^{ns}	62.41±0.71^{ns}
IDAR-04	100±6.38	90.06±4.27*	83.41±4.16***	85.70±10.71*	74.23±8.37***	65.65±12.29***
IDAR-05	100±6.38	90.06±4.27*	83.41±4.16***	85.70±10.71**	74.23±8.37***	65.65±12.29***
IDAR-06	100±6.38	91.27±7.64*	81.99±6.62**	83.60±8.75*	62.80±13.28***	35.65±1.71^{ns}
IDAR-07	100±5.49	88.08±3.15***	89.07±3.20**	80.07±4.40***	53.38±3.49^{ns}	35.47±0.90^{ns}
IDAR-08	100±9.35	79.48±6.29**	71.95±10.82**	74.5±5.38***	78.47±5.92**	71.72±17.79*
IDAR-09	100±8.21	76.71±3.53***	74.51±11.42**	83.42±8.00*	98.68±6.76^{ns}	26.40±1.93^{ns}

Table 4.23: Cell survivals of PC3 cells treated with various concentrations of methanol extract of nine isolates of *Isaria* spp., collected from different ecological region of Darjeeling hills. Data are representative as a mean ±SD of four independent experiments indicates *p <0.05, **p<0.01, ***p<0.001 as compared to control using two paired t-test.

Isolate	**Cell viability on PC3 Cell Line**					
	Control	**1µg**	**3µg**	**10µg**	**30µg**	**100µg**
IDAR-01	100±1.35	84.32±9.20*	77.08±16.94***	84.23±18.89*	79.10±12.23**	64.77±17.54***
IDAR-02	100±14.98	$96.19±4.05^{ns}$	$91.01±16.08^{ns}$	$94.00±9.87^{ns}$	$96.57±3.18^{ns}$	$87.25±16.88^{ns}$
IDAR-03	100±4.31	$97.34±7.83^{ns}$	87.46±6.91***	84.75±8.39**	73.86±5.59***	$44.07±5.33^{ns}$
IDAR-04	100±6.38	89.84±11.92*	86.61±3.09***	89.59±6.89**	$95.92±6.57^{ns}$	$56.74±5.18^{ns}$
IDAR-05	100±4.31	$97.34±2.00^{ns}$	87.46±6.91***	84.75±8.39***	73.86±5.59***	$44.07±5.33^{ns}$
IDAR-06	100±6.33	$98.74±2.97^{ns}$	96.71±7.28*	$93.00±8.36^{ns}$	90.02±7.61*	60.95±20.41**
IDAR-07	100±7.84	93.10±8.59*	82.93±4.28***	$95.54±4.63^{ns}$	91.87±5.84*	72.02±20.49*
IDAR-08	100±3.94	$99.17±6.66^{ns}$	$97.19±3.51^{ns}$	93.17±3.15*	86.96±5.06***	$67.28±5.18^{ns}$
IDAR-09	100±8.26	$97.23±6.94^{ns}$	$97.72±3.44^{ns}$	76.01±11.43*	68.87±18.38*	$40.65±2.10^{ns}$

4.38 IC_{50} values in Cell Viability Assay

The cell viability potential of the methanol extracts assayed herein the present study were summarised in Table 4.24., and the results were normalized and expressed as IC_{50} values (µg/ml) for comparison. Effectiveness of the extracts in cell viability properties is inversely correlated with IC_{50} value. With regard to IC_{50} values in cell viability assay methanolic extracts were much more effective towards HeLa cells than HepG2 and PC3 cells. It was recorded in the present study lowest IC_{50} was recorded with the methanolic extracts of IDAR-01 3.32±0.09 µg/ml in Hela cells followed by IDAR-07 and IDAR-05 with 3.35±0.03 µg/ml and 3.07±0.18 µg/ml in HepG2 and PC3 cells respectively. Nonetheless, the methanol extract from the majority of isolates at 100µg/ml did not show maximum relative activity in HepG2 and PC3 cells.

Table 4.24: IC_{50} of methanol extracts of nine isolates on HeLa, HepG2 and PC3 cell line.

S/No	Isolates	IC50 (µg/ml)		
		HeLa	HepG2	PC3
1	IDAR-01	3.32±0.09	>100	>100
2	IDAR-02	3.99±0.45	>100	>100
3	IDAR-03	3.72±0.11	>100	3.07±0.21
4	IDAR-04	3.90±0.55	>100	>100
5	IDAR-05	3.77±0.74	>100	3.07±0.18
6	IDAR-06	>100	3.37±0.25	>100
7	IDAR-07	3.50±0.27	3.35±0.03	>100
8	IDAR-08	3.81±0.68	>100	>100
9	IDAR-09	7.33±1.42	>100	3.10±0.16

4.39 Principal Component Analysis (PCA) of antiproliferative parameters of nine *Isaria* isolates

In the present study, the results of the phytochemical content and antiproliferative activity of methanol extracts from nine isolates of *Isaria* spp are considered variables. PCA was performed to understand the interrelationships based on the content of total phenol, total

flavonoid, total tannin, total carotene, total lycopene, beauvercin and antiproliferative MTT assay of methanol extracts from nine isolates of *Isaria*. Two factor loadings were obtained that accounted for the PC1 of 45.13 % and PC2 of 30.02 % of the total variability of the methanol extracts, chosen on the basis of their matrix eigen values (>1) using Varimax factor rotation.

The cumulative percentage of variance-covariance of the first two principal components is 75.15% and its linear combination is characteristic of all the variables because it represents more than 50%. Therefore, the first two principal components are suitable for representing the information of all variables in general. HeLa cell inhibition was highly loaded on PC1 with loadings 0.987, whereas HepG2 cell inhibition was loaded on PC 2 with loadings 0.853 and PC3 cell inhibition on PC 3 with loadings of 0.821 which indicated the three cancer cell properties are not in close relationship with respect to their antiproliferative activity (Figure 4.26).

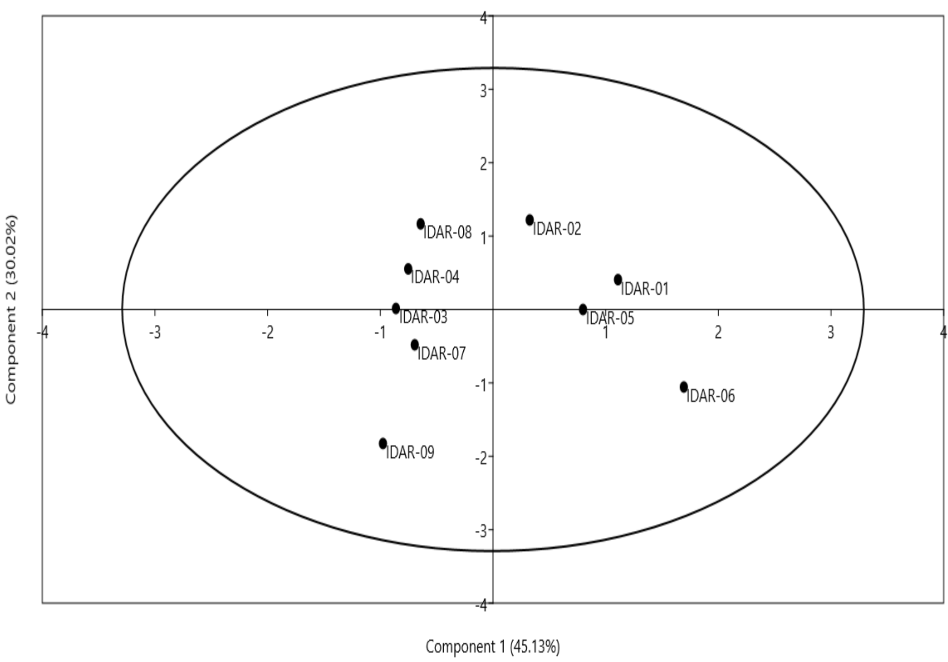


Figure 4.26: Principal component analysis of nine *Isaria* isolates collected from Darjeeling hills based on the content of total phenol, total flavonoid, total tannin, total carotene, total lycopene, beauvercin and antiproliferative MTT assay on three cancer cell lines (HeLa, HepG2 and PC3).

Figure 4.27., shows the percentage of Eigen values and variance explained by the principal components. From the figure it is found that three principal components carry over 95.85 % of the information contained in primary variables. The scree plot also shows that there are four values on its slope, while the other four are off the slope. In the present study, three principal components were selected for representation of statistical behaviour which accounts to 95.85 % of their variance for a set of analysed variables in the methanol extracts of nine isolates of *Isaria*.

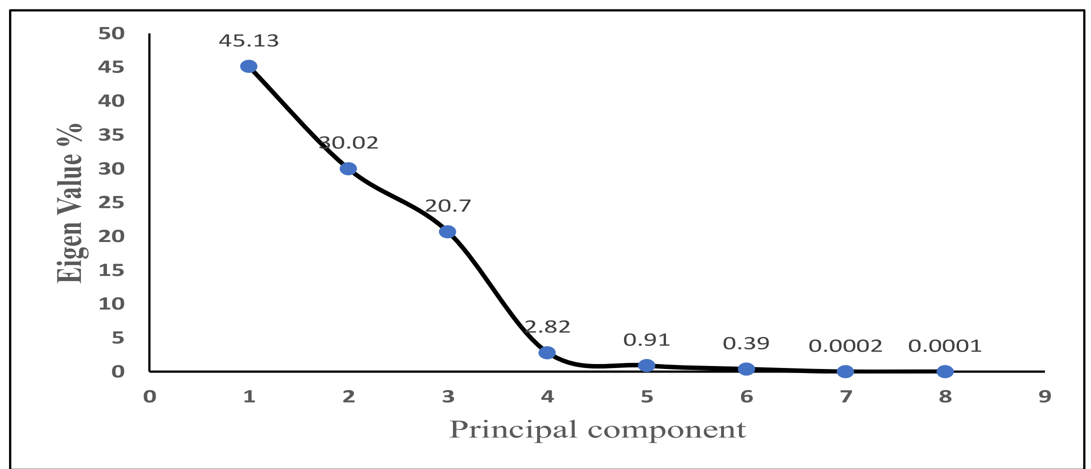


Figure 4.27: The scree plot of nine *Isaria* isolates collected from Darjeeling hills based on the content of total phenol, total flavonoid, total tannin, total carotene, total lycopene, beauvercin and antiproliferative MTT assay on three cancer cell lines (HeLa, HepG2 and PC3).

4.40 Evaluation of apoptotic induction potential of Beauvercin

A compound capable of inducing apoptosis is generally considered as a potent candidate in development of novel therapeutics for treating cancer. A few natural compounds used in the treatment of diseases have been identified from *Isaria* and we tried to identify one such anti-proliferative component in methanol extracts. We conducted HPLC with standard marker compound beauvercin and the resulting HPLC profile revealed that among the nine isolates *I. farinosa* (IDAR-02) and *I. tenuipes* (IDAR-04) was found to contain beauvercin at 6.02 ±0.36 and 6.71±0.31 µg/ml respectively. The identification was achieved by

comparing their retention time with retention time on chromatogram of marker compound beauvercin (Figure 4.28).

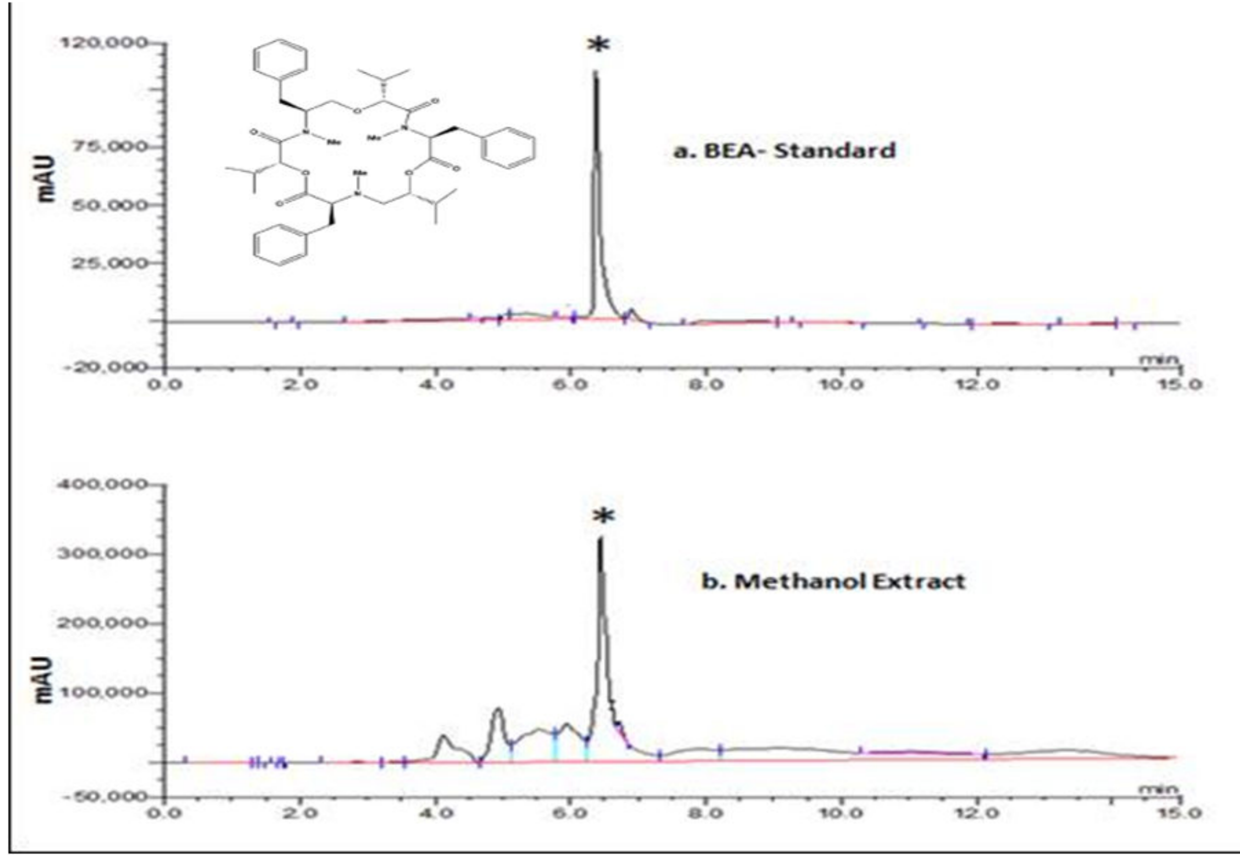


Figure 4.28: (a) HPLC profile representing signal generated at 210nm by injecting 20 µl of 20µg/ml beauvericin (BEA) standard; (b) methanol extract from mycelia of (*Isaria tenuipes* IDAR04).

4.41 Cell death assessment by flow cytometry assay using PI staining

To elucidate the effect of beavurecin positive methanol extracts particularly of *I. farinosa* IDAR-02 and *I. tenuipes* IDAR-02 on cell apoptosis we examined cell death of HeLa cell lines using propidium iodide (PI) single staining. Flow cytometric method was used to determine the cause of cell death. Since the methanolic extract exhibited promising results with HeLa cell line in the MTT assay, we conducted the propidium iodide (PI) staining to

evaluate apoptosis in the HeLa cells. In the present study, the result of PI staining demonstrated that the percentage of apoptosis in HeLa cells treated with 1, 3, 10, 30 and 100 μg/ml concentrations of methanolic extract from *I. tenuipes* IDAR-04 was 5.90% 6.85%, 10.67% 24.59% and 23.83% respectively (Figure 4.29).

After treatment with various increasing concentration of methanolic extracts (1-100μg/ml) for 72 hours the percentage of apoptosis demonstrated by *I. farinosa* IDAR-2 was found to be 2.56, 7.08, 5.70, 5.83, and 10.67% respectively (Figure 4.30). Comparatively the potential of *I. tenuipes* in bringing about cell death through apoptotic means was far superior to that of the ability exhibited by *I. farinosa* IDAR-02. Besides, the control sample without treatment exhibited that 0.54% of cells were undergoing apoptosis in contrast to the cells treated with DMSO used as a vehicle control, it was 3.43%. These results implied that beauvercin positive isolates are likely to be linked to anti-cell proliferation of HeLa cells via activation of apoptosis, indicating its presence in the methanolic extracts. It may be a good choice for enhancing cytotoxic cell death potency of beauvercin to seek more potent pro-apoptotic natural products.

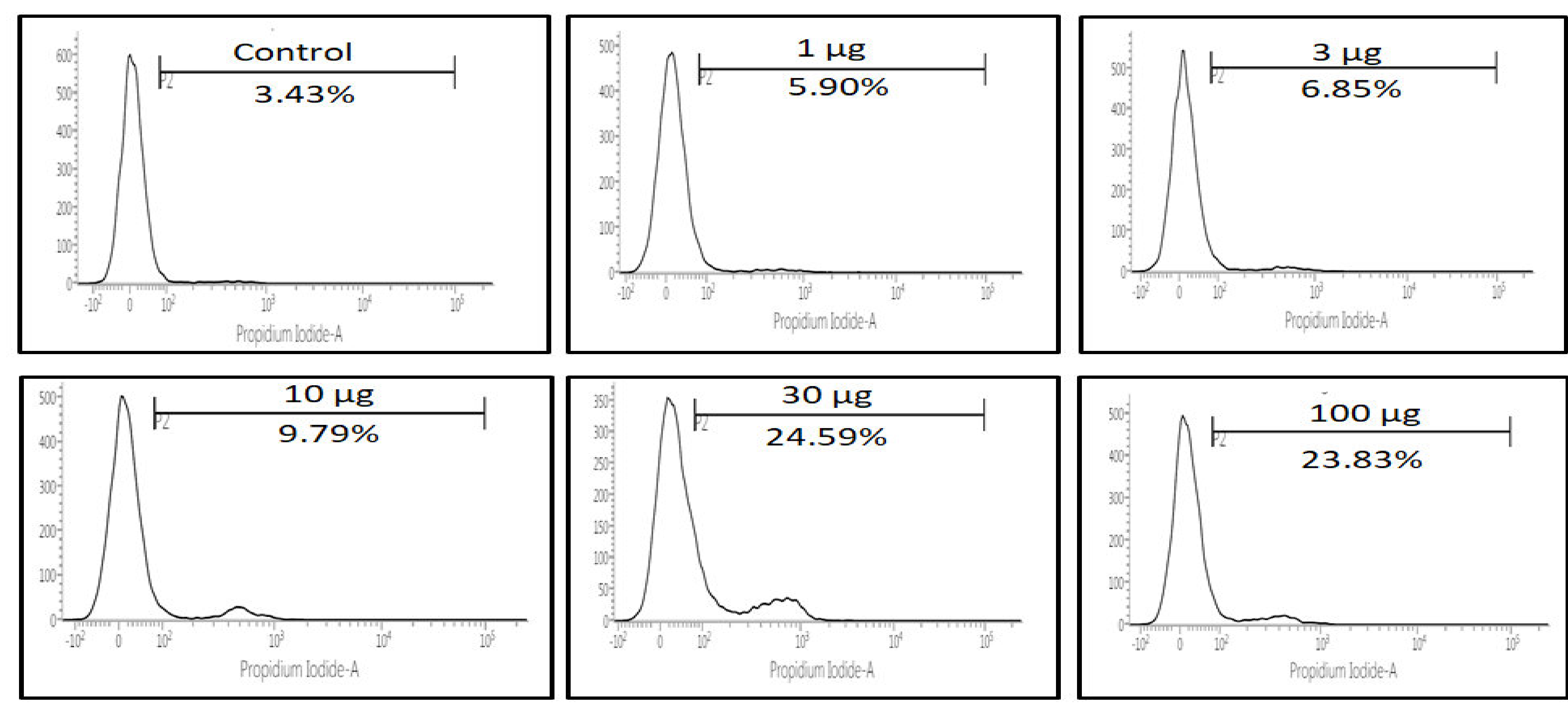


Figure 4.29: Cell death was assessed by flow cytometry of PI staining. HeLa cells were incubated with untreated and 1-100 µg/ml methanolic extracts for 72 h. Exposure to 30 and 100µg/ml methanol extract from *Isaria tenuipes* (IDAR-04), results in an increased level of cell death.

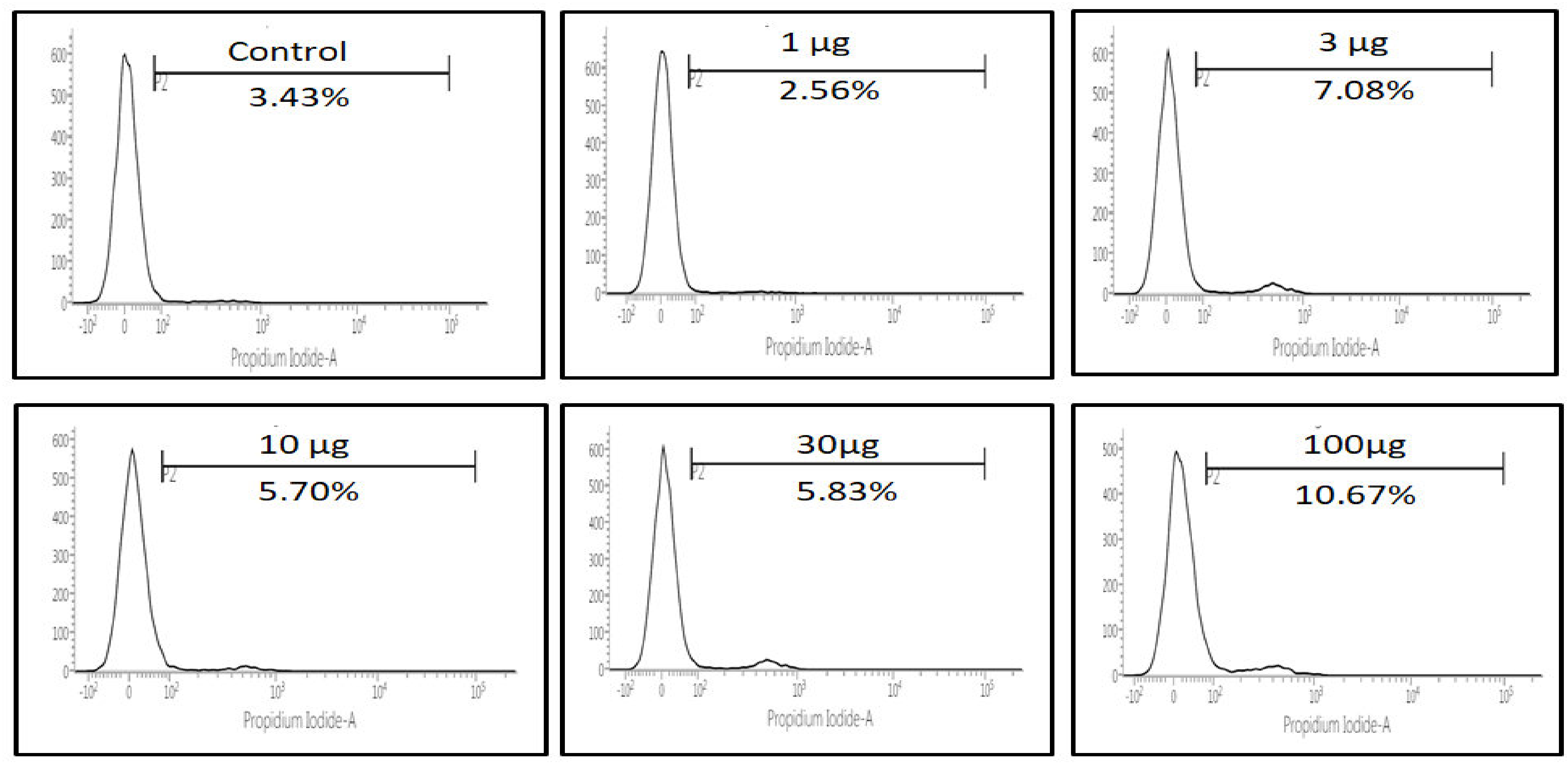


Figure 4.30: Cell death assessment by flow cytometry of PI staining. Cells were incubated with untreated and 1-100 µg/ml methanol extracts for 72 h. Exposure to 30 and 100µg/ml methanol extract of *Isaria farinosa* (IDAR-02) results in an increase level of cell death.

4.42 Morphological Evaluation

The significant inhibition of HeLa cells proliferation by IDAR-04 in comparison to IDAR-02 further led us to check the effect of IDAR-04 methanol extract on cell apoptosis. For morphological evaluation of apoptosis, HeLa cells were cultured in 24 well plates and treated with highest concentrations of IDAR-04 methanol extract (100µg/ml). We found that the dose-dependent treatment of methanol extract caused the change in cellular morphology of HeLa cells which were observed to be rounding up, shrunken, with irregular boundaries. In addition, presence of apoptotic bodies, decrease in cell concentration along with presence of few detached cells from the substrate compared to control well was clearly noticeable in HeLa cells (Figure 4.31).

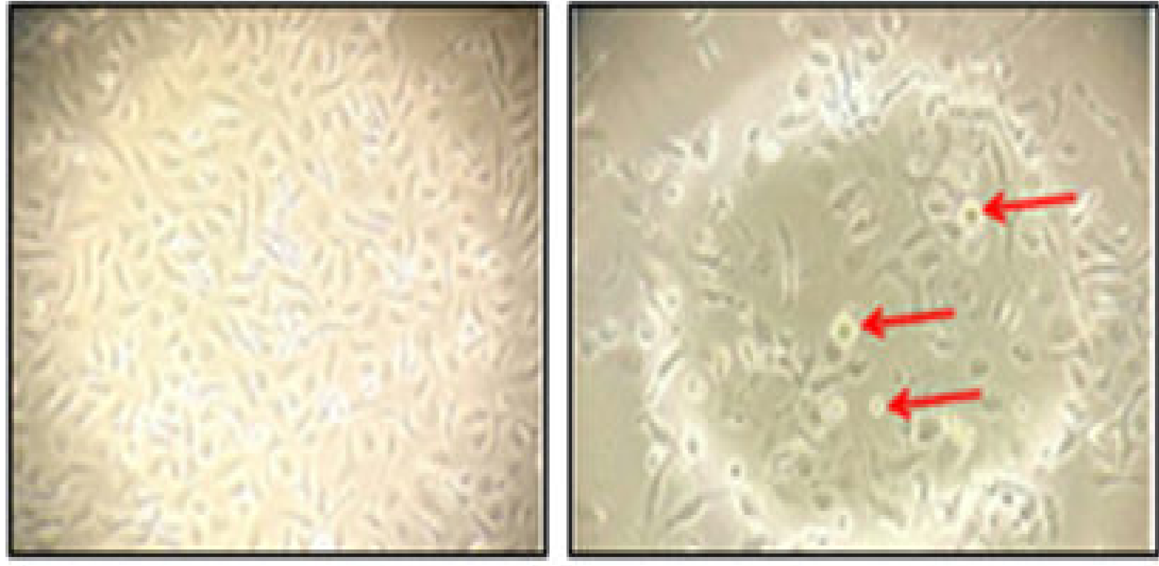


Figure 4.31: Light micrograph image of untreated HeLa cells (left) 70 % Methanol extract of *Isaria tenuipes* (IDAR-04) (100µg/ml) (right) treated HeLa cells after 72 hours, arrow showing apoptotic bodies.

4.43 Quantitative estimation of apoptotic cells

To confirm the apoptotic effect of HeLa cells after treatment with methanolic extracts of *I. tenuipes* IDAR-04, the *in vitro* FACS method was used to detect live, early apoptotic, late apoptotic and necrotic cells as well as to evaluate quantitatively estimate the number of apoptotic cells. As shown in the representative pictograms figure 4.32 of Hela cells, it was found that the number of intact cells in the lower left quadrant was 47.49±6.27% at higher concentration tested (100µg/ml). The lower right quadrant consisted of early apoptotic cells and the upper right quadrant contained late-apoptotic cells. After treatment with methanol extracts from *I. tenuipes* IDAR-04 (Table 4.25), the percentages of both early and late apoptotic cells gradually increased in a dose-dependent fashion reaching 42.205±19.03% at 100µg/mL with the concomitant decrease in the percentage of viable cells (43.85%). The percentages of Hela cells undergoing apoptosis following treatment with 0, 1, 3, 10, 30 and 100 µg/mL methanol extracts (including the early and late apoptotic cells) were 3.54±1.22, 7.09±3.73, 6.52±3.47, 8.86±4.36, 17.365±7.99 and 42.205±19.03% respectively as compared to 3.5% (early apoptotic+ late apoptotic population) in vehicle negative cells control (Table 4.25). From this result, it is clear that higher concentration at 30 and 100µg/mL resulted in an increasing number of double (+) cells indicating late apoptotic cells. Whereas the upper right quadrant in the pictogram also quantifies the number of cells that have entered the necrotic phase which in the present study was found to be 5.12 to 10.30±0.19% at the range of increasing concentrations 0 to 100µg/ml of methanol extracts tested. In this study we have observed dose-dependent effects of *I. tenuipes* IDAR-04 methanol extract on apoptosis (early and late) and the data has been statistically analysed with one way ANOVA to compare between means with standard deviation for three parallel experiments (Figure 4.33). Thus, FACS scan analysis of the treated cells revealed that the Hela cells were positive for Annexin V-FITC and PI at a very low concentration of test sample.

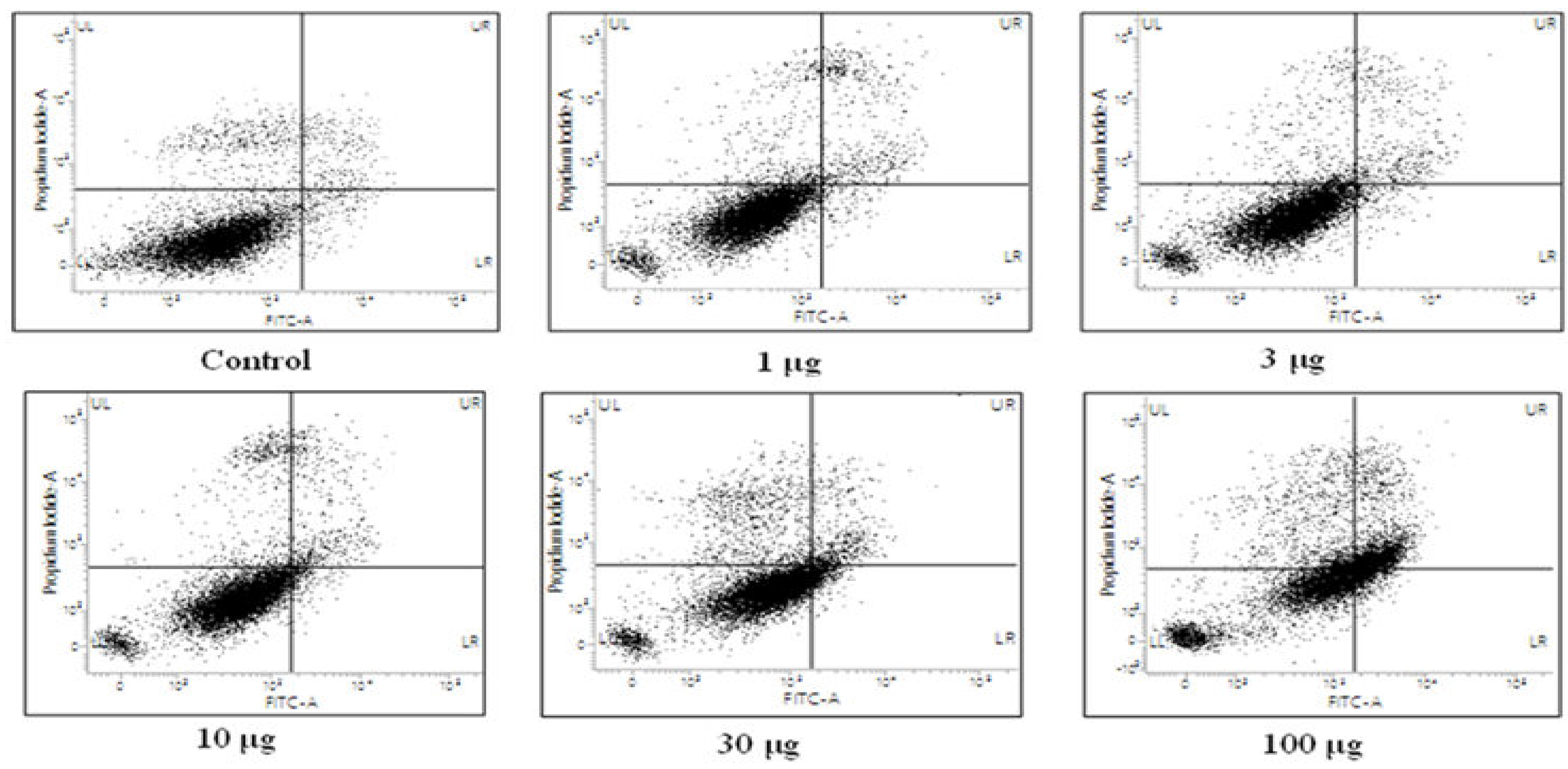


Figure 4.32: Evaluation of apoptosis induction in Hela cells after 48h of treatment with methanolic extract. The histograms show the percentage of early and late apoptosis for one of the experiments.

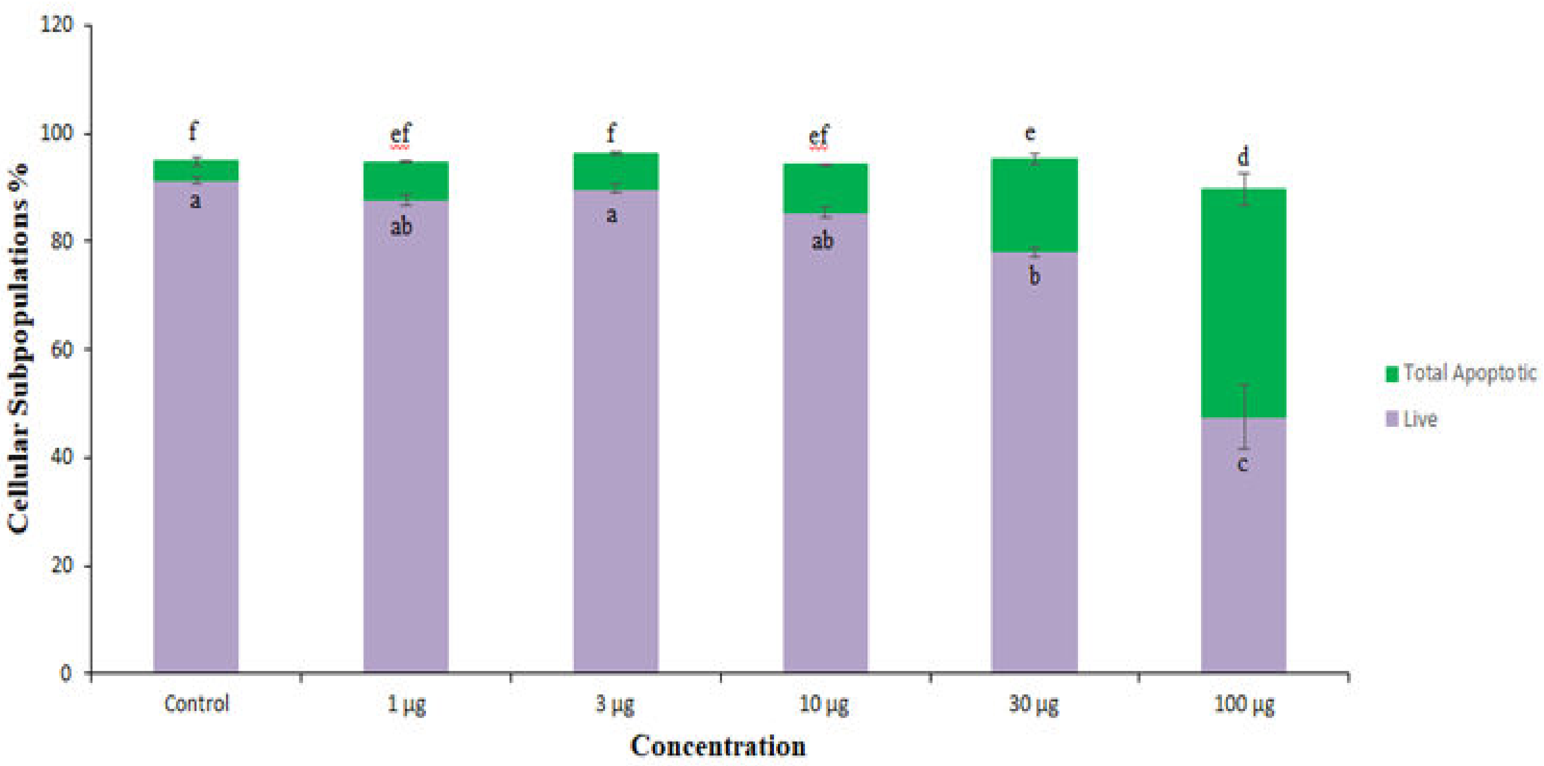


Figure 4.33: The percentage of total live, early and late apoptoctic population of Hela cells after 48h of treatment with 70% methanolic extract of *Isaria tenuipes* IDAR-04. The data are presented as the mean ± the SD (n=3), [a~c] and [e~f] values with different letters were significantly different at $p < 0.05$, as analysed by one-way Anova followed by Tukeys post hoc test of significance.

Table 4.25: The table shows the mean standard deviation (SD) percentage of live, early and late apoptotic cells from three independent experiments on the dried mycelial methanol extract of *Isaria tenuipes* (IDAR-04).

Features	**Control**	**1 µg**	**3 µg**	**10 µg**	**30 µg**	**100 µg**
Live	91.34±0.39	87.675±0.54	89.815±0.39	85.33±0.08	78.03±1.74	47.49±6.27
Total Apoptotic	3.54±1.22	7.09±3.73	6.52±3.47	8.86±4.36	17.365±7.99	42.205±19.03
Dead	5.12±0.95	5.23±1.52	3.67±0.17	5.81±1.13	4.60±0.99	10.30±0.19

4.44 Colony formation assay

Determination of cell colony formation is one of the important methods of cancer cell studies. To evaluate anti-proliferation activity in long-term cell culture and the ability of the HeLa cells to form colonies in the presence of aqueous methanolic mycelial extracts, colony formation assay was performed in varied dose in different time interval for up to 10 days. The outcome of the experiment was under the results obtained in the MTT assay and thus, a reduced clonogenic growth was observed in a dose-dependent manner with extracts of *Isaria tenuipes* IDAR-04, as shown in Figure 4.31. Densitometry analysis of no of colonies in each well with different doses of extracts of *Isaria tenuipes* IDAR-04 was calculated by using Image J software as shown in Figure 4.31. The results showed maximum colony formation of HeLa cells in negative control. The colony numbers were found to be significantly reduced after treatment with methanol extracts with increasing concentrations. Compared with those in the control group, the colony numbers in the methanolic extract treated cells were reduced by over >99% at 100µg/ml in a dose dependent manner. Thus, the cultured methanol extracts of *Isaria tenuipes* IDAR-04 decreases the proliferation capacity of HeLa cells.

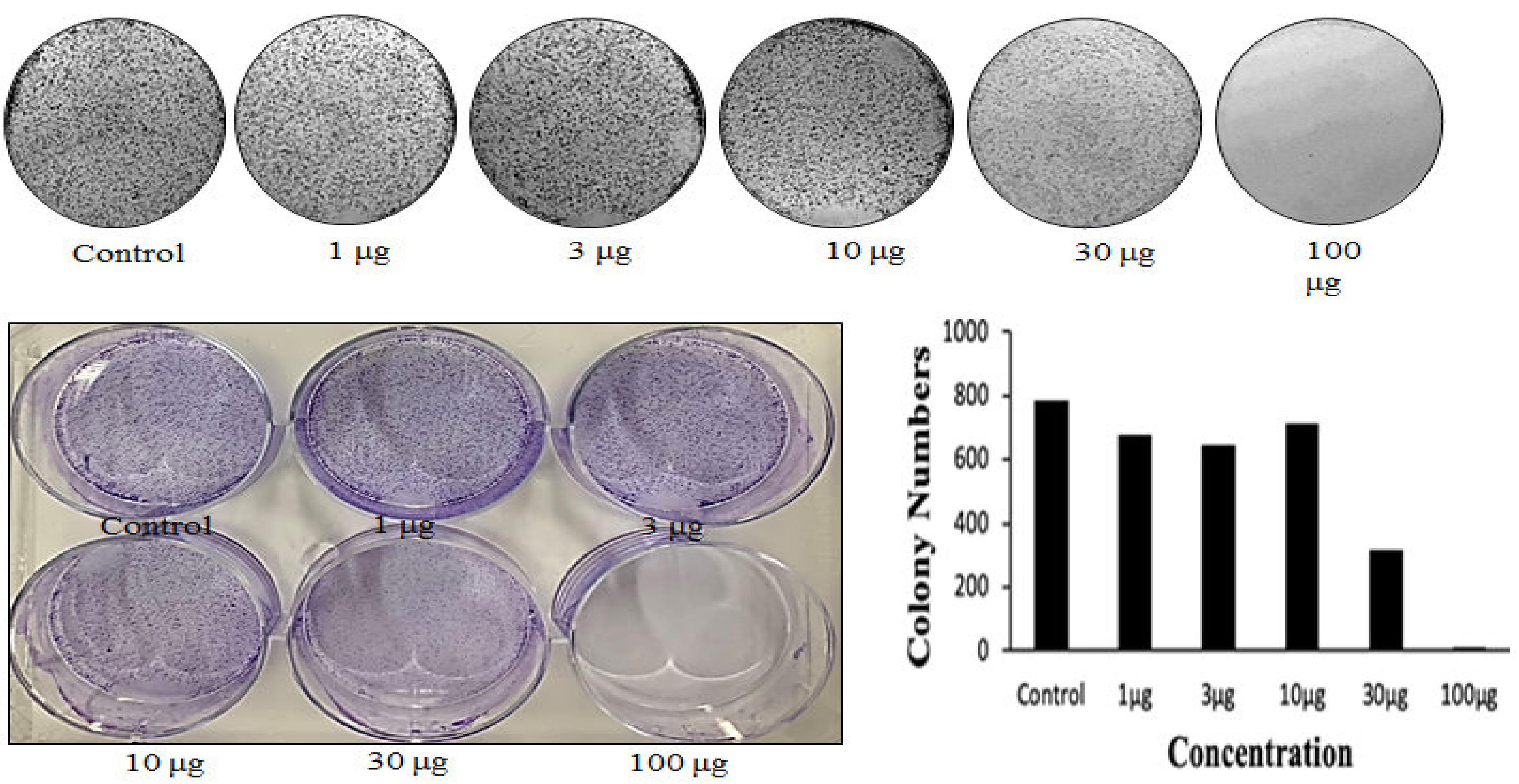


Figure 4.31: Evaluation of colony formations by clonogenic assay where cells were treated with indicated concentrations of the *Isaria tenuipes* (IDAR-04) extract and the colonies were quantified using ImageJ.

5. DISCUSSION

This study contributes to the goal of understanding the response of entomopathogenic fungi of the genus *Isaria* spp., to laboratory variables which is essential to improve their use as therapeutic and biocontrol agents. The work presented in this study used molecular taxonomy, cultural characterisation, biomass yield studies, antimicrobial, antioxidant activity and *in vitro* antiproliferative assays against three cancer cell lines to establish a base of knowledge useful for the future development of therapeutic agents.

This is the first study to report the presence of naturally occurring entomopathogenic fungi of the genus *Isaria* spp., from the temperate region of Darjeeling hills. In the present study, four species viz., *Isaria javanica, Isaria farinosa, Isaria tenuipes* and *Isaria fumosorosea* are reported from genus *Isaria*. Macroscopic and microscopic characteristics used for delineation of the genus of nine naturally occurring fungal isolates are shown in Table 4.2.

Species delineation was conducted with the internal transcribed spacer of rDNA (ITS-4 and ITS-5 sequences). In the present work, *Isaria* isolates from different locations and samples from around Darjeeling hills were identified to species level using the complete sequences of the ITS-4 and ITS-5. The sequences obtained were compared to GenBank database using the nucleotide BLAST and phylogenetic analysis was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). Sequences from the ITS-4—ITS-5 region using primers ITS4 and ITS5 generated an amplicon of 543 to 556 nucleotides in length specific to entomopathogenic fungus *Isaria* (Table 4.3). When the sequences of all the nine isolates were BLASTN searched against the sequences of *Isaria* species already

submitted in the NCBI GenBank it was found that the ITS sequences of the present samples had a maximal identity of 98-100 % homology with *Isaria* spp. The ITS phylogeny in the present study also supported the species identifications based on morphology (Figure 4.1). From this work, it was concluded, that the ITS sequences were clearly efficient to resolve lineages of *Isaria* isolates collected from different localities and various insect hosts.

The temperature is one of the most important physical factors that directly influence the mycelial growth. In the present study the suitable temperature for mycelial growth of all the nine isolates was found to be between 12°C and 28°C. This could partly be attributed to the adaptability of fungi in the wild to survive in a range of temperatures and a demonstration of their versatility to survive and flourish. The optimum temperature for the mycelial growth of the members of genus *Isaria* spp., was found to be approximately 20°C (Table 4.5). All the isolates clearly revealed its mesophilic nature and supported the earlier observations (Ban et al., 1998; Liu et al., 2018; Yamanaka et al., 1998; Liu et al., 2019). Therefore, present results also substantiate the findings of previous workers. However, some of the reported entomopathogenic fungi have a wider range of temperature tolerance of i.e., 0-40°C (Cabanillas, & Jones, 2009). In addition, strains of *Isaria farinosa* and *Isaria fumosorosea* were capable of exhibiting growth up to 35°C (Borisade, & Magan, 2014). While for the best mycelial growth of *Isaria japonica* the desired temperature range was between 22 and 27°C with the optimum temperature being 25°C (Yamanaka, & Inatomi, 1997). Shimazu and Takatsuki (2010), reported that in case of *Isaria javanica* the growth occurs in between 10-30°C with the optimum at 25°C. Furthermore, the optimal temperature for the growth of *Isaria cicadae* was reported to be 25°C (Liu et al., 2019). The present result shows relatively narrower temperature

range than that of other entomopathogenic fungi such as *Beauveria bassiana, Cordyceps militaris*, *Metarhizium anisopliae* and *Paecilomyces farinosus*, each having a broad temperature range from 1 to 25°C (Harada et al., 1995), 5 to 35°C, 5 to 35°C, and 5 to 40°C (Hallsworth, & Magan, 1999), respectively. More importantly, the *in-vitro* mycelial growths of all the isolates were most sensitive to increase in temperature. Normally in Darjeeling hills, the temperature does not exceed above 30°C even in the summer months. In the present experiment increase in temperature above 30°C was found to be suppressive for the mycelial growth. However, it was observed in different isolates of *Isaria* spp., exhibited different temperature optima for its growth. Therefore, it may be emphasized that higher mycelial production could be achieved by growing these isolates under controlled temperature as per specific requirements.

The study on fungal physiology has revealed that the optimum pH range for the growth of filamentous fungi is between pH 3.8 – 6, however; reasonable growth may be observed between pH 2 – 9 (Smith & Onions 1983; Bellettini et al., 2019). Since utilization of nutrients provided in the culture media depends chiefly on the pH of the media, it is imperative to determine the pH optima of genus *Isaria* members, if it has to be cultivated in artificial conditions. It is clear from the results that the pH range between 6.0 and 8.0 is optimal for the favourable mycelial growth with abundant density of all nine Indian isolates of genus *Isaria*. The present results with respect to pH optima seem to differ widely with the works of Ban et al., 1998 and Yamanaka et al., 1998, constituting one sharp pH optimal level of 7.0 in the different strains of *Isaria*. This difference in pH levels may be due to ecological variations. However, the present result is compatible with *Paecilomyces fumosoroseus*, as it grows well in the broad range of pH 6.0—9.0 (Shim et al., 2003). Shim et al., (2003)

also reported that pH optimum for maximum mycelial growth was 8.0, in *Paecilomyces sinclairii*, while in case of *Cordyceps militaris* the initial pH of 9.0 was recorded to be an optimum for maximal mycelial biomass yield (Kim et al., 2003) and pH values of 5-8 for fruiting body production (Wen et al., 2014). Furthermore, the optimal initial pH for the growth of *Isaria japonica* was reported to be in the range of 6.5 to 8.0 (Yamanaka, & Inatomi, 1997) which is in accordance with the present study. In general fungi have the ability to tolerate acidic pH (Booth, 1971). Therefore, the result obtained for members of this fungi exhibited growth at slightly acidic, at neutral and at strong alkaline levels although the pH level of substratum soil in the temperate region of Darjeeling hills was recorded to be 5.6 to 6.5. In the present studies, the growth of mycelia in higher pH range could be due to not using any buffer system to maintain a constant pH of the potato dextrose agar medium during the growth. The pH was kept uncontrolled since addition of any buffering ions could have drifted the overall composition of the media. Moreover, it is established in case of fungi that if the nutrient requirement is satisfied then most of the fungi are capable of exhibiting growth over a broad range of pH (Carlile, et al., 2001). It may be safely inferred that a range of pH optima between slightly acidic to slightly alkaline was eminently suitable in the nine isolates of *Isaria* spp., from Darjeeling hills.

Light plays a major role in the growth of fungi. Light has been reported to influence colony growth and airborne structures in certain members of fungi, but it is not involved in the morphogenesis of synnemata in the members of genus *Isaria* (Taber, & Vining, 1959). Colony diameter, mycelial density, texture and pigmentation of fungus in *in-vitro* conditions are sometimes influenced by light (Shrestha et al., 2006; Liu et al., 2019). Moreover, the common metabolic effect of light on fungal growth is the induction of carotenoid biosynthesis (Carlile, et al., 2001). However, in

the present investigation it has been observed that light had no significant effect on the colony diameter and mycelial density of *Isaria* isolates. Therefore, the growth of test isolates appeared to be independent of light. Contrarily, comparable growth was observed when the fungus was incubated in complete darkness. Furthermore, no pigmentation of the mycelium was observed under light conditions. Similar results have been reported earlier for *Isaria* (Yamanaka et al., 1998; Liu et al., 2018). It is interesting to note that the alternate light and dark conditions of twelve hours supported the abundant mycelial density of all the nine isolates of genus *Isaria* (Figure 4.2). The present experimental results are in consonance with the results on *Cordyceps militaris* isolates (Shrestha et al., 2006), on *Ophiocordyceps longissima* (Sung et al., 2011), as well as with *Isaria cicadae* (Liu et al., 2019).

Of the different culture media, the MCM medium was found to be most promising in terms of colony diameter and mycelial density for the growth of five isolates of *Isaria* spp., (Table 4.7). As glucose is the main carbon source of MCM (2 %, w/v glucose), it is believed that the presence of this carbon source could have helped in expediting mycelial growth at an early stage compared to other tested media. The sources of nitrogen in MCM are yeast extract and peptone which are considered to be the best nitrogen sources for the members of most fungi. The MCM contains possibly all low molecular weight soluble organic substances plus mineral nutrients needed for fungal growth. Potentially, this is the primary reason for such excellent growth irrespective of test isolates. Present work corroborated the findings with other entomopathogenic fungi and justified the nutritional requirements for abundant mycelial growth of the nine isolates (Kang et al., 2014; Shim et al., 2003; Wen et al., 2014). This result is also in consonance with that of *Shimizuomyces paradoxus* which had been reported by Sung et al., (2010). Even though the mycelial

growth in fifteen different media showed wide range of variations (17.0~60.0 mm in colony diameter), the mycelial density was compact in 9 of 10 culture media tested (Table 4.7). However, the observation of growth variation is probably due to the fact that the growth requirements for each fungus may vary from strain to strain. Cultures of the same species and genera usually grow well on similar media (Smith, & Kolkowski, 1996), the very interesting observation that sodium chloride (7.5%, w/v) was found to exhibit growth inhibitory effect in four strains of *Isaria tenuipes*, while few other strains could survive the effect of sodium chloride, although salinity affected erratically in their growth with very thin mycelial density. However, the result was anticipated and the source of an isolate can give us an indication of suitable growth conditions (Smith, & Kolkowski, 1996). These results indicated that *Isaria* isolates showed large variation in mycelial growth and texture in response to the media tested. This may be because each *Isaria* species has its own physiology and therefore its own specificity with respect to the components of the nutrient media.

Screening of fungal isolates from temperate region of Darjeeling Hills for twelve carbon sources has led to the selection of a favourable one. There were significant effects of carbon source supplementation to the basal media on the mycelial growth of all nine isolates (Table 4.8). Among the carbon sources tested, the highest or the longest radial colony mycelial growth was obtained with maltose, followed by sucrose and dulcitol. The greater and efficient utilization of maltose may be related to the hydrolysis of maltose during the autoclaving process generating glucose residues causing the initial mycelial growth rate. Favourability of most fungi toward glucose is perhaps due to its common availability in nature (Griffin, 1994). The present results are in agreement with the work on *Ophiocordyceps sinensis* (Dong and Yao, 2005) and with the work on *Ophiocordyceps longissima* (Sung et al., 2011).

Moreover, the results are also consistent with the work carried out by Liu et al., (2018) on *Isaria farinosa*. It also appeared that disaccharides had comparatively stronger effects on mycelial radial growth than the monosaccharides and polysaccharides tested. This result is in accordance with the findings of Kim et al., (2003) on *Cordyceps militaris* NG3, Shin et al., (2004), on *Cordyceps pruinosa* and Sung et al., (2010), on *Metacordyceps yongmunensis*. All the aforesaid workers have observed a high level of mycelial growth in media supplemented with disaccharides. The present experiment also revealed that between the two monosaccharides used, the ketohexose was more preferred than aldohexose for growth of mycelium which may be related to efficient incorporation of fructose directly in the respiratory pathway after phosphorylation (Cochrane, 1958; Chandra, & Purkayastha, 1977). Conversely, xylose and Na-acetate proved to be a very poor source of carbon for the growth of all nine isolates. The incapability of xylose to produce larger colony diameter may be due to its conversion to furfural form during autoclaving process which was sufficient to restrict the mycelial growth (Cochrane, 1958). Assimilation of disaccharides or polysaccharides by the fungi generally depends upon the ability to produce necessary hydrolytic enzymes. The present isolates were able to use polysaccharides in the form of soluble starch and mannose and it could be due to all nine isolates have the ability to produce starch hydrolysing enzymes under *in vitro* conditions. Amylase is one such enzyme which can hydrolyze starch into maltose and glucose and as a result may utilize complex carbon sources quite efficiently. The results indicate that sucrose could also produce comparable colony diameter and texture with respect to maltose which was the best carbon source determined.

Fungi are non-diazotrophic and do not fix atmospheric nitrogen into bioavailable forms. But they can utilize combined form of nitrogen. In the present

experiment, it was found that the test isolates had equal affinity to almost all types of nitrogen sources. When the organic nitrogen in the form of amino acids is tested, glutamic acid was found to be the most suitable for the mycelial growth of *Isaria javanica* (IDAR-01), *Isaria tenuipes* (IDAR-03) and *Isaria tenuipes* (IDAR-04). This is in accordance with the findings of Liu et al. (2016). This result points to the fact that single amino acid in the form of glutamic acid can produce all the other essential amino acids by transamination reactions (Deacon, 2006). Similarly, complex organic nitrogenous compound, yeast extract was found to be an excellent source of nitrogen for *Isaria farinosa* (IDAR-02), *Isaria tenuipes* (IDAR-06), *Isaria tenuipes* (IDAR-07) and *Isaria farinosa* (IDAR-08) respectively (Table 4.9). The superiority of yeast extract over other nitrogenous substance is that it contains a mixture of amino acids, peptides and water-soluble vitamins, especially B vitamins (Difco Manual 11th Edition) and it could be the reason for better growth of test isolates. Moreover, it has also been reported that organic nitrogen sources are absorbed easily by the cells in comparison to inorganic ones (Jung et al., 1997; Kang et al., 1997; Kim et al., 2003; Wang et al., 2008). This result substantiated the previous findings of Ali et al., (2009) on *Isaria fumosoroseus* and Liu et al., (2018) on *Isaria farinosa*. It is clear from the Table 4.9., that nitrate nitrogen yielded excellent mycelial growth of *Isaria farinosa* (IDAR-05) and *Isaria fumosorosea* (IDAR-09) with $NaNO_3$ and KNO_3 respectively. Such growth with nitrate nitrogen is presumably due to its ability to convert nitrate into ammonium by the expression of enzymes nitrate and nitrite reductase (Deacon, 2006). The ability to utilize nitrate as a nitrogen source in absence of other assimilable ammonium source is in concordance with other findings (Wang et al., 2008; Sung et al., 2010; Liu et al., 2018), Although Liu et al., (2018), concluded that urea could be a better source of nitrogen than nitrate for the growth of *Isaria farinosa*. The result of

present study was not in agreement with the view and urea as nitrogen source was found a poor supporter of growth in al isolates, except *Isaria javanica* (IDAR-01), Isaria tenuipes (IDAR-03) and *Isaria farinosa* (IDAR-05) exhibiting a moderate growth with somewhat compact mycelial density. Such exceptional use of urea by three isolates could be explained due to the ability of these isolates to produce adequate amounts of urea hydrolysing enzyme urease under cultural conditions. It also seems probable that urea requirements are specific for specific isolates. Similar observations were put forward by earlier workers such as Dong and Yao (2005) on *Ophiocordyceps sinensis* and Sung et al., (2010), on *Cordyceps cardinalis*. The different nitrogen sources except urea supported relatively appreciable growth as well as mycelial texture in different isolates of *Isaria*. It is striking to note that the metabolic potential of using organic and inorganic nitrogen sources may be accounted due to the peculiar life cycle pattern they carry out in the natural environment.

Studies show that a balanced nutrient medium will contain about ten times as much carbon as nitrogen. Therefore, C: N ratios of 10:1 or less will ensure a high protein content and a C: N ratio greater than this will favour accumulation of alcohol, acetate derived secondary metabolites, lipids or extracellular polysaccharides (Carlile et al., 2001). In the present study it was revealed that the effect of C/N ratio was strain dependent in which an optimum C/N ratio of 50: 1 was observed for *Isaria javanica* (IDAR-01), *Isaria tenuipes* (IDAR-03) and *Isaria fumosorosea* (IDAR-09) while 60:1 for *Isaria tenuipes* (IDAR-04), *Isaria tenuipes* (IDAR-06) and *Isaria farinosa* (IDAR-08), respectively and a very low C/N ratio of 5:1 for *Isaria farinosa* (IDAR-05). It is noteworthy to mention that if the percentage of nitrogen used is higher than carbon, a super mycelial growth inhibition occurs in most fungi (Bellettini et al., 2019). The results shown in Table 4.10 suggest that all the test isolates could utilize a maximum

amount of carbon when the percentage of carbon and nitrogen is 6% and 1% respectively.

In addition to various elements, fungi require very minute-amounts of vitamins not as an energy source (Kavanagh, 2011), mostly as organic coenzymes for various groups of enzymes (Campbell, & Farrell, 2009). It is clear from the results depicted in Table 4.11 that among the tested vitamins, biotin proved to be the best for *Isaria tenuipes* (IDAR-04), *Isaria tenuipes* (IDAR-06) and *Isaria farinosa* (IDAR-02) whereas *Isaria fumosorosea* (IDAR-09) preferred thiamine-HCl. It is also reported that the species *Isaria farinosa* grew well on B vitamins supplemented media (Liu et al., 2018; Liu et al., 2016; Yang et al., 2005). It appears from the present investigation that the B vitamins mainly the biotin and thiamine exhibited relatively better growth (maximum 34.66±0.57 mm on *Isaria farinosa* (IDAR-08) and 41±1 mm on *Isaria fumosorosea* (IDAR-09) colony diameter respectively).

Metals are directly or indirectly involved in all aspects of fungal growth, metabolism and differentiation (Gadd, 1986). Thus, indispensability of some mineral-salts that constitutes essential elements for the growth of nine isolates was investigated. It is interesting to note that from the experimental results that the macroelements such as potassium (KH_2PO_4), magnesium ($MgSO_4.7H_2O$), calcium ($CaCl_2.2H_2O$), iron ($FeSO_4.7H_2O$), zinc ($ZnSO_4.7H_2O$) and manganese ($MnSO_4.7H_2O$), promoted a luxuriant mycelial growth. It is reasonable to speculate the importance of potassium and magnesium in the central metabolic pathways and its role in fungal growth physiology. In most fungi, these mineral salts are necessary for the production of proteins even in the presence of organic carbon compounds (Masse, 1906). It was also reported that the potassium has **a** stimulative effect in the glycolytic cycles and its deficiency may inhibit sugar metabolism (Wang et al., 2008).

While the scarcity of magnesium can interfere with normal oxidation process of carbon source as it makes up an integral component of number of co-enzymes (Feng et al., 1995). There have been a few studies regarding the inhibition of mycelial growth by these elements and the present results are in consonance with these works (Feng et al., 1995; Sung et al., 2002; Park et al., 2004; Wang et al., 2008; Liu et al., 2018). However, the calcium ions are generally regarded as non-essential element but it has shown some effects on the mycelial growth of nine isolates on agar plate medium. As shown in the result the highest mycelial growth was recorded with *Isaria farinosa* (IDAR-08) and *Isaria fumosorosea* (IDAR-09) in the medium containing $FeSO_4.7H_2O$, while with $ZnSO_4.7H_2O$ supplementation the highest growth was observed for *Isaria farinosa* (IDAR-08) which was the highest growth recorded in the present experiment. On the contrary, it was observed that addition of $CuSO_4.5H_2O$ in the MPDA medium was a suppressor of growth in *Isaria javanica* (IDAR-01) (12.33±1.52mm) and IDAR-09 (15±1mm) (Table 4.13). Though copper is an essential element for fungal growth, however, even slight increase in Cu concentration has a toxic effect on fungi (Gruhn, & Miller, 1991). Excessive amounts of Cu can cause a drastic decrease in mycelial dry weight and diameter of mycelial growth in ectomycorrhizal species on agar media (Gruhn, & Miller, 1991; Tam, 1995). Our findings were also in line with these findings. The essentiality of iron and zinc in fungal growth was reported by Kim et al., (2010) for *Tricholoma matsutake*. Indispensability of iron in the mycelial growth is in agreement with the report of Liu et al., (2018) for *Isaria farinosa* and by Dong and Yao (2005) for *Ophiocordyceps sinensis*. In addition, zinc is also required in small amounts by the majority of fungi for being co-factors of a variety of enzymes (Garraway, & Evans, 1984). Furthermore, it was reported that the manganese ions are coenzyme of superoxide

dismutase, and are essential ions for enzyme that utilize ATP, and synthesize DNA and RNA (Reeslev, & Jensen, 1995; Park et al., 1995; Kim et al., 2010).

The nutritional and physiological analysis of *Isaria* reliably adds to the understanding of its adaptation in the climatic conditions prevailing in Darjeeling Hills. Thus, the present findings could also be used in selecting strains for future studies.

Phenolic compounds are mostly aromatic hydroxylated compounds, found in vegetables, fruits and many food sources. As a kind of secondary metabolites, phenolic compounds are generally categorized as phenolic acids and flavonoids, (Ferreira et al., 2017; Liu et al., 2022). Total phenolics content determined colorimetrically in the nine isolates were found in ranging from 115.58 µg GAE/g for methanol extract of *Isaria javanica* (IDAR-01) to 304.47µg GAE/g for *Isaria farinosa* (IDAR-02) methanol extract (Figure 4.9). The present results are in conformity with the trend documented by Chunyu et al., 2019 and Sharma et al., 2021 in *Isaria cicadae.* The total flavonoid content values of the extracts ranged from 7.39 µg QEs/g for methanol extract of *Isaria tenuipes* (IDAR-06) to 16.22 µg GAE/g for *Isaria tenuipes* (IDAR-03) methanol extract (Figure 4.10) and they decrease in the following relative order: IDAR-03 > IDAR-01 > IDAR-09> IDAR-08 > IDAR-02 > IDAR-04 > IDAR-05 > IDAR-07> IDAR-06 > methanol. Boonsong et al., (2016) have reported a flavonoid content in the water extract of an edible macro fungi such as *Lentinus edodes* (3.75±0.28 mg QE/g dw) and *Volvariella volvacea* (7.29±0.21 mg QE/g dw), but in the present study such high level of flavonoid was not recorded (Figure 4.10). The total tannin content values of the extracts range from 0.257 mg TAEs/g for methanol extract of *Isaria farinosa* (IDAR-08) to 0.483 µg TAEs/g for *Isaria farinosa* (IDAR-05) methanol extract (Figure 4.11) and they increase in the

following relative order: IDAR-05 < IDAR-07 <IDAR-06< IDAR-02 < IDAR-04 < IDAR-03< IDAR-01< IDAR-09< IDAR-08 < methanol. The amount of β carotene ranged from 0.055 in *Isaria javanica* (IDAR-01) to 0.208 mg/100g extracts in *Isaria fumosorosea* (IDAR-09) (Figure 4.12) whereas lycopene ranged from 0.002 in IDAR-01 to 0.041 mg/100g extracts in IDAR-09 (Figure 4.13). These results are also in line with those reported by Sharma et al., (2021) on the content of β carotene and lycopene content in *Isaria cicadae* to be 0.39±0.05µg/g and 0.29±0.05µg/g respectively.

The screening of antioxidant activity of the mycelial extracts was carried out in the current study. In the present work, seven widely accepted laboratory assessment parameters, viz. DPPH□ scavenging, ABTS□ scavenging, hydroxyl radical scavenging, hydrogen peroxide scavenging reducing power (RP), FRAP, and metal-ion chelation (MC) capacities of the cultured mycelium from nine isolates of *Isaria* spp., in addition to their total phenolics, flavonoids, tannins along with carotenoid contents were chosen for in vitro screening.

Figure 4.14., illustrates that in a concentration-dependent, radical-scavenging ability at a series of concentration of BHT and methanol extract from nine isolates, the lowest recorded scavenging effects was 3.3% in case of *I. javanica* (IDAR-01) at 2 mg ml^{-1} against the highest recorded 77.63% at the dose of 2 mg mL^{-1} for *Isaria fumosorosea* (IDAR-09). This activity of DPPH□ scavenging was closely matched by results of *Isaria cicadae* (Ren et al., 2014; Sharma et al., 2021), where the pattern of result exhibited higher DPPH activity with increasing concentration. Although, in the present study the isolates showed a weaker antiradical scavenging in comparison to that of *Isaria japonica* which scavenged the DPPH□ radicals by 49% at 0.1mg/ml (Yahagi et al., 1999). However, the present result was comparable to the one reported by Xiao et al., (2016), for alkali soluble and alkali refined polysaccharide from

Cordyceps taii where they scavenged DPPH radicals with EC_{50} values of 9.80±0.66 and 13.60±1.95 mg/mL respectively. From other medicinal fungi such as *Ophiocordyceps sinensis* showed >80% scavenging potential from hot water extracts of natural mycelia and cultured mycelia (Dong, & Yao, 2008), whereas for *Tolypocladium* sp. the maximum scavenged value was 80.1% at 4.5mg/ml (Zheng et al., 2008). While in case of non-entomopathogenic fungi it was found that at 10 mg/ml, the methanolic extracts of *Agrocybe cylindracea* and *Ganoderma tsugae* mycelia scavenged 91.4% and 95.6% of DPPH radicals, respectively (Tsai, 2002).

The degree of antiradical activity in among the nine isolates has been shown on the basis of determined IC_{50} value (Table 4.18). No remarkable scavenging activity were noted at lower concentrations while all the isolates exhibited radical scavenging activity in higher concentrations of methanolic extract which may be due to accumulation of various phytochemicals. However, this needs to be further explored in the future work.

The findings from this study suggest that at a 10 mg/ml concentration, the methanol extract of *Isaria javanica* (IDAR-01) exhibited the highest radical scavenging activity (83.65±0.37%) when reacted with ABTS* radicals. This isolate showing >80% scavenging capacity was considered as an excellent isolate. This activity was followed by methanol extracts of *Isaria tenuipes* (IDAR-03) and *Isaria farinosa* (IDAR-02) at 62.53±0.80% and 60.47±2.47%, respectively. These two isolates exhibiting > 60% activity was categorised as good isolates. The lowest scavenging activity was exhibited by *Isaria tenuipes* (IDAR-07) at 35.92±0.96% which was classified as moderate isolate in terms of its ability to scavenge free radicals. In the present study, it was also observed that the mycelium extracts efficiently scavenged the ABTS radical in a dose dependent manner indicating its

possible usage as a scavenger of peroxyl radicals. This, results have correlated with an earlier study of antioxidant effects generated by exopolysaccharides of two entomopathogenic fungi *Isaria tenuipes* and *Isaria sinclairii* showing ABTS scavenging activity with IC_{50} values of 5.43 ±0.13 mg/ml and 6.29 ± 0.13 respectively (Sharma, 2015). In the same study the ABTS scavenging ability of the intracellular polysaccharides was reported with IC_{50} values of 3.11±0.19 and 4.25±0.19 respectively (Sharma, 2015). Chhetri et al., (2020) found that methanolic extracts of mycelia of *Isaria tenuipes* scavenged 44.42%of ABTS radicals at 10 mg/ml. However, the present result is quite different from those reported by Sharma et al., (2021) in *Isaria cicadae* that showed a very high level of ABTS scavenging activity of 93±0.17%. However, the ABTS radical scavenging activity was inferior to that of methanolic extract from other medicinal mushrooms. Thus, Gursoy et al., (2009) found that a methanolic extract of the fruiting bodies of *Morchella conica* scavenged 78.66% of ABTS□ radicals at a 40 µg/ml concentration, while, de Bruijn et al., (2009), found the scavenging effect of an ethanol extract of *Grifolia gargal* to be 94.5%. On the basis of these comparisons, it is suggested that cultured extracts of nine isolates evaluated here could be of use as a natural source of antioxidants only at higher doses (8-10mg/mL). Methanolic extracts of all nine isolates may possibly react with free radicals, which are major initiators in the autoxidation of lipid, thereby terminating the chain reaction.

The hydroxyl scavenging activity of the mycelial extract in the present study showed moderate response that increased with concentration of the extract (Figure 4.16). In a study on *Isaria farinosa* reported elsewhere, the scavenging activity was around 46.48% when 3.2mg of its water-soluble polysaccharide was used (Jiang et al., 2008). In the present study, a similar magnitude of effect of about 50% inhibition of

hydroxyl radical was obtained only at 10mg/ml test solution. However, these results are in agreement with recent report on *Isaria* spp., (Chhetri et al., 2019, 2020). Similarly, an investigation carried out by Zheng et al. (2008), reported that the purified polysaccharides from *Cordyceps taii* demonstrated good activity at EC_{50} value of 2.72±0.80. Furthermore, hot water extracts from *Cordyceps militaris* showed 60% of hydroxy radical scavenging activity at 2mg/mL (Zhan et al., 2006). A similar finding was also reported from natural mycelia of *Ophiocordyceps sinensis* again showing 60% antioxidant activity at 2mg/ml (Dong & Yao, 2008). However, at 16 mg/ml, methanolic extracts from other medicinal mushrooms, such as *Coriolus versicolor, Ganoderma lucidum,* and *Ganoderma tsugae,* scavenged hydroxyl radicals by 38.0–52.6% (Mau et al., 2002; a, b). Thus, nine isolates of *Isaria* spp., in the present study may be considered a moderate scavenger of hydroxyl radicals suggesting it could be helpful in preventing the damage to important macromolecules.

Hydrogen peroxide scavenging effects of methanolic extracts from nine isolates of *Isaria* spp., increased with the increased concentrations from 15.23 to 56.82% at 2-10 mg/ml (Figure 4.17). The scavenging effect on hydroxyl free radicals was the highest by *Isaria tenuipes* (IDAR-06) showing 57.48±0.58% followed by *Isaria tenuipes* (IDAR-04) 56.82±1.41% activity at 10 mg/ml respectively. The rest of the extracts did not scavenge free radicals in >50% level even at the highest tested concentration (10mg/ml). Jiang et al., (2005), reported that a scavenging ability of water soluble exo and intra polysaccharides from *Isaria farinosa* B05 at 10.24 mg on hydrogen peroxide radical was 100 % respectively. In the present study, absolute scavenging was not demonstrated by any of the nine isolates. While in other mushroom such as *Agaricus bisporus* it was found that the hydroalcoholic extracts had high inhibiting activity with low EC_{50} value of 2.75 ± 0.2 mg/ml (Vamanu, 2012).

Vishwakarma et al., (2017) found that hydroalcoholic extracts of the fruiting bodies of *Pleurotus cystidiosus, Pleurotus flabellatus* and *Pleurotus florida* scavenged hydrogen peroxide radicals with EC_{50} values of 2.4, 3.4, 1.5 and 0.4 mg/ml respectively. The present results indicated that *Isaria* spp., showed dose dependent manner in scavenging hydrogen peroxide radicals and are moderate scavengers of such radicals.

In the present study, on the isolates at increasing concentration (2-10mg/mL), *Isaria javanica* (IDAR-01) demonstrated strongest reducing power at 1.39±0.09 (10mg/mL), which was followed by *Isaria fumosorosea* (IDAR-09) at 1.002±0.33. Sharma (2015), reported an excellent reducing property of exo and intra polysaccharides of *Isaria tenuipes* (IC_{50} 6.36 ± 0.22 and 2.43 ± 0.27). A relatively high reducing power of *Cordyceps taii* have been previously reported with EC_{50} values at 3.23±0.47 mg/ml for alkali soluble refined polysaccharides (Xiao et al., 2016). With regard to the antioxidant activities, crude methanolic extracts from present study were found to be much lower than those reported from polysaccharides of aforementioned species. However, the present study demonstrated comparable reducing activity to those of *Ophiocordyceps sinensis* and *Cordyceps militaris* (Zhan et al., 2006; Lin et al., 2012). Moreover, the results found in mycelial methanolic extracts in the present study are even higher than those reported by previous authors for *Cordyceps militaris, Ophiocordyceps sinensis* and *Tolypocladium* sp. (Zhan et al., 2006; Lin et al., 2012). The reducing power may be attributed to their hydrogen donating ability, identifying the presence of antioxidants (Shimada et al., 1992). Accordingly, the nine isolates of *Isaria* spp., might contain higher amounts of reductone, which could react with free radicals to stabilise and terminate radical chain reactions. Although no methanol extract could give similar reductive capability at the

same concentration as BHT, but similar activities to that of BHT was observed in few isolates (Figure 4.18).

Mycelial extracts in methanol of nine isolates used in this study exhibited more or less similar iron chelating ability as functions of their concentration (Figure 4.19). Methanol extracts of *Isaria tenuipes* (IDAR-04) showed the highest ferrous iron chelating effect (86.73±1.3%) at 10 mg/ml while *Isaria javanica* (IDAR-02) showed the lowest chelating ability (71.72±1.6%). The metal chelating activity of *Isaria farinosa* was determined at around 97.44% at 12.8mg/ml (Jiang et al., 2008). Fe^{2+}chelating effect to the tune of about 66% was shown by 8mg/ml extract of *Cordyceps militaris*, another entomopathogenic fungus (Zhan et al., 2006). The aqueous extract from *C. taii* was an excellent ferrous chelator, chelated 85.4% at 1.0mg/ml (Xiao et al., 2016). The present findings are also in agreement with the results found by Sharma (2015), using exopolysaccharides and intrapolysaccharides isolated from *Isaria tenuipes*. The present results have shown comparable inhibition of Fe^{2+} and therefore, the present work may be considered in sync. The chelating activity in the present study from nine isolates of *Isaria* spp., sharply increased with increased dose from 2.0 mg/mL to 10.0 mg/ml, and reached a plateau of 75.39% to 86.73% in between a concentration of 6.0 mg/ml and 10.0 mg/ml. This result meant that methanolic extracts had a stronger chelating capacity but not as much as the positive control EDTA. Chelating agents might function as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ions (Gordon, 1990). In general, methanol extracts from the cultured mycelium in the current study demonstrated antioxidant efficiencies comparable to the other species reported in the literature.

Antimicrobial resistance (AMR) is a natural prevalence that is connected to a rise in "mortality, morbidity and economic burden" of nations worldwide (Zhen et al., 2019). Additionally, the strains exhibiting Multi Drug Resistance (MDR) are increasingly spreading as a contagious pathogen throughout the globe. Owing to these negative health trends and the need of new antimicrobial agents to reduce threat of further resistance has emboldened us to evaluate the effect of methanol extracts of nine isolates of *Isaria* spp. as antimicrobial agents. In the current study fungal crude extract demonstrated promising *in vitro* anti-infective result by exhibiting maximum antibacterial activity against human bacterial pathogens. It was observed that the all of the crude extracts inhibited at least one of the microorganisms studied (Table 4.19 & 4.20). Among the tested extracts, *Isaria farinosa* (IDAR-02), *Isaria tenuipes* (IDAR-03), *Isaria tenuipes* (IDAR-05) and *Isaria fumosorosea* (IDAR-09) extract inhibited the growth of the all six human pathogens; *Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and have shown broad spectrum activity. Antibacterial activity of each extract was consistent to results obtained with mycelial extract from *Isaria farinosa* (Brel et al., 2020). However, the other extracts displayed significantly smaller inhibition zones when compared to a positive standard Streptomycin at 100µg/ml. The mycelial crude extracts have shown largest zone of inhibition against *Klebsiella pneumoniae*, 16.33±0.57mm (*Isaria tenuipes* IDAR-06) and against *Bacillus subtilis,* 16±1.0mm (*Isaria fumosorosea* IDAR-09) respectively. Moreover, it was observed that the cultured mycelia were capable in restoration of anti-infective activity in particularly problematic Gram-negative bacteria mainly the streptomycin resistant strain the *Pseudomonas aeruginosa*. Like Gram-negative bacteria the Gram-positive bacteria was also found to remain highly susceptible towards all the tested

concentration of methanol extract and the positive control. In addition, no growth was noticeable in any of the wells containing EDTA which was used as a negative control. Therefore, it is believed that cultured methanol extract constitutes a potential anti-infective agent for the treatment of problematic bacteria residing as commensals within the mammalian cells. This in particular constitutes Gram-negative bacteria which has the maximum bioavailability problems in comparison to Gram-positive bacteria (Graef et al., 2016). Components from crude fungal extracts have demonstrated antimicrobial activity against wide spectrum of human pathogens and its antimicrobial properties have been proven by many studies (Idris et al., 2013). Although antimicrobial properties of higher medicinal fungi are available, however, there has been a lacuna of research on the antibacterial action of *Isaria* species. The genus *Isaria* has received considerable attention in recent years, because of its wide range of chemically novel metabolites (Weng et al., 2019; Zhang et al., 2019). The current study suggests that the antimicrobial action of isolates from *Isaria* spp., may be mediated by bioactive metabolites.

The goal for a successful cancer therapy demands the screening and identification of highly efficient therapeutic molecules (Venkatachalam, & Nadumane, 2021). The high toxicity of the available drugs and their various other side effects necessitates the search for novel drugs active against diverse kinds of tumours (Demain and Sanchez 2009). It is estimated that out of the 175 small molecules utilized clinically for cancer therapy from 1940s to 2014, 131 (approx. 75%) drugs are either being natural products or those directly derived therefrom (Newman & Cragg, 2010; 2014).

Fungi-derived natural products are considered to be one of the most important sources of pharmaceutical compounds. Surprisingly however, no fungi-derived agent

has been approved as an anticancer drug so far. The genus *Isaria* spp., has been demonstrated to possess various biological and pharmacological properties, including growth inhibitory effects on various cancer cells. Fungal extract from *Isaria japonica* could significantly decrease tumour weights and volumes, in mice inoculated with Sarcoma-180 tumour cells (Shin et al., 2003). The fungal extract at 50mg/kg caused inhibition of the tumour volume by 28.1% (3749 ± 279.3 mm^3) and a decrease in the tumour weights by 42.3% (5.1 ± 0.4 g) (Shin et al., 2003). The methanolic extracts from fruiting body of *Isaria tenuipes* DGUM 32001 has been reported to possess significant cytotoxicity against human cancer cell lines: MCF-7 and HepG2 (Shim et al., 2001). The ethyl acetate fraction of this fungus showed cytotoxicity against HepG2 and MCF-7 with IC_{50} values of 40 and 9.6 µg/mL, respectively (Shim et al., 2001). It was further demonstrated that MCF-7 breast carcinoma was inhibited by 44.83 ± 6.88% with ethyl acetate extract of *Isaria tenuipes* VHI-2 fruiting bodies (Pham et al., 2020). In *Isaria tenuipes*, phytochemical constituents were attributed for antiproliferative ability and the presence of beavurecin was thought be responsible for antiproliferative activity also exhibited apoptosis inducing ability on HeLa cells (Chhetri et al., 2020). In this study, methanol extracts from cultured mycelia showed effective antiproliferation on 3 types of cancer cells as reported in the result section (Table 4.21; 4.22; 4.23).

MTT assay of the methanol extracts on HeLa, HepG2 and PC-3 cell lines indicated potent cytotoxicity of the test samples against the cancer cell lines (Figure 4.23, 4.24 & 4.25). In the antiproliferative test, within 72 hours of exposure, the methanol extracts demonstrated reduction in cell viability by more than 95% (at 100µg/ml of extract) in a dose-dependent manner especially on HeLa cells (Figure 4.23). Of all the fungal extracts tested, the methanol extract from *I. tenuipes* IDAR-

03, *I. tenuipes* IDAR-04 and *I. fumosorosea* IDAR-09 exhibited the most promising results after 72 hours of treatment where the viability of the HeLa cells decreased to 6.74, 6.91 and 5.52% respectively at the highest test concentration (100µg/mL). Thus, the observed cytotoxicity reported in this study can be attributed to the presence of various phytochemical compounds in the fungal extracts. Indeed, many researchers have confirmed that the extracts from entomopathogenic fungi are excellent producers of strong cytotoxic phytochemicals (Sun et al., 2017; Wang et al., 2016). According to the United States National Cancer Institute Plant Screening Program, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} values is<30-40µg/ml (Talib, & Mahasheh, 2010). Taken together, methanol extract can, therefore, be regarded as a good source of phytochemicals with cytotoxic potential. In this context, the cytotoxic activity of the mycelial extracts established a fact that the entomopathogenic fungi are a potential source of natural anticancer compounds. Similar studies where the percentage of viability of the cells decreased with an increase in concentration of the test extract have been reported from mycelium of medicinal mushroom *Ganoderma tsugae* used in traditional folk medicine where the viability of Hep 3B and HL-60 cells decreased to 25.5% and 21.00 % at 4.00mg/ml after 72 hours of treatment respectively (Chien et al., 2015).

Further, the lead compounds possible in the fungus present could also play a significant role in disease prevention and treatment. However, the antiproliferative effects may differ markedly based on different cancer cell types (Ogawa et al., 2014). In brief, it is worth noting that the HeLa cells exhibited noticeable inhibition even at a lower concentration of extract tested. The inhibition of HeLa cells was found to be higher in the present study as compared to that of *Isaria amoenerosea* which exhibited about 53% inhibition (Chhetri et al., 2019). It should be stressed, however,

that in the HepG2 and PC3 cells, methanol extracts were shown to only induce low antiproliferative activity.

Defining mechanism responsible for antiproliferative potential of the methanol extract is crucial to understand the features associated with cancer. Necrosis and apoptosis are two different mechanisms of cell death with some specific features. Apoptosis is believed to be highly regulated at the morphological level and is well characterised by membrane blebbing, chromatin condensation, cell shrinkage, nuclear cytoplasmic fragmentation and the formation of apoptotic bodies (Sadananda et al., 2016). Apoptosis usually takes place during normal development and aging processes and as a homeostatic mechanism to maintain cell populations in tissues (Elmore, 2007).

The alternative to apoptotic death is necrosis which is a passive form of cell death that mainly is induced by toxic or physical damage and is considered to be a toxic process (Edinger et al., 2004). The morphological changes that occur with necrosis involve the process in which damaged cells are aggregated and clustered, the shape and function of mitochondria are changed and nuclear chromatin becomes plexiform (Wyllie et al., 1980). Owing to eventual disruption of membrane integrity results in swelling and then rupture of the cells releasing the cytoplasmic contents into the surrounding tissue, with eventual recruitment of inflammatory cells (Elmore, 2007). In the case of apoptotic cells, it does not release any of their cellular constituents into the surrounding interstitial tissue and are quickly phagocytosed or disposed of, which prevents the development of an inflammatory reaction (Savill and Fadok, 2000; Kurosaka et al., 2003).

Apoptosis or necrosis may confer the growth inhibition of HeLa, HepG2 and PC3 cells in response to methanol extract treatment. Morphological investigation of the methanolic extract at 100 μg/ml concentration on HeLa cells revealed cell death

through the mechanism of apoptosis (Figure 4.23). In the current study, the microscopic observations revealed that as compared to the untreated cells, the treated cells became compact, had shrunken irregular margins, a high degree of membrane blebs, formation of apoptotic bodies with rounded shape, improved brightness and reduced cellular volumes indicating apoptosis. From such evaluation, it is presumed that the methanol extracts may be responsible for bringing about apoptosis under *in-vitro* conditions. Recent studies suggest that *Isaria tenuipes* is rich in various secondary metabolites making it an important fungal species with potential pharmacological functions (Zhang et al., 2019).

Anticancer effects by inducing apoptosis may be a valuable mechanistic approach for cancer chemotherapy while preventing unfavourable side effects and drug resistance (Shafi et al., 2009). In this study, first we confirmed the cytotoxic effects of methanol extracts using cell viability assay and tried to identify physiologically active molecule beauvercin in the nine isolates. Cyclic peptides, such as beauvericin isolated from fungal sources are capable of exerting anticancer effects (Wang & Xu 2012). Studies have attributed the anticancer activities of beauvericin on several cancer cell lines including human epidermoid carcinoma cell lines KB and KBv200 with an IC_{50} values of 5.76±0.55 and 5.34±0.09 μM, respectively (Tao et al., 2015). In the present study, the presence of beauvericin was identified using HPLC. Studies have suggested that beauvericin had remarkable activity against diverse cancer cell lines with the potential for use as medicine (Wang & Xu, 2012). The most important species of the genus *Isaria* which are reported to be a beauvericin producer includes *Isaria fumosorosea, Isaria japonica, Isaria cicadae*, and *Isaria tenuipes* (Luangsa-ard et al., 2009). Furthermore, beauvericin isolated from *Isaria* has been reported to inhibit the migration activity of PANC-1 cells by upregulating the

expression of the *E-cadherin* gene and reducing *N-cadherin* and *Snail* genes (Yahagi et al., 2020).

Further validation of the possibility of beauvercin positive isolates *Isaria farinosa* (IDAR-02) and *Isaria tenuipes* (IDAR-04) to ensure whether this cytotoxic effect was from apoptosis induction or through necrosis, flow cytometric analysis of apoptosis was performed. The flow cytometric analysis of cell cycle showed that methanol extracts from *Isaria farinosa* (IDAR-02) and *Isaria tenuipes* (IDAR-04) promoted HeLa cell apoptosis in a dose dependent manner. HeLa cells were undergoing preliminary cell cycle arrest in the G0/G1-phase followed by increased in apoptotic cells that could explain the morphological changes in the cell and eventual decrease in cell viability. The percentage of HeLa cells undergoing apoptosis following treatment with methanolic extract of *Isaria tenuipes* (IDAR-04) exhibited up to a maximum of 24.59% (Figure 4.29). Similarly, the apoptosis rates of HeLa cells were found up to 10.67% by *Isaria farinosa* (IDAR-02) extract treatment (Figure 4.30).

Determination of cell apoptosis by the propidium iodide (PI) flow cytometric assay is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Thus, flow cytometric method may reveal the cause of cell death and in this technique the hypodiploid nuclei from apoptotic cells are detected using DNA-binding fluorochrome, propidium iodide. The technique determines the DNA content which could indicate the number of apoptotic cells. It is noteworthy here to mention that sometimes necrotic cells also display a certain degree of DNA fragmentation that may eventually result in hypodiploid nuclei. Therefore, the presence of a hypodiploid

DNA peak in flow cytometric studies does not provide unambiguous proof of apoptotic cell death (Vermes et al., 2000).

To further investigate the apoptotic activity of methanol extract from *I. tenuipes* IDAR-04 on HeLa cells, we examined cell death and observed it with double positive staining using Annexin-V-FITC/PI to distinguish the dead cell population as apoptosis or necrosis. Koopman et al., (1994) for unravelling the regulation process of apoptosis developed a flow cytometric assay using Annexin V conjugated to FITC to measure Annexin V binding to apoptotic cells. The Annexin V/PI staining is mostly used to discriminate between apoptotic and viable cells. Apoptotic cells are stained positively for Annexin V that binds to phosphatidylserine (PS) but are negative for staining with PI, whereas viable cells are negative for both Annexin V and PI staining (Arora & Tandon, 2014; van Engeland et al., 1998).

To quantify the extent of apoptosis, Hela cells treated with methanol extract were stained with Annexin V-FITC/PI followed by flow cytometry analysis. In the representative pictograms of Hela cells, it was found that the number of intact cells in the lower left quadrant was 47.49±6.27% at higher concentration tested (100µg/ml). The lower right quadrant consisted of early apoptotic cells and the upper right quadrant contained late-apoptotic ones. After treatment with methanol extracts of *Isaria tenuipes* (IDAR-04) (Figure 4.32), the percentages of both early and late apoptotic cells gradually increased in a dose-dependent fashion reaching 42.205±19.03% at 100µg/ml. From this result it is clear that treatment of higher concentrations at 30 and 100µg/mL resulted in an increasing number of double (+) cells indicating late apoptotic cells. Whereas the upper right quadrant in the pictogram also quantifies the number of cells that have entered the necrotic phase which in the present study was found to be 5.12 to 10.30±0.19% at the range of increasing

concentrations 0 to 100µg/mL of methanol extracts tested (Table 4.25). Thus, FACS scan analysis of the treated cells revealed that the Hela cells were positive for Annexin V-FITC and PI at a very low concentration of test sample. Studies on *Cordyceps militaris* and *Ophiocordyceps sinensis* have revealed that cordycepin at 15.34 µM could induce 24.3% early and late apoptosis in H1975 cell (Wang et al., 2016). A similar effect was observed with nucleoside rich ethanol extracts of *Cordyceps militaris* that increased the early apoptosis of a human colorectal cancer-derived cell line, RKO to 8.48% at 100µg/mL (Lee at al., 2015). Most importantly, the present findings in Hela cell lines are in agreement with the apoptosis rates of powdered extract from *Isaria cicadae* demonstrated by Sun et al., (2017). They have reported that the apoptotic rates including the early and late apoptotic Hela cells were found to be 0.60 ± 0.19%, 22.0 ± 0.67%, 31.60 ± 1.58% and 92.70 ± 3.25%, respectively at 0, 40, 80 and 160 µg/mL of *Isaria cicadae* broken conidia powder (ICBCP) (Sun et al., 2017). Therefore, the present result is comparable and in agreement with a number of fungi-based phytochemicals capable of demonstrating apoptosis in the test cell lines.

Finally, the colony forming ability of Hela cells was investigated in presence of methanol extracts of *Isaria tenuipes* (IDAR-04) for ten days. The colony numbers were found to be significantly reduced after treatment with methanol extracts with increasing concentrations (1, 3, 10, 30, 100 µg/mL). Compared with those in the control group, the colony numbers in the methanolic extracts treated cells were reduced by over >99% at 100µg/mL in a dose dependent manner (Figure 4.34). Thus, the cultured methanol extracts of *Isaria tenuipes* (IDAR-04) decreases the proliferation of HeLa cells. The ability to form colony is well correlated with the *in vivo* oncogenic potential of cancer cells (Mori et al., 2009). The present results are in

accordance to that of ganoderic acid from medicinal mushroom *Ganoderma lucidum*, which demonstrated inhibition on colony formation of invasive breast cancer cells (Jiang et al., 2008). This drastic reduction in the colony forming ability of Hela cells reveal that apart from exerting direct cytotoxic effects, the extracts also demonstrated anti-proliferative effects on the cancer cell tested and thus proved to be a potential anticancer agent.

The present experiments showed that all the methanol extracts exhibited cytotoxic or cytocidal effects on three cancer cell lines tested. The cytocidal properties of test extracts was confirmed by cell viability assay which have thrown some light on the mechanism of action of the extracts. Furthermore, it was demonstrated that the fungal extract treated cells showed *in-vitro* cytocidal activity which was determined using Annexin V-FITC/PI and clonogenic cell survival assay on HeLa cells (Figure 4.26). The clonogenic assay confirmed that the methanolic extract of *Isaria tenuipes* IDAR-04 was cytotoxic in its mode of action thereby hindering the multiplication of the HeLa cells in an irreversible manner. The results were consistent and in conformity with those of *Cordyceps militaris* against human breast and bladder cancer cells (Park et al., 2009), *Cordyceps militaris* against human colorectal RKO cells (Lee et al., 2015), *Isaria cicadae* against gynaecological carcinoma, MCF-7 and Hela cells (Sun et al., 2017), *Isaria farinosa* against MRC-5 cells etc. (Brel et al., 2020).

One limitation of this study is that the assays were executed in only three cancer cell lines using extract from only one solvent system. It would be important in prospecting experiment to evaluate the effect of *Isaria* spp extracts on a larger number of tumour and non-tumour cell lines using multiple solvent systems and de-replicate extracts. Furthermore, the effect of pure metabolites isolated from *I. tenuipes* extracts,

such as beauvercin, and others, has not, to our knowledge, been tested. Therefore, the observations comprise a first approach to investigate the possible use of extracts of *Isaria* spp as chemo preventive natural resource against cancer. Nonetheless, study of their cytotoxicity and molecular mechanisms is subject to further investigations to determine the anticancer components. The result indicated that the methanol extract specifically reduces the proliferation of HeLa cells. It was confirmed through, colony morphological evaluation, FACS cytometric analysis and clonogenic assay that revealed that the cell death is mediated via apoptosis.

In conclusion, nine isolates of *Isaria* spp., exhibited excellent mycelial growth across an extended spectrum of growth and nutritional conditions. Specific phytochemicals were detected in the aqueous mycelial extract (70:30, Me-OH: H_2O) and this finding was verified by performing antioxidant activity *in vitro*. Biological activity was tested on human cancer cell lines and on bacterial test strains. The presented results suggests that, aqueous methanol mycelial extracts from nine isolates may confer potential antioxidant, antibacterial and antiproliferative activities in the cancer cell lines and bacterial strains tested with clinical relevance to humans. Due to its distinctive biotherapeutic profiles, the genus *Isaria* has the potential to be a major addition to the recently established safe mycomedicine class. The demonstrated antiproliferative activity of nine isolates in this study is reported here for the first time in India.

These findings provide base line information on the biological activity of *Isaria* spp., and suggest their potential use as an alternative or complementary therapy against some forms of cancer.

6. CONCLUSION AND FUTURE PERSPECTIVE

The findings of this study provided a valuable scientific landscape showing the genus *Isaria* spp., in all probability having novel bioactive metabolites. The original aims and objectives, as outlined in the introduction, were meticulously accomplished. One of the important outcomes of this study involves the generation of ITS-4 and ITS-5 DNA data sequences because several known species reported from India lack such sequence data, but these DNA sequences may be useful in future metagenomics study to address the entire community of the organism. The current study has made a significant contribution in understanding the physiological and nutritional requirements for the mycelial cultivation of genus *Isaria* of which there is a lack of documented research. This study is a baseline study to fill these lacunae using nine *Isaria* isolates from Darjeeling Hills. The methanol extracts exhibited the most potent *in vitro* antibacterial, antioxidant and antiproliferative activity which can be used in further study for its application. At least a 90-day subchronic toxicity study using animal model is desired for all species related to genus *Isaria* to be approved as new food ingredient. The active component or the major marker compounds could be considered to be used as quality control indicators. As more studies report antioxidant and antiproliferative activity using *in vivo* models, a clearer picture may emerge as to proceed adequately to drug development through clinical trials and studies related to eliminate the toxic side-effect of its components.

7. SUMMARY

1. A total of nine entomopathogenic fungal samples representing genus *Isaria* were collected from Darjeeling Hills, India and its adjoining temperate forests. The fungus was aseptically brought to the laboratory and single spore isolation for raising axenic culture was done on potato dextrose agar media.

2. Taxonomic identification of the collected fungus was made according to taxonomic keys and monographs following the standard protocol described by in literature. Two micromorphological characters length-width of conidia and phialides were observed to be significant with respect to the description of nine isolates.

3. For molecular identification, genomic DNA was extracted from pure axenic cultures grown on potato dextrose agar (PDA) for one week at 25°C under dark condition, by a simple and rapid DNA extraction protocol. Molecular characterization of all the nine isolates was done using ITS4 and ITS5 primers.

4. Based on BLAST analysis of the nuclear rDNA ITS sequence, the fungus was successfully identified as *Isaria* (IDAR01-09) with 98-100% similarity covering 543 to 556 sequence base pairs.

5. Nine isolates with four species delimitation were proposed using the concordance between morphological characters, ecology, cultural characteristics and molecular phylogeny ITS data for the first time on Indian collections.

6. In culture all the nine isolates of *Isaria* showed a temperature optimum of 20°C and the growth was retarded with increase of temperature beyond 30°C.

7. The optimum pH for mycelium growth was close to 7.0 for all the fungal isolates. However, all the test isolates could tolerate a pH range from 4.0 to 9.0.

8. Mushroom complete medium was an ideal medium for all the species excepting for *I. tenuipes* (IDAR-04) and *I. farinosa*, (IDAR-05). Among the semisynthetic media, potato-dextrose (PDA) and Sabouraud dextrose agar (SDA) was a good medium for all nine fungal isolates. Of the synthetic: media, such as glucose peptone and Capek's agar medium (CDA) was found to be overall satisfactory in growing all isolates under consideration.

9. Among the carbon sources tested most of the isolates (IDAR-04, IDAR-05, IDAR-06, IDAR-07 and IDAR-08) best grew vegetatively on media when the carbon source was disaccharides like maltose and sucrose. Monosaccharides such as dulcitol excreted positive growth influence on some isolates (IDAR-02 and IDAR-05). Very weak mycelial growth was recorded in the media supplemented with xylose or sodium acetate as the carbon source. Organic acids were poorer sources of carbon than carbohydrates for all the nine isolates. Glutamic acid as nitrogen source among amino acids tested was superior in accelerating the mycelial growth of all isolates. Similarly, yeast extracts as organic nitrogenous source and sodium nitrate was found to be superior sources of nitrogen with respect mycelial growth and density.

10. An optimum C/N ratio of 50: 1 (12.5 g D-Glucose or 5% C and 3.84 g Proteose Peptone) was observed for IDAR-01, IDAR-03 and IDAR-09 while 60:1(15 g D-Glucose or 6% C and 4.61 g Proteose Peptone), for IDAR-04, IDAR-06 and IDAR-08, respectively and a very low C/N ratio of 5:1(1.25 g D-Glucose or 0.5% C and 0.769 g Proteose Peptone) was suitable for IDAR-05.

11. Among the vitamins tested, thiamine-HCl showed the best results on the radial mycelial growth of all the test isolates in vitro.

12. Liquid static fermentation (LSF) was employed using potato dextrose broth supplemented with 0.01% yeast and meat extracts which proved to be a promising method for the production of fungal biomass and bioactive functional compounds.

13. The quantitative estimation of phytochemicals of cultured mycelium extracts showed a high degree of total phenolics in *Isaria farinosa* (IDAR-02) at 304.47μgGAEsg^{-1}, flavonoid in *Isaria tenuipes* (IDAR-03) at 16.22μgQEsg^{-1}, tannin in *Isaria farinosa* (IDAR-05) at 0.483mgTAEsg^{-1}, β-carotene in *Isaria farinosa* (IDAR-02) at 0.255mg/g and lycopene in *Isaria tenuipes* (IDAR-04) at 0.0413mg/g.

14. The methanol extract was fingerprinted with FTIR from 400-4000 cm^{-1}. Crude methanol 70% (v/v) cultured mycelial extract demonstrated reasonably good antibacterial activity against pathogenically significant Gram positive and Gram-negative bacteria.

15. Methanol extract was further explored for its antioxidant potential using a wide range of *in vitro* protocols (DPPH, ABTS$^{\bullet}$-scavenging, H_2O_2, OH-scavenging, reducing power ability, FRAP, and metal chelating ability). Methanol extracts from the cultured mycelium of each of the selected isolates were demonstrated as effective antioxidants. Additionally, it was also investigated that antioxidant activity increases in concentrations of fungal extract from 2.0 to 10.0 mg/ml.

16. IC_{50} values was used to compare the efficiency of reductant present in the methanolic extracts of nine isolates (IDAR-01-IDAR-09). The lowest IC_{50} values

recorded in each assay includes 2.54 mg/ml for DPPH assay with *I. fumosorosea* IDAR-09, 2.9 mg/ml in *Isaria javanica* (IDAR-01) for ABTS assay, 3.44 mg/mL in *Isaria fumosorosea* (IDAR-09) for hydroxyl activity, 3.60 mg/ml for hydrogen peroxide scavenging assay followed by 2.05 mg/ml in *Isaria farinosa* (IDAR-02) for iron chelating assay respectively.

17. MTT assay of all methanol extracts were conducted individually on three human cancerous cell lines (HeLa: cervical cancer; HepG2: hepatocarcinoma and PC-3: prostate cancer). It prevented proliferation of all three carcinoma cells. The 70% methanolic extracts showed strongest cytotoxic effects against PC3 cells with a lowest IC_{50} value of 3.10±0.16µg/ml from methanolic extract of *I. fumosorosea* IDAR-09 followed by cervical cancer cell line with IC_{50} value of 3.32±0.09µg/ml in *I. javanica* IDAR-01 respectively. The result was significant when the means were compared with two tailed t-test at 0.05% level with untreated sample.

18. The antiproliferative property of the extract may be due to its constituent secondary metabolites in the form of beauvercin. Among the nine isolates *I. farinosa* (IDAR-02) and *I. tenuipes* (IDAR-04) was found to contain beauvercin at 6.02±0.36 and 6.71±0.31 µg/ml respectively. The identification was achieved by comparing their retention time with retention time on chromatogram of marker compound (pure-beauvercin) using HPLC.

19. The two beauvercin positive isolates were further analysed for the determination of cell apoptosis using HeLa cell lines. The percentage of HeLa cells undergoing apoptosis following treatment with 1, 3, 10, 30 and 100 µg/ml concentrations of methanolic extract were 5.90% 6.85%, 10.67% 24.59% and 23.83% respectively for *I. tenuipes* IDAR-04. Similarly, the apoptosis rates of

HeLa cells were 2.56, 7.08, 5.70, 5.83, and 10.67% respectively in *I. farinosa* IDAR-02.

20. *I. tenuipes* (IDAR-04) responded well in PI single staining and this isolate was selected as the candidate for measuring apoptosis by flow cytometry with double positive staining using Annexin-V-FITC/PI to distinguish the dead cell population as apoptosis or necrosis. The percentages of Hela cells undergoing apoptosis following treatment with 0, 1, 3, 10, 30 and 100 µg/ml methanol extracts (including the early and late apoptotic cells) were 3.54±1.22, 7.09±3.73, 6.52±3.47, 8.86±4.36, 17.365±7.99 and 42.205±19.03% respectively. These results were well supported by morphological observation under microscope. Thus, the cultured mycelial extracts demonstrated that combination of multiple chemical constituents along with beauvercin may have yielded favourable biological activities.

21. Finally, we have investigated the colony formation ability of Hela cells in presence of methanol extracts from *I. tenuipes* (IDAR-04) for ten days. The colony numbers were found to be significantly reduced after treatment with methanol extracts with increasing concentrations. Compared with those in the control group, the colony numbers in the methanolic extract treated cells were reduced by over 90% at 100µg/ml in a dose dependent manner.

8. REFERENCES

Aamir, S., Sutar, S., Singh, S.K., & Baghela, A. (2015). A rapid and efficient method of fungal genomic DNA extraction, suitable for PCR based molecular methods. *Plant Pathology and Quarantine,* 5, 74–81.

Adejoye, O. D., Adebayo-Tayo, B. C., Ogunjobi, A. A., Olaoye, O. A. & Fadahunsi, F. I. (2006). Effect of carbon, nitrogen and mineral sources on growth of *Pleurotus florida*, a Nigeria edible mushroom. *African Journal of Biotechnology,* 5, 1355-1359.

Aghajanzadeh, S., & Mallik, B. (2007). Sampling and distribution pattern of citrus rust mite, *Phyllocoptruta oleivora* Ashmead (Acari, Eriophyidae) using adhesive tape method. *International Journal Agriculture and Biology,* 9, 329–332.

Agrawal, Y., Khatri, I., Subramanian, S., & Shenoy, B.D. (2015). Genome sequence, comparative analysis, and evolutionary insights into chitinases of entomopathogenic fungus *Hirsutella thompsonii*. *Genome Biol Evol*., 25, 916-930. doi: 10.1093/gbe/evv037.

Ahn, M.Y., Ryu, K.S., Jee, S.D., Kim, I., Kim, J.W., Kim, Y.S., Kim, H.S., Kim, I.S., Kang, S.C., Koo, H.J., Park, Y.A., Choi, S.M., Yoo, E.J., Kwack, S.J., Yoo, S.D., & Lee, B.M. (2004). Genotoxicity evaluation of *Isaria sinclairii* (ISE) extract. *J Toxicol Environ Health,* 67, 2037-2044. doi: 10.1080/15287390490514796.

Ahn, M.Y., Jee, S.D., & Lee, B.M. (2007). Antiobesity effects of *Isaria sinclairii* by repeated oral treatment in obese Zucker rats over a 4-month period. *J Toxicol Environ Health,* 70, 1395-401. doi: 10.1080/15287390701428556.

Ahn, M.Y., Heo, J.E., Ryu, J-H., Jeong, H., Ji, S.D., Park, H.C., & Sim, H.S (2008). Antioxidant Activity of N-hydroxyethyl Adenosine from *Isaria sinclairii*. *Int. J. Indust. Entomology,* 17, 197-200.

Ahn, M.Y., Jee, S.D., Lee, B.M., Yeon, J.H., Park, K.K., Hwang, J.S., & Yu, E.Y. (2010). Anti-diabetic effect and gene expression profiling in obese mice

treated with *Isaria sinclairii* over a 6-month period. *J. Toxicol. Environ. Health Part A.* 73, 1511–1520.

Ahn, M.Y., Jung, Y.S., Jee, S.D., Han., J.W., Hwang, J.S., Yun, E.Y., & Lee, B.M. (2013). Blood-pressure-lowering effect of glycosaminoglycan derived from *Isaria sinclairii* in spontaneously hypertensive rats. *J Toxicol Environ Health,* 2;76(6):391-9.doi: 10.1080/15287394.2013.765370.

Aime, M.C., Miller, A.N., Aoki, T., Bensch, K., Cai, L., Crous, P.W., Hawksworth, D.L., Hyde, K.D., Kirk, P.M., Lücking, R., May, T.W., Malosso, E., Redhead, S.A., Rossman, A.Y., Stadler, M., Thines, M., Yurkov, A.M., Zhang, N., & Schoch, C.L. (2021). How to publish a new fungal species, or name, version 3.0. *IMA Fungus*. 3;12(1):11. doi: 10.1186/s43008-021-00063-1.

Alexopoulus, C.J., & Beneke, E.S. (1964). Laboratory manual for introductory mycology, Minneapolis, Burgess Pub. Co.

Ali, S., Huang, Z., & Ren, S. (2009). Media composition influences on growth, enzyme activity, and virulence of the entomopathogen hyphomycete *Isaria fumosoroseus. Entomologia Experimentalis et Applicata,* 131, 30–38. doi:10.1111/j.1570-7458.2009.00833.x

Alonso-Díaz, M.A., & Fernández-Salas, A. (2021). Entomopathogenic Fungi for Tick Control in Cattle Livestock From Mexico. *Fronteirs in Fungal Biology,* 2:657694. doi: 10.3389/ffunb.2021.657694.

Alves, S.B., Tamai, M.A., Rossi, L.S., & Castiglioni, E. (2005). *Beauveria bassiana* pathogenicity to the citrus rust mite *Phyllocoptruta oleivora*. *Experimental and Applied Acarology,* 37, 117–122.

Amin, S.M.R., Alam, N., Tania, M., & Khan Md.A. (2008). Study of Mycelial Growth of *Cordyceps sinensis* in Different Media, at Different pH level and Temperature. *Bangladesh J. Mushroom,* 2(2): 43-48.

Ansari, M.A., Pope, E.C., Carpenter, S., Scholte, E-J., & Butt, T.M. (2011). Entomopathogenic Fungus as a Biological Control for an Important Vector

of Livestock Disease: The *Culicoides* Biting Midge. *PLoS ONE,* 6(1): e16108. doi:10.1371/journal.pone.0016108.

Araújo, J.P.M., & Hughes, D.P. (2016). Chapter One - Diversity of Entomopathogenic Fungi: Which Groups Conquered the Insect Body?, Editor(s): Brian Lovett, Raymond J. St. Leger, *Advances in Genetics*, Academic Press, Volume 94, 2016, Pages 1-39, ISSN 0065-2660, ISBN 9780128046944, https://doi.org/10.1016/bs.adgen.2016.01.001.

Arora, S., & Tandon, S. (2014). *Achyranthes aspera* root extracts induce human colon cancer cell (COLO-205) death by triggering the mitochondrial apoptosis pathway and S phase cell cycle arrest. *Scientific World Journal,* 1-7. doi: 10.1155/2014/129697.

Asaia, T., Yamamoto, T., Chung, Y-M., Chang, F-R., Wu, Y-C., Yamashita, K., & Oshima, Y. (2012). Aromatic polyketide glycosides from an entomopathogenic fungus, *Cordyceps indigotica. Tetrahedron Letters,* 53, 277–280.

Ashraf, S.A., Elkhalifa, A., Siddiqui, A.J., Patel, M., Awadelkareem, A.M., Snoussi, M., Ashraf, M.S., Adnan, M., & Hadi, S. (2020). Cordycepin for Health and Wellbeing: A Potent Bioactive Metabolite of an Entomopathogenic *Cordyceps* Medicinal Fungus and Its Nutraceutical and Therapeutic Potential. *Molecules,* *25*(12), 2735. https://doi.org/10.3390/molecules25122735.

Asghar, A., Tan, Y.C., Shahid, M., Yow, Y.Y., & Lahiri, C. (2021). Metabolite Profiling of Malaysian *Gracilaria edulis* Reveals Eplerenone as Novel Antibacterial Compound for Drug Repurposing Against MDR Bacteria. *Front Microbiol*ogy, 30;12:653562. doi: 10.3389.

Asante-Appiah, K., & Kennedy, B.P. (2003). Protein tyrosine phosphatases: the quest for negative regulators of insulin action. *Am. J. Physiol. Endocrinol. Metab.,* 284, 663–670.

Atkinson, G.F. (1894). Artificial Cultures of an Entomogenous Fungus. Botanical Gazette, Vol. 19, No. 4 pp. 129-135Published by: The University of Chicago Press Stable URL: https://www.jstor.org/stable/2464336.

Aung, O.M., Soytong, K., & Hyde, K.D. (2008). Diversity of entomopathogenic fungi in rainforests of Chiang Mai Province, Thailand. *Fungal Diversity,* 30, 15-22.

Avery, P.B., Hunter, W.B., Hall, D.G., Jackson, M.A., Powell, C.A., & Rogers, M. (2009), 'Diaphorina citri (Hemiptera: Psyllidae) Infection and Dissemination of the Entomopathogenic Fungus *Isaria fumosorosea* (Hypocreales: Cordycipitaceae) Under Laboratory Conditions'. *Florida Entomologist,* 92, 608-618.

Avery, P.B., Wekesa, V.W., Hunter, W.B., Hall, D.G., McKenzie, C.L., Osborne, L.S., Powell, C.A., & Rogers, M.E. (2011). 'Effects of the fungus *Isaria fumosorosea* (Hypocreales: Cordycipitaceae) on Reduced Feeding and Mortality of the Asian Citrus Psyllid, *Diaphorina citri* (Hemiptera: Psyllidae)'. *Biocontrol Science and Technology,* 21, 1065-1078.

Awang MA, Daud N.M, Ismail NIM, Cheng PG, Ismail MF, Ramaiya SD. (2021). Antioxidant and cytotoxicity activity of *Cordyceps militaris* extracts against human colorectal cancer cell line. *J Appl Pharm Sci.,* 11, 105–109.

Bao L, Wang Y, Ma R, et al. (2015). Apoptosis-inducing effects of lentinan on the proliferation of human bladder cancer T24 cells. *Pak J Pharm Sci.* 28, 1595–1600.

Ban, K-W., Park, D-K., Shim, J-O., Lee, Y-S., Park, C-H., Lee, J-Y., Lee, T-S., Lee, S-S., & Lee, M-W. (1998). Cultural Characteristics for Inducing Fruting-body of *Isaria japonica*. *Korean Journal of Mycology*, 26, 380-386.

Baral, B. (2017). Entomopathogenicity and Biological Attributes of Himalayan Treasured Fungus *Ophiocordyceps sinensis* (Yarsagumba). *Journal of Fungi*, 3, 1-24. doi:10.3390/jof3010004

Barros, L., Baptista, P., & Ferreira, I. C. F. R. (2007). Effect of Lactarius piperatus fruiting body maturity stage on antioxidant activity measured by several biochemical assays. *Food and Chemical Toxicology,* 45, 1731-1737.

Barros, L., Cruz, T., Baptista, P., Estevinho, L. M., & Ferreira, I. C. F. R. (2008). Wild and commercial mushrooms as source of nutrients and nutraceuticals. *Food and Chemical Toxicology,* 46, 2742-2747.

Barta, M. (2010). Preliminary evaluation of insect-pathogenic *Hypocreales* against *Leptoglossus occidentalis* (Heteroptera: *Coreidae*) in laboratory conditions. – *Folia Oecologica,* 37, 137-143.

Barta, M., Lalík, M., Rell, S., Kunca, A., Horáková, M.K., Mudrončeková, S., & Galko, J. (2019). "Hypocrealean Fungi Associated with *Hylobius abietis* in Slovakia, Their Virulence against Weevil Adults and Effect on Feeding Damage in Laboratory." *Forests,* 10 (8): 634. https://doi.org/10.3390/f10080634.

Bates, S.L., Borden, J.H., Kermode, A.R., Bennett, R.G. (2000). Impact of *Leptoglossus occidentalis* (Hemiptera: Coreidae) on Douglas-fir seed production. *Journal of Economic Entomology,* 93, 1444- 1451.

Bedding, R.A., & Molyneux, A.S. (1982). Penetration of insect cuticle by infective juveniles of *Heterorhabditis* Spp. (Heterorhabditidae: Nematoda). *Nematologica,* 28, 354–359.

Bellettini, M.B., Fiorda, F.A., Maieves, H.A., Teixeira, G.L., Ávila, S., Hornung, P.S., Júnior, A.M., & Ribani, R.H. (2019). Factors affecting mushroom *Pleurotus* spp. *Saudi J Biol Sci.,* 26(4):633-646. doi: 10.1016/j.sjbs.2016.12.005

Bensky, D., Clavey, S., & Stoger, E. (2004). In: Chinese herbal medicine: materia medica, 3rd edn. Eastland Press, Seattle, p 770, 2004.

Benzie, I.F., & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry,* 239, 70–76.

Bernardinelli, I., & Zandigiacomo, P. (2001). *Leptoglossus occidentalis* Heidemann (Heteroptera, *Coreidae*): a conifer seed bug recently found in northern Italy. – *J. Forest Science,* 47, 56–58.

Biedermann, P.H.W., & Vega, F.E. (2020). Ecology and Evolution of Insect–Fungus Mutualisms. *Annual Review of Entomology, 65(1), annurev-ento-011019-024910–.* doi:10.1146/annurev-ento-011019-024910

Blanford, S., Jenkins, N.E., Read, A.F., & Thomas, M.B. (2012). Evaluating the lethal and pre-lethal effects of a range of fungi against adult *Anopheles stephensi* mosquitoes. *Malar J*., 5;11:365. doi: 10.1186/1475-2875-11-365.

Blumthaler, M. (1993). UV-B Radiation and Ozone Depletion: Effects on Humans, Animals, Plants, Microorganisms, and Materials (pp. 71-94), Boca Raton: Lewis Publishers.

Boh, B., Berovic, M., Zhang, J., & Zhi□Bin, L. (2007). Ganoderma lucidum and its pharmaceutically active compounds. *Biotechnol. Annu. Rev.,* 13, 265–301, doi:10.1016/s1387□2656(07)13010□6

Boomsma J. J., Jensen A. B., Meyling N. V., & Eilenberg J. (2014). Evolutionary interaction networks of insect pathogenic fungi. *Annu. Rev. Entomol.,* 59, 467–485. 10.1146/annurev-ento-011613-162054.

Boonsong, S., Klaypradit, W., Wilaipun, P. (2016). Antioxidant activities of extracts from five edible mushrooms using different extractants, Agriculture *and Natural Resources,* 2, 89-97,

Boettger, D. et al., 2012. Evolutionary imprint of catalytic domains in fungal PKS–NRPS hybrids. *ChemBioChem,* 13, 2363–2373.

Booth, C. (1971). Chapter II Fungal Culture Media, Editor(s): C. Booth, Methods in Microbiology, Academic Press, Volume 4,Pages 49-94, ISSN 0580-9517, ISBN 9780125215046, https://doi.org/10.1016/S0580-9517(09)70007-8.

Bove, J.M. (2006), 'Huanglongbing: A Destructive, Newly-Emerging, Century-Old Disease of Citrus', *Journal of Plant Pathology,* 88, 7-37.

Bracalini, M., Benedettelli, S., Croci, F., Terreni, P., Tiberi, R., & Panzavolta, T. (2013). Cone and seed pests of *Pinus pinea*: Assessment and characterization of damage. *J Econ Entomol.*, 106 (1), pp. 229- 234.

Brand-Williams, W,, Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity'. *LWT Food Sci Technol.,* 28:25–30.

Braga, G.Ú.L., Flint, S.D., Miller, C.D., Anderson, A.J., & Roberts, D.W. (2001). Variability in response to UV-B among species and strains of *Metarhizium* isolated from sites at latitudes from 61°N to 54° S. J. *Invertebr. Pathol.* 78, 98–108.

Braga, G.U.L., Flint, S.D., Miller, C.D., Anderson, A.J., & Roberts, D.W. (2007). Both solar UVA and UVB radiation impair conidial culturability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. *Photochem. Photobiol*., 74, 734–739.

Brel, O., Touré, S., Levasseur, M., Lechat, C., Pellissier, L., Wolfender, J.L. wet al. (2020). Paecilosetin Derivatives as Potent Antimicrobial Agents from *Isaria farinosa*. *Journal of Natural Products,* 23, 2915-2922. doi: 10.1021/acs.jnatprod.0c00444. Epub 2020 Oct 6. PMID: 33021377.

Brown, A.H., & Smith, G. (1957). *Paecilomyces javanicus* (Friederichs and Bally) comb. nov. *Transactions of the British Mycological Society,* 40, 65-67.

Bruns, T., White, T., & Taylor, J. (1991). Fungal molecular systematics. *Annual Review of Ecology and Systematics,* 22, 525-564.

Bruns, T., & Shefferson, R. (2004). Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. *Canadian Journal of Botany,* 82, 1122-1132.

Bugeme, D.M., Maniania, N.K., Knapp, M., & Boga, H.I. (2008). Effect of temperature on virulence of Beauveria bassiana and Metarhizium anisopliae isolates to *Tetranychus evansi*. *Exp Appl Acarol.,* 46, 275–285.

Bunyapaiboonsri, T., Yoiprommarat, S., Intereya, K., Rachtawee, P., Hywel-Jones, N.L., & Isaka, M. (2009). Isariotins E and F, Spirocyclic and Bicyclic Hemiacetals from the Entomopathogenic Fungus *Isaria tenuipes* BCC 12625. *J. Nat. Prod.,* 72, 756–759.

Bunyapaiboonsri, T., Yoiprommarat, S., Srisanoh, U., Choowong, W., Tasanathai, K., Hywel-Jones, N.L. et al. (2011). Isariotins G-J from cultures of the Lepidoptera pathogenic fungus *Isaria tenuipes*. *Phytochem Lett*ers, 4, 283–286.

Butler, E.J., & Bisby, G.R. (1931). The fungi of India. Imp. Coun. of Agri. Res. lndia, Sci. Mono. I 18, 237.

Cabanillas, H.E., & Jones, W.A. (2009). Effects of temperature and culture media on vegetative growth of an entomopathogenic fungus *Isaria* sp. (Hypocreales: Clavicipitaceae) naturally affecting the whitefly, *Bemisia tabaci* in Texas. *Mycopathologia,* 167, 263-71. doi: 10.1007/s11046-008-9176-2.

Campbell, M.K., & Farewell, S.O. (2009). Biochemistry, 6th Edition. Thomson Brooks/Cole, Belmont, CA. ISBN: 9780495390411

Carlile, M M.K., &., Watkinson, A., & Gooday G. (2001). The Fungi. 2nd edition.

Castrillo, L.A. (2020). USDA-ARS Collection of Entomopathogenic Fungal Cultures. USDA-*ARS Emerging Pests and Pathogens Research Unit,* 1-60.

Corallo, A.B., Pechi, E., Bettucci, L. et al., (2021). Biological control of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) by Entomopathogenic fungi and their side effects on natural enemies. *Egypt J Biol Pest Control,* 31**,** 15 https://doi.org/10.1186/s41938-020-00358-2.

Chandra, A., & Purkayastha, R. P. (1977). Physiological studies on Indian edible mushrooms. *Transactions of the British Mycological Society*, 69, 63–70.

Charnley, A.K. (1989). Mycoinsecticides; present use and future prospects. *BCPC Mono. No. 43. Progress and Prospects o f Insect Control,* pp. 165- 181.

Chen, C., Han, Y., Li, S., Wang, R., & Tao, C. (2021). Nutritional, antioxidant, and quality characteristics of novel cookies enriched with mushroom (*Cordyceps militaris*) flour, *CyTA - Journal of Food,* 19, 137-145. DOI: 10.1080/19476337.2020.1864021.

Cheng, Y., Schneider, B., Riese, U., Schubert, B., Li, Z., Hamburger, M. (2004). Farinosones A-C, Neurotrophic Alkaloidal Metabolites from the Entomogenous Deuteromycete *Paecilomyces farinosus. Journal of Natural Products, 67, 1854–1858.* doi:10.1021/np049761w.

Cheng, C.,Chang, K., & Lee, Y. (2009). Antiproliferative effect of beauvericin on retinoblastoma. *Fu-Jen J. Med.,* 7, 167–169.

Cheng, J.W., Wang, Y.B., He, L., Qian, H., & Fu, L.Z., et al., (2012). Optimization of fermentation process for the production of intracellular polysaccharide from *Paecilomyces cicadae* and the immuno-stimulating activity of intracellular polysaccharide. *World J Microb Biot.,* 28, 3293–3299.

Cheng, W-ming., Zhang, Q-lin., Wu, Z-hua., Zhang, Z-yong., Miao, Y-ru., Peng, F., & Li, C-ru. (2017). Identification and determination of myriocin in *Isaria cicadae* and its allies by LTQ-Orbitrap-HRMS. *Mycology,* 8, 286-292, DOI: 10.1080/21501203.2017.1383319.

Chen, Y., Yang, S.H., Hueng, D.Y., Syu, J.P., Liao, C.C., & Wu, Y.C. (2014). Cordycepin induces apoptosis of C6 glioma cells through the adenosine 2A receptor-p53-caspase-7-PARP pathway. *Chem Biol Interact*., 5, 216:17-25. doi: 10.1016/j.cbi.2014.03.010.

Chen, Y.P., Yang, C.G., Wei, P.Y., Li, L., Luo, D.Q., Zheng, Z.H., & Lu, X.H. (2014). Penostatin derivatives, a novel kind of protein phosphatase 1b inhibitors isolated from solid cultures of the entomogenous fungus *Isaria tenuipes*. *Molecules,* 29;19(2):1663-71. doi: 10.3390/molecules19021663.

Chen, C., Han, Y., Li, S., Wang, R., & Tao, C. (2021). Nutritional, antioxidant, and quality characteristics of novel cookies enriched with mushroom (*Cordyceps*

militaris) flour, *CyTA - Journal of Food,* 19, 137-145, DOI: 10.1080/19476337.2020.1864021.

Chen, W.-H., Liu, C., Han, Y.-F., Liang, J.-D., & Liang, Z.-Q. (2018). *Akanthomyces araneogenum*, a new *Isaria*-like araneogenous species. *Phytotaxa*, 379, 66–72.

Chen, W.H., Han, Y.F., Liang, J.D., Tian, W.Y., & Liang, Z.Q. (2020). Morphological and phylogenetic characterizations reveal three new species of *Samsoniella* (Cordycipitaceae, Hypocreales) from Guizhou, China. *MycoKeys,* 74, 1–15.

Chien, R.C., Tsai, S.Y., Lai, E.Y., & Mau, J.L. (2015). Antiproliferative Activities of Hot Water Extracts from Culinary-Medicinal Mushrooms, *Ganoderma tsugae* and *Agrocybe cylindracea* (Higher Basidiomycetes) on Cancer Cells. *Int J Med Mushrooms,* 17, 453-62. doi: 10.1615/intjmedmushrooms. v17.i5.50.

Cheek, M., NicLughadha, E., & Kirk, P., *et al.,* (2020). New scientific discoveries: Plants and fungi. *Plants, People, Planet*, 2, 371–388. doi.org/10.1002/ppp3.10148.

Chhetri, P.K., Pradhan, B.K. & Chhetri, B. First Record of *Ophiocordyceps dipterigena* Berk. & Broome (Ophiocordycipitaceae) in the Himalayas. *Natl. Acad. Sci. Lett.,* 43, 367–369 (2020). https://doi.org/10.1007/s40009-019-00857-3.

Chhetri, A., Pokhrel, Y.R., Shahi, N., Lama, D., Chhetri, D.R.(2019). Antioxidant and cytotoxic activities of *Isaria amoenerosea* Henn.: An entomopathogenic fungus from Darjeeling Hills, Eastern Himalaya. *Indian J Nat Prod Resour.,* 10, 111–118.

Chhetri, D.R., Chhetri, A., Shahi, N., Tiwari, S., Karna, S., Lama, D., & Pokharel, Y.R. (2020). *Isaria tenuipes* Peck, an entomopathogenic fungus from Darjeeling Himalaya: Evaluation of in-vitro antiproliferative and antioxidant

potential of its mycelium extract. *BMC complementary medicine and therapies*, 20(1), 185. https://doi.org/10.1186/s12906-020-02973-w.

Chiba, K., Kataoka, H., Seki, N., Shimano, K., Koyama, M., Fukunari, A., Sugahara, K., & Sugita, T. (2011). Fingolimod (FTY720), sphingosine 1-phosphate receptor modulator, shows superior efficacy as compared with interferon-β in mouse experimental autoimmune encephalomyelitis. *Int Immunopharmacology,* 11, 366-72. doi: 10.1016/j.intimp.2010.10.005.

Chiba K, & Adachi K. (2012). Discovery of fingolimod, the sphingosine 1-phosphate receptor modulator and its application for the therapy of multiple sclerosis. *Future Med Chem.,* 4, 771-781. doi: 10.4155/fmc.12.25.

Chiba, K. (2020). Discovery of fingolimod based on the chemical modification of a natural product from the fungus, *Isaria sinclairii. The Journal of Antibiotics, 1-7.* doi:10.1038/s41429-020-0351-0

Chihara, G. (1969). The antitumor polysaccharide lentinan: an over-view. In T. Aoki, et al. (Eds.), Manipulation of host defense mechanism. *Nature,* 222:687–694

Chiu, C.P., Hwang, T.L., Chan, Y., El-Shazly M., Wu, T.Y., Lo, I.W. et al. (2016). Research and development of *Cordyceps* in Taiwan. *Food Science and Human Wellness*, 5: 4; 177-185, ISSN 2213-4530,2016. https://doi.org/10.1016/j.fshw.2016.08.001

Chunyu,Y.J., Lu,Z-M., Luo,Z-S., Li,S-S., Li,H., Geng, Y. et al. (2019). Promotion of Metabolite Synthesis in *Isaria cicadae*, a Dominant Species in the Cicada Flower Microbiota, by Cicada Pupae. *J. Agric. Food Chem.*, 67, 8476−8484.

Clarkson, J. M., & Charnley, A. K. (1996). New insights into the mechanisms of fungal pathogenesis in insects. *Trends in Microbiology,* 4, 197-203.

Cochrane, V. W. (1958). Physiology of fungi. New York: John Wiley.

Corallo, A.B., Pechi, E., Bettucci, L. & Tiscornia S. (2021). Biological control of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) by

Entomopathogenic fungi and their side effects on natural enemies. *Egypt J Biol Pest Control,* 31, 15 (2021). https://doi.org/10.1186/s41938-020-00358-2

Cook, R.J. (1988). Biological control and holistic plant health care in agriculture. *Am. J. Alt. Agric.,* 3, 51-62.

Cook, N.C., & Samman, S. (1996). Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry,* 7: (2) 66-76.

Cooke, M.C. (1892). Vegetable wasps and plant worms. A popular history of entomogenous-fungi, or fungi parasitic upon insects. *Society for promoting Christian knowledge*:London.

Crespo, R., Patricia Juárez, M., & Cafferata, L.F.R. (2000). Biochemical interaction between entomopathogenous fungi and their insect-hostlike hydrocarbons. *Mycologia,* 92, 528–536.

Crisan, E.V. (1973). Current concepts of thermophilism and the thermophilic fungi. *Mycologia,* 65, 1171–1198. doi:10.2307/3758296

Cooke, R.C. & Rayner, A.D.M. (1984). Ecology of Saprotrophic Fungi. Longman, London, UK.

Cummings, N.J. (2009). Entomopathogenic fungi in New Zealand native forests: the genera *Beauveria* and *Isaria* PhD Thesis University of Canterbury.

D'Alessandro, C.P., Jones, L.R., Humber, R.A., López Lastra, C.C., & Sosa-Gomez, D.R. (2014). Characterization and phylogeny of *Isaria* spp. strains (Ascomycota: Hypocreales) using ITS1-5.8S-ITS2 and elongation factor 1-alpha sequences. *Journal of Basic Microbiology,* 543: 1-11. doi: 10.1002/jobm.201300499.

Dai, R., Liu, M., Nabil, W.N.N., Xi, Z., & Xu, H. (2021). Mycomedicine: A Unique Class of Natural Products with Potent Anti□Tumour Bioactivities. *Molecules*, 26, 1113:1-23. https://doi.org/10.3390/molecules26041113.

Das, P., Hazarika, L.K., Bora, D.S., Puzari, K.C., & Dutta, P. (2011). Efect of temperature on radial growth, sporulation, germination, colony forming unit and biomass production of different strains of *Beauveria bassiana* Vuill. (Bals.). *Pestology,* 35, 50–55

Das, P., Hazarika, L.K., Kalita, & S., Roy, S. (2019). Current State of Fungal Antagonists with Special Emphasis on Indian Scenario. In: Khan, M., Ahmad, W. (eds) Microbes for Sustainable Insect Pest Management. *Sustainability in Plant and Crop Protection.* Springer, Cham. https://doi.org/10.1007/978-3-030-23045-6_14

Das, K., Das, P., Parveen, A. *et al.* (2021). Molecular characterization of indigenous entomopathogenic fungus *Beauveria bassiana*, isolate and its virulence to *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae). *Int J Trop Insect Sci.,* 41, 2443–2451. https://doi.org/10.1007/s42690-020-00421-3.

Das, P., Borah, B., Saikia, P. *et al.* (2022). Molecular characterization of an *Isaria fumosorosea* (Wize) native strain, and its pathogenicity on *Eublemma amabilis* (Lepidoptera: Noctuidae). *Egypt J Biol Pest Control*, 32, 122. https://doi.org/10.1186/s41938-022-00624-5

Das, G., Shin, H-S., Leyva-Gómez, G., Prado-Audelo, M.L.D., Cortes, H., Singh, Y.D. et al. (2021). *Cordyceps* spp.: A Review on Its Immune-Stimulatory and Other Biological Potentials. *Front. Pharmacology,* 11:602364. doi: 10.3389/fphar.2020.602364.

Davies, J, & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev.*, 74, 417–433.

Demain, A.L., & Sanchez, S. (2009). Microbial drug discovery: 80 years of progress. *Journal of Antibiotics,* 62(1):5-16. doi: 10.1038/ja.2008.16.

Devi, K.U., Sridevi, V., Mohan, C.M., & Padmavathi, J. (2005). Effect of high temperature and water stress on in vitro germination and growth in isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin. *J Invertebr Pathology,* 88, 181–189.

de Bruijn, J., Loyola, C., Aqueveque, P., Canumir, J., Cortez, M., & France, A. (2009). Antioxidant properties of extracts obtained from *Grifolia* gargal mushrooms. *Micologia Aplicada Int*., 21, 11–18.

de Faria, M.R., & Wraight, S.P. (2007). "Mycoinsecticides and Mycoacaricides: A Comprehensive List with Worldwide Coverage and International Classification of Formulation Types." *Biological Control,* 43 (3): 237–56. https://doi.org/10.1016/j.biocontrol.2007.08.001.

de Hoog, G. (1972). The genera *Beauveria, isaria, Tritirachium* and *Acrodontium* gen. nov. *Studies in Mycology,* 1, 1-41.

de Queiroz, K. (1998). The general lineage concept of species, species criteria and the process of speciation: A conceptual unification and terminological recommendations. *Pp. 57−75 (Chapter 5) in Endless forms: Species and Speciation*, D.J., Howard and S.H. Berlocher (eds.) Oxford University Press, Oxford, England.

de Queiroz, K. (2007). Species concepts and species delimitation. *Systematic Biology,* 56, 879−886.

De Silva, D.D., Rapior, S., & Hyde, K.D. *et al.* Medicinal mushrooms in prevention and control of diabetes mellitus. *Fungal Diversity*, 56, 1–29 (2012a). https://doi.org/10.1007/s13225-012-0187-4

De Silva, D.D., Rapior, S., & Fons, F. *et al.* (2012b). Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. *Fungal Diversity,* 55, 1–35. https://doi.org/10.1007/s13225-012-0151-3

De Silva, D.D., Rapior, S., Sudarman, E. *et al.* (2013). Bioactive metabolites from macrofungi: ethnopharmacology, biological activities and chemistry. *Fungal Diversity,* 62, 1–40. https://doi.org/10.1007/s13225-013-0265-2.

Deacon, J.W. (2006). Fungal Biology, 4th Edition. Wiley-Blackwell, ISBN: 978-1-118-68507-5.

Doberski, J.W. (1981), 'Comparative Laboratory Studies on Three Fungal Pathogens of the Elm Bark Beetle *Scolytus scolytus:* Effect of Temperature and Humidity on Infection by *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus'*. *Journal of Invertebrate Pathology,* 37, 195-200.

Dolinski, C., & Lacey, L. (2007). Microbial control of arthropod pests of tropical tree fruits. *Neotropical Entomology,* 36, 161–179.

Dong, C.H., & Yao, Y.J. (2005). "Nutritional Requirements of Mycelial Growth of *Cordyceps sinensis* in Submerged Culture," *Journal of Applied Microbiology*, 99, 483-492.

Dong, C. H., & Yao, Y. J. (2008). In vitro evaluation of antioxidant activities of aqueous extracts from natural and cultured mycelia of *Cordyceps sinensis*. *Lebensmittel-Wissenschaft + [i.e. und] Technologie. Food science + technology. Science + technologie alimentaire*, 41, 669–677. https://doi.org/10.1016/j.lwt.2007.05.002

Dong, C., Guo, S., Wang, W., & Liu, X. (2015). *Cordyceps* industry in China. *Mycology,* 21;6(2):121-129. doi: 10.1080/21501203.2015.1043967.

Downes, S., Chen, Y-J., Kyles, A., & Gregory, C.(2007). Oral pharmacokinetic and pharmacodynamic effects of FTY720 in cats. *J. vet. Pharmacol. Therap.,* 30, 55–61,. doi:10.1111/j.1365-2885.2007.00815.x.

Du, L., & Lou, L. (2010). PKS and NRPS release mechanisms. *Nat. Prod. Rep.*, 27, 255–278.

Dutta, P., Pegu, J., Puzari, K.C. (2013). Current status and future prospects of entomopathogenic fungi in North East India. *Kavaka*, 41, 75-86.

Edinger, A.L., & Thompson, C.B. (2004). Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol.,* 16, 663–69.

Egerton, F. N. (2012). History of ecological sciences, part 44: phytopathology during the 1800s. *ESA Bulletin,* 93, 303–339.

Ejelonu, O.C., Akinmoladun, A.C., Elekofehinti, O.O., & Olaleye, M.T. (2013). Antioxidant profile of four selected wild edible mushrooms in Nigeria. *Journal of Chemical and Pharmaceutical Research,* 5, 286-295.

Ejikeme, C.M., Ezeonu, C.S., & Eboatu, A.N. (2014). "Determination of physical and phytochemical constituents of some tropical timbers indigenous to Niger Delta Area of Nigeria," *European Scientific Journal*, 18, 247–270.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology*, *35*(4), 495–516. https://doi.org/10.1080/01926230701320337

Ezeonu, C.S., & Ejikeme, C.M. (2016). Qualitative and Quantitative Determination of Phytochemical Contents of Indigenous Nigerian Softwoods. *New Journal of Science*, 1-9. http://dx.doi.org/10.1155/2016/5601327

Ekesi, S., Maniania, N.K., & Ampong-Nyarko, K. (1999). Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Sci. Technol*., 9, 177–185.

Esmaillzadeh, A., & Azatbakth, L. (2008). Food intake patterns may explain the high prevalence of cardiovascular risk factors among Iranian women. *J. Nutr. Nutrit. Epidemiol*., 138, 1469–1475.

Evans, H.C. (1974). Natural control of arthropods, with special reference to ants (Formicidae), by fungi in tropical high forest of Ghana. *Journal of Applied Ecology,* 11, 37-49.

Evans, H.C. (1982). Entomogenous fungi in tropical forest ecosystems - an appraisal. *Ecological Entomology,* 7, 47-60.

Evans, R.C., & Samson, R.A. (1982a). *Cordyceps* species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems 1. The *Cephalotes* (Myrmicinae) complex. *Transactions of the British Mycological Society,* 79, 431-453.

Evans, H.C., & Samson, R.A. (1982b). Entomogenous fungi from the Galapagos Islands. *Canadian Journal of Botany,* 60, 2325-2333.

Evans, RC. & Samson, R.A. (1984). *Cordyceps* species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems II. The *Camponotus* (Formicinae) complex. *Transactions of the British Mycological Society,* 81, 127-150.

Evans, H.C. (1989). Mycopathogens of insects in epigeal and aerial habitats. In *Insect-fungus interactions* (eds. N. Wilding, N. Collins, P. Hammond & J. Webber) pp. 205-237. Academic Press: London.

Evans, H.C., & Hywel-Jones, N.L. (1997). 2.1 Entomopathogenic Fungi. World Crop Pests. Vol. 7. Elsevier B.V. https://doi.org/10.1016/S1572-4379(97)80075-9.

Fargues, J., & Remaudiere, G. (1977). Considerations on the specificity of entomopathogenic fungi. *Mycopathologia,* 62, 31-37.

Fargues, J., Maniania, Nk., Delmas, Jc., & Smits, N. (1992). Influence de la température sur la croissance *in vitro* d'hyphomycètes entomopathogènes. Agronomie, *EDP Sciences*., 12 (7), pp.557-564. ffhal00885500f

Fargues, J., Goettel, M.S., Smits, N., Ouedraogo, A., Vidal, C., Lacey, L.A. et al. (1996). Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. *Mycopathologia,* 135, 171–181.

Fargues, J, & Bon, M.C. (2004). Influence of temperature preferences of two *Paecilomyces fumosoroseus* lineages on their co-infection pattern. *J Invertebr Pathology,* 87, 94-104. doi: 10.1016/j.jip.2004.07.001.

Farinha, A.O., Branco, M., Pereira, M.F., Auger□Rozenberg, M.A., Maurício, A., Yart, A. et al. (2018). Micro X-ray computed tomography suggests cooperative feeding among adult invasive bugs *Leptoglossus occidentalis* on mature seeds of Stone pine *Pinus pinea. Agricultural and Forest Entomology,* 20, 18-27

Fent, M., & Kment, P. (2011). First record of the invasive western conifer seed bug *Leptoglossus occidentalis* (Heteroptera: Coreidae) in Turkey. *North-Western Journal of Zoology,* 7, 72-80.

Fernandes, É.K.K., Rangel, D.E.N., Moraes, Á.M., Bittencourt, V.R.E.P., & Roberts, D.W. (2007). Variability in tolerance to UV-B radiation among *Beauveria* spp. isolates. *J. Invertebr. Pathol.,* 96, 237–243.

Fernandes, É.K.K., Rangel, D.E.N., Moraes, Á.M., Bittencourt, V.R.E.P., & Roberts, D.W. (2008). Cold activity of *Beauveria bassiana* and *Metarhizium*, and thermotolerance of *Beauveria*. *J Invertebr Pathol.*, 98, 69–78.

Fernandes, E.K.K., Angelo, I.C., Rangel, D.E.N., Bahiense, T.C., Moraes, A.M., Roberts, D.W., & Bittencourt, V.R. (2011). An intensive search for promising fungal biological control agents of ticks, particularly *Rhipicephalus microplus*. *Veterinary Parasitology,* 182, 307–318. doi:10.1016/j.vetpar.2011.05.046

Fernández-Blanco, C., Frizzell, C., Shannon, M., Ruiz, M. J., & Connolly, L. (2016). An in vitro investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B1 and beauvericin. *Toxicol. Lett.,* 57, 1–10. doi: 10.1016/j.toxlet.2016.05.021

Ferreira, I.C., Barros, L., Abreu, R.M. (2009). Antioxidants in wild mushrooms. *Curr Med Chem.,* 16, 1543–1560.

Ferreira, I.C.F.R., Martins, N., & Barros. L. (2017). Phenolic Compounds and Its Bioavailability: In Vitro Bioactive Compounds or Health Promoters? *Adv Food Nutr Res.,* 82, 1-44. doi: 10.1016/bs.afnr.2016.12.004.

Ferron, P. (1981). Pest control by the fungi *Beauveria* and *Metarhizium*. In: *In: Microbial control of Pests and Plant Diseases* 1970-1980 Edited by (Editor) BH: Academic Press, London.

Feng, X.L., Gao, W.Y., Feng, P.S., & Su, Z.G. (1995). Studies on submerged process of *Ganoderma lucidum* mycelium. *Chinese J. Biochem. Pharm.*, 5, 210–213.

Fisch, K.M. et al., 2011. Rational domain swaps decipher programming in fungal highly reducing polyketide synthases and resurrect an extinct metabolite. *J. Am. Chem. Soc.,* 133, 16635–16641.

Flieger, J., & Flieger, M. (2020). The [DPPH●/DPPH-H]-HPLC-DAD Method on Tracking the Antioxidant Activity of Pure Antioxidants and Goutweed (*Aegopodium podagraria* L.) Hydroalcoholic Extracts. *Molecules*, *25*(24), 6005. https://doi.org/10.3390/molecules25246005

Foster, R.N., Jaronski, S., Reuter, K.C., Black, L.R., Schlothauer, R., Harper, J., & Jech, L.E. (2011). Simulated aerial sprays for field cage evaluation of *Beauveria bassiana* and *Metarhizium brunneum* (Ascomycetes: Hypocreales) against *Anabrus simplex* (Orthoptera: Tettigoniidae) in Montana. *Biocontrol Science and Technology,* 21, 1331–1350. doi:10.1080/ 09583157.2011.620080.

Fotakis, G., & Timbrell, J.A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicological Letter,* 5;160(2):171-7. doi: 10.1016/j.toxlet.2005.07.001.

Fries, E.M. (1821). Systema Mycologicum; Ex Officina Berlingiana: Lund & Greifswald, Sweden, Volume 1, pp. 1–726.

Fujita, T., Inoue, K., & Yamamoto, S., et al. (1994). Fungal metabolites: Part 11, a potent immunosuppressive activity found in *Isaria sinclairii* metabolite. *Journal of Antibiotics,* 47, 208–215.

Fukatsu, T., Sato, H., & Kuriyama, H. (1997). Isolation, inoculation to insect host, and molecular phylogeny of an entomogenous fungus Paecilomyces tenuipes. *Journal of Invertebrate Pathology,* 70, 203–208.

Gadd, G.M. (1986). *Fungal responses towards heavy metals.* In: Microbes in Extreme Environments (Herbert. R.A. and Codd, G.A.. Eds.). Academic Press. London. pp.83-110.

Gallou, A., Serna-Domínguez, M.G., Berlanga-Padilla, A.M., Ayala-Zermeño, M.A., Mellín-Rosas, M.A., Montesinos-Matías, R., & Arredondo-Bernal, H.C. (2016). Species clarification of *Isaria* isolates used as biocontrol agents against *Diaphorina citri* (Hemiptera: Liviidae) in Mexico. *Fungal Biology,* 120, 414-423. doi: 10.1016/j.funbio.2015.11.009.

Gams, W. (1971). *Cephalosporium-artige Schimmelpilze.* Gustav Fischer Verlag: Stuttgart.

Gams, W., Hodge, K.T., Samson, R.A., Korf, R.P., & Seifert, K.A. (2005). (1684) Proposal to conserve the name *Isaria* (anamorphic fungi) with a conserved type. *Taxon,* 54: 537.

Gibson, D.M., Donzelli, B.G., Krasnoff, S.B., & Keyhani, N.O. (2014). Discovering the secondary metabolite potential encoded within entomopathogenic fungi, *Nat. Prod. Rep*., 31, 1287–1305.

Giles, J. R., Peterson, A. T., Busch, J. D., Olafson, P. U., Scoles, G. A., Davey, R. B., et al. (2014). Invasive potential of cattle fever ticks in the southern United States. *Parasites Vectors,* 7:189. doi: 10.1186/1756-3305-7-189.

Guerzoni, M.E., Lanciotti, R., & Cocconcelli, P.S. (2001). Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology,* 147, 2255–2264.

Glare, T.R., Inwood, AJ., van der Wielen, P. & Nelson, T. (1996a). Identification of novel starins of *Beauveria* and *Metarhizium* in New Zealand. In *Proceedings of the Third International Workshop on Microbial Control of Soil Dwelling Pests* (eds. T.A Jackson & T.R. Glare) pp. 39-49.

Glare, T.R., Milner, R.J., & Beaton, C.D. (1996b). Variation in *Metarhizium,* a genus of fungal pathogens attacking Orthoptera: is phialide morphology a useful taxonomic criterion? *Journal of Orthopteran Research,* 5, 19-27.

Glare, T.R., & Inwood, A.J. (1998). Morphological and genetic characterisation of *Beauveria* spp. from New Zealand. *Mycological Research,* 102, 250-256.

Gogala, A. (2003). Listonožka (Leptoglossus occidentalis) že v Sloveniji (Heteroptera: Coreidae) [A leaffooted conifer seed bug (*Leptoglossus occidentalis*) in Slovenia already (Heteroptera: Coreidae)]. *Acta Ent. Slovenica,* 11, 189–190.

Goos, R.D. (1956) "Classification of the Fungi lmperfecti," *Proceedings of the Iowa Academy of Science,* 63, 311-320.

Goettel, M.S., Inglis, G.D., & Wraight, S.P. (2000). Fungi. In: Lacey LA, Kaya HK, editors. *Field manual of techniques in invertebrate pathology.* Norwell, MA: Kluwer Academic Publishers. p. 255–82.

Goettel, M.S., Eilenberg, J., & Glare, T.R. (2005). Entomopathogenic fungi and their role in regulation of insect populations, pp 361–405 In: Gilbert LI, Iatrou K, Gill S [eds.], *Comprehensive Molecular Insect Science*, Volume 6. Pergamon Press, Oxford, United Kingdom.

Goettel, M.S., Eilenberg, J., & Glare, T.R. (2010), 'Entomopathogenic Fungi and Their Role in Regulation of Insect Populations', in Insect Control: *Biological and Synthetic Agents*, eds. L.I. Gilbert and S. Gill, London: Academic Press, pp. 387-432.

Gordon, M.H. (1990). The Mechanism of Antioxidant Action in vitro. In: Food Antioxidants, B.J.F. Hudson (Ed.), Elsevier, London, UK, pp. 1–18.

Graef, F., Gordon, S., & Lehr, C-M. (2016). Anti-infectives in Drug Delivery—Overcoming the Gram-Negative Bacterial Cell Envelope. *Current Topics in Microbiology and Immunology*, 398: 475–496 DOI 10.1007/82_2016.

Griffin, D. H. (1994). Fungal Physiology. Wiley-Liss, Inc. New York.

Gruhn, C.M., & Miller, O.K. (1991). Effect of Cu on tyrosinase activity and polyamine content of some ectomycorrhizal fungi. *Mycological Research,* 95, 268-272.

Guillamon, E., García-Lafuente, A., Lozano, M., D'Arrigo, M., Rostagno, M. A., Villares, A., et al. (2010). Edible mushrooms: role in the prevention of cardiovascular diseases. *Fitoterapia,* 81, 715-723.

Guo, Y.Y., An, Z., & Shi, W.P., 2012. Control of grasshoppers by combined application of *Paranosema locustae* and an insect growth regulator (IGR) (Cascade) in Rangelands in China. J. *Econ. Entomol.,* 105, 1915–1920.

Gursoy, N., Sarikürkçü, C., Cengiz, M., & Solak, M.H. (2009). Antioxidant activities, metal contents, total phenolics and flavonoids of seven Morchella species. *Food Chem Toxicol.,* 47, 2381–2388.

Haeckel, E. (1866). Generelle Morphologie der Organismen: Allgemeine Grundzuge der organischen Formen-Wissenschaft, mechanisch begrundet durch die von Charles Darwin reformirte Descendenz-Theorie. Georg Rierner, Berlin.

Hall, J.E., Crook, E.D., Jones, D.W., Wofford, M.R., & Dubbert, P.M. (2002). Mechanisms of obesity-associated cardiovascular and renal disease. *Am. J. Med. Sci*., 324:127–137.

Hallsworth, J.E., & Magan, N. (1999), 'Water and Temperature Relations of Growth of the Entomogenous Fungi *Beauveria bassiana, Metarhizium anisopliae*, and *Paecilomyces farinosus. Journal of Invertebrate Pathology,* 74, 261-266.

Hajek, A.E., & St Leger, R.A. (1994). Interactions between fungal pathogens and their hosts. *Annual review of Entomology*, 39, 293-322.

Hajek, A., & Eilenberg, J. (2018). Fungal Pathogens of Invertebrates. In Natural Enemies: An Introduction to Biological Control (pp. 229-242). Cambridge: Cambridge University Press. doi:10.1017/9781107280267.013.

Halo, L.M. et al., (2008). Authentic heterologous expression of the tenellin iterative polyketide synthase nonribosomal peptide synthetase requires coexpression with an enoyl reductase. *ChemBioChem.,* 9, 585–594.

Hammer, Ø., Harper, D.A.T., & Ryan, P.D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica,* 4(1): 9pp.

Hamill, R.L., Higgens, C.E., Boaz, H.E., & Gorman, M. (1969). The structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia salina*. *Tetrahedron Letters,* 49, 4255–4258.

Han, J, Sohn, E.J., Kim, B., Kim, S., Won, G., Yoon, S. et al. (2014). Upregulation of death receptor 5 and activation of caspase 8/3 play a critical role in ergosterol peroxide induced apoptosis in DU 145 prostate cancer cells. *Cancer Cell Int.,* 30;14(1):117. doi: 10.1186/s12935-014-0117-5.

Haritakun, R., Srikitikulchai, P., Khoyaiklang, P., & Isaka, M. (2007). Isariotins A–D, Alkaloids from the Insect Pathogenic Fungus *Isaria tenuipes* BCC 7831 *J. Nat. Prod.*, *70,* 1478–1480.

Harborne, J.B. (1973). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, Chapman and Hall, London, UK.

Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., Santos-Buelga, C., Ferreira, I.C.F.R. (2011). Targeted metabolites analysis in wild *Boletus* species. *LWT - Food Sci Technol*ogy, 44, 1343–1348.

Heleno, S.A., Barros, L., Martins, A., Queiroz, M.J.R.P., Santos-Buelga, C., & Ferreira, I. C.F.R. (2012). Fruiting body, spores and *in vitro* produced mycelium of *Ganoderma lucidum* from Northeast Portugal: A comparative study of the antioxidant potential of phenolic and polysaccharidic extracts. *Food Research International*, 46, 135–140.

Heilos, D., Rodríguez-Carrasco, Y., Englinger, B., Timelthaler, G., van Schoonhoven, S., Sulyok, M. et al. (2017). The Natural Fungal Metabolite Beauvericin Exerts Anticancer Activity *In Vivo:* A Pre-Clinical Pilot Study. *Toxins,* 24;9(9):258. doi: 10.3390/toxins9090258.

He, Y.T., Zhang, X.L., Xie, Y.M., Xu, Y.X., & Li, J.R. (2013). Extraction and Antioxidant Property in Vitro of Cordycepin in Artificially Cultivated

Cordyceps militaris. Advanced Materials Research, 750-752;1593–1596. doi:10.4028/www.scientific.net/amr.750-752.1593

Holliday, J. (2017). *Cordyceps*: A Highly Coveted Medicinal Mushroom. In: Agrawal D., Tsay HS., Shyur LF., Wu YC., Wang SY. (eds) Medicinal Plants and Fungi: Recent Advances in Research and Development. *Medicinal and Aromatic Plants of the World*, vol 4. Springer, Singapore. https://doi.org/10.1007/978-981-10-5978-0_3.

Hoy, M., Singh, R., & Rogers, M.E. (2010), 'Evaluations of a Novel Isolate of *Isaria fumosorosea* for Control of the Asian Citrus Psyllid, *Diaphorina citri* (Hemiptera: Psyllidae)', Florida Entomologist, 93, 24-32.

Hibbett, D.S., Binder, M., Bischoff, 1.F., Blackwell, M., Cannon, P.F., Eriksson, O.E. et al. (2007). A higher-level phylogenetic classification of the Fungi. *Mycological Research,* 111**,** 509-547.

Hibbett, D.S., Binder, M., & Bischoff, J.F., *et al.,* (2007). A higher-level phylogenetic classification of the Fungi. *Mycological Research,* 111, 509-47. doi: 10.1016/j.mycres.2007.03.004.

Hobbs, C. (2003). Medicinal mushrooms: An exploration of tradition, healing & culture. Botanica Press.

Hodge, K.T., Gams, W., Samson, R.A, Korf, R.P. & Seifert, K.A. (2005). Lectotypification and status of *Isaria* Pers.: Fr. *Taxon,* 54, 485-489.

Hong, I.P., Nam, S.H., Sung, G.B., Chung, I.M., Hur, H., Lee, M.W. et al. (2007). Chemical Components of *Paecilomyces tenuipes* (Peck) Samson. *Mycobiology,* 35(4):215-8. doi: 10.4489/MYCO.2007.35.4.215.

Hywel-Jones, N.L., & Sivichai, S. (1995). *Cordyceps cylindrica* and its association with *Nomuraea atypicola* in Thailand. *Mycological Research,* 99, 809- 812.

Hywel-Jones, N. (1995a). *Cordyceps sphecocephala* and a *Hymenostilbe sp.* infecting wasps and bees in Thailand. *Mycological Research,* 99, 154- 158.

Hywel-Jones, N.L. (1995b). *Cordyceps brunneapunctata* sp. nov. infecting beetle larvae in Thailand. *Mycological Research,* 99, 1195- 1198.

Hywe1-Jones, N.L. (1996). *Cordyceps myrmecophila-like* fungi infecting ants in the leaflitter of tropical forest in Thailand. *Mycological Research,* 100**,** 613- 619.

Hywel-Jones, N. (1997). The biological diversity of invertebrate pathogenic fungi. In *Biodiversity o/tropical microfimgi* (ed. K. Hyde) pp. 107-119. Hong Kong University Press: Hong Kong.

Hywel-Jones, N. (1997a). The biological diversity of invertebrate pathogenic fungi. In *Biodiversity of tropical microfungi* (ed. K. Hyde) pp. 107-119. Hong Kong University Press: Hong Kong.

Hywel-Jones, N. (1997b). *Torrubiella petchii,* a new species of scale insect pathogen from Thailand. *Mycological Research,* 101**,** 143-145.

Hywel-Jones, N. (2001). A review of invertebrate pathogenic Clavicipitaceae from Thailand. *BRT Research Reports,* 34-41.

Hywel-Jones, N.L. (2002). The importance of invertebrate-pathogenic fungi from the tropics. *Tropical Mycology*, 2:133-144.

Hsu, J. H., Jhou, B. Y., Yeh, S. H., Chen, Y. L., & Chen, C. C. (2015). Healthcare functions of *Cordyceps cicadae*. *J. Nutrit. Food Sci.,* 5:100432. doi: 10.4172/2155- 9600.1000432

Huang, B.M., Ju, S.Y., Wu, C.S., Chuang, W.J., Sheu, C.C., & Leu, S.F. (2001). *Cordyceps sinensis* and its fractions stimulate MA-10 mouse Leydig tumor cell steroidogenesis. *J Androl.,* 22(5):831-7.

Hughes, S. (1953). Conidiophores, conidia, and classification. *Canadian Journal of Botany,* 31, 577-659.

Humber, R.A. (2000). Fungal pathogens and parasites of insects. In *Applied Microbial Systematics* (eds. F. Preist & M. Goodfellow) pp. 203-230. Kluwer Academic Publishers:Dordrecht.

Humber, R.A. (2008). Evolution of entomopathogenicity in fungi. *Journal of Invertebrate Pathology,* 98(3):262-6. doi: 10.1016/j.jip.2008.02.017.

Humber, R.A., & Hansen, K.S. (2005), 'USDA-ARS Collection of entomopathogenic fungal cultures (ARSEF)', ARSEF-Catalog: Host by Fungus. http://arsef.fpsnl.cornell.edu.

Hung, L.T. (2010). Entomopathogenic fungi in the Southern area of Cat Tien National Park, Vietnam and Cordycepin production in selected species from Japan, Thailand and Vietnam. Master Thesis (MSc) Kasetsart University.

Hunter, W.B., Avery, P.B., Pick, D., & Powell, C.A. (2011). 'Broad Spectrum Potential of *Isaria fumosorosea* on Insect Pests of Citrus', *Florida Entomologist,* 94, 1051-1054.

Hur, H. (2008). Chemical Ingredients of *Cordyceps militaris*. *Mycobiology, 36*, 233–235. https://doi.org/10.4489/MYCO.36.4.233.

Hussein, H.M., Habuštová, S., Uža, O.P., & Zemek, R. (2016). Laboratory evaluation of *Isaria fumosorosea* CCM-8367 and *Steinernema feltiae* Ustinov against immature stages of the Colorado potato beetle. *PLoS ONE,* 11:e0152399

Huijsduijnen, R.H.V., Bombrun, A., & Swinnen, D. (2002). Selecting protein tyrosine phosphatases as drug targets. *Drug Discovery Today,* 7, 1013–1019.

Hyde KD, Bahkali AH, Moslem MA. (2010) Fungi–an unusual source for cosmetics. *Fungal Diversity*, 43, 1–9.

Hyde, K.D., Xu, J.C., Rapior, S., Jeewon, R., Lumyong, S., Niego, A.G.T, et al, (2019). The amazing potential of fungi: 50 ways we can exploit fungi industrially. *Fungal Diversity*, 97, 1–136, 2019. doi: 10.1007/s13225-019-00430-9.

Idris, A., Ietidal, A., & Idris, M. (2013). Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia africana*. *Egypt Acad J Biol Sci.,* 5, 1–9.

Ignoffo, C.M. (1973). Effects of entomopathogens on vertebrates. *Ann N Y Acad Sci*. 22;217:141-72. doi: 10.1111/j.1749-6632.1973.tb32756.x.

Inglis, G. D., Goettel, M. S., Butt, T. M., & Strasser, H. (2001). Use of hyphomycetous fungi for managing insect pests. In T. M. Butt, C. Jackson & N. Magan (Eds.), Fungi as Biocontrol Agents: Progress, Problems and Potential (pp. 23-69). Wallingford: CABI.

Inglis, P., & Tigano-Milani, M. (2006). Identification and taxonomy of some entomopathogenic *Paecilomyces* spp. (Ascomycota) isolates using rDNA-ITS Sequences. *Genetics and Molecular Biology,* 29, 132-136.

Isaka, M., Kittakoop, P., Kirtikara, K., Hywel-Jones, N.L., & Thebtaranonth Y. (2005). Bioactive substances from insect pathogenic fungi. *Acc Chem Res*., 38, 813-823. doi: 10.1021/ar040247r.

Islam, Tahidul., Yu, Xiaoming., & Baojun, X. (2016). Phenolic profiles, antioxidant capacities and metal chelating ability of edible mushrooms commonly consumed in China. *LWT - Food Science and Technology, 72, 423–431*. doi:10.1016/j.lwt.2016.05.005

Ishiguro, S., Shinada, T., Wu, Z., Karimazawa, M., Uchidate, M., Nishimura, E., et al. (2021). A novel cyclic peptide (Naturido) modulates glia–neuron interactions *in vitro* and reverses ageing-related deficits in senescence-accelerated mice. *PLoS ONE* 16(1): e0245235. https://doi.org/10.1371/journal. pone.0245235.

Ishikawa, T., & Kikuhara, Y. (2009): *(Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae), a presumable recent invader to Japan). *Japanese Journal of Entomology,* 12,115– 116. [in Japanese, English abstract].

Jacobson, P.B., Morgan, S.J., Wilcox, D.M., Nguyen, P., Ratajczak, C.A., Carlson, R.P. et al. (1999). A new spin on an old model: in vivo evaluation of disease progression by magnetic resonance imaging with respect to standard inflammatory parameters and histopathology in the adjuvant arthritic rat. *Arthritis Rheum*., 42, 2060-73. doi: 10.1002/1529-0131(199910)42:10<2060::AID-ANR6>3.0.CO;2-L.

James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., Cox, C.J. et al. (2006). Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature,* 19;443(7113):818-22. doi: 10.1038/nature05110.

Jaronski, S.T. (2010). Ecological factors in the inundative use of fungal entomopathogens. *Biological Control,* 55, 159-185.

Jedrejko, K.J., Lazur, J., & Muszynska, B. (2021). *Cordyceps militaris*: An Overview of Its Chemical Constituents in Relation to Biological Activity. *Foods,* 10, 2634. https://doi.org/ 10.3390/foods10112634

Jee, S.H., Sull, J.W., Park, J., Lee, S.Y., Ohrr, H., Gualler, E., Samet, J.M. (2006). Body-mass index and mortality in Korean men and women. *N. Engl. J. Med*., 335, 779–787.

Jiang, P. (1987). Pharmacology constituent and function of *Cordyceps sinensis*. *Journal of Westnorthern Medicine,* 2, 43–44.

Jiang, Y.H., Jiang, X.L., Wang, P., & Hu, X.K. (2005). *In vitro* antioxidant activities of water-soluble polysaccharides extracted from *Isaria farinosa* B05. *J Food Biochem.,* 29, 323–335. doi: 10.1111/j.1745-4514.2005.00040.x.

Jiang, Y-H., Jiang, X-L Wang, P., Mou, H-J., Hu, X-K., & Liu, S-Q. (2008). The antitumor and antioxidative activities of polysaccharides isolated from *Isaria farinosa* B05. *Microbiological Research,* 163, 424-430.

Jiang ,J., Grieb, B., Thyagarajan, A., & Sliva, D. (2008). Ganoderic acids suppress growth and invasive behavior of breast cancer cells by modulating AP-1 and NF- κB signaling. *International Journal of Molecular Medicine,* 21, 577-584.

Jin, C.-Y., Kim, G.-Y., & Choi, Y.-H. (2008). Induction of Apoptosis by Aqueous Extract of *Cordyceps militaris* through Activation of Caspases and Inactivation of Akt in Human Breast Cancer MDA-MB-231 Cells. *J. Microbiol. Biotechnol*., 18, 1997–2003.

Ji, S.D., Sung, G.B., Kang, P.D., Kim, K.Y., Choi, Y.S., Kim, N.S. et al. (2011). Synnemata Production Using Silkworm Variety, Female Yangwonjam by *Isaria tenuipes*. *Mycobiology,* 39, 158-63. doi: 10.5941/MYCO.2011.39.3.158.

Jing, T., Chi, H., & Li, L., & Rui□Yan, M.A. (2014). Effects of temperature and relative humidity on conidial germination of *Isaria fumosorosea* (Hypocreales: Cordycipitaceae) IF□1106 and pathogenicity of the fungus against *Bemisia tabaci* (Homoptera: Aleyrodidae). *Mycosystema,* 33, 668-679. DOI: 10.13346/j.mycosystema.130168.

Johnson, C.K. (2006). Calmodulin, conformational states, and calcium signaling. A single-molecule perspective. *Biochemistry*, 48, 14233-14236.

Johnson, D., Sung, G.H., Hywel-Jones, N., Luangsa-Ard, J.J., Bischoff, J.F., Kepler, R. & Spatafora, J.W. (2009). Systematics and evolution of the genus *Torrubiella* (Hypocreales, Ascomycota). *Mycological Research,* 113, 279-289.

Jung, I.C., Kim, S., Kwon, Y.I., Park K.S., & Lee J.S. (1997). Cultural condition for the mycelial growth of *Phellinus iginarius* on chemically defined medium and grains. *Korean Journal of Mycology,* , 25, 133–142.

Kang, T.S., Lee, D.G., & Lee, S.Y. (1997). Isolation and mycelial cultivation submerged of *Phellinus* sp. *Korean Journal of Mycology*, 25, 257–267.

Kang, P. D., Sung, G. B., Kim, K. Y., Kim, M. J., Hong, I. P., & Ha, N. G. (2010). Breeding of a Silkworm Variety for Synnemata Production of *Isaria tenuipes*. *Mycobiology, 38*(3), 180–183.

Kang, C., Wen, T.C., Kang, J.C., Meng, Z.B., Li, G.R., & Hyde, K.D. (2014). Optimization of large-scale culture conditions for the production of cordycepin with *Cordyceps militaris* by liquid static culture. *Scientific World Journal,* 510627. doi: 10.1155/2014/510627.

Khachatourians, G.G. (1986). Production and use of biological pest control agents. *Trends Biotechnol.,* 4, 120-124.

Khachatourians, G.G. (1991). Physiology and genetics of entomopathogenic fungi. In: *Handbook o f Applied Mycology. Volume* 2. *Humans, Animals and Insects.* (Edited by Arora, D.K., Ajello, L. and Mukeiji, K.G.). Marcel Dekker, New York. pp. 613-663.

Kaczmarek, A., & Bogu´s, M.I. (2021). The Impact of the Entomopathogenic Fungus *Conidiobolus coronatus* on the Free Fatty Acid Profile of the Flesh Fly *Sarcophaga argyrostoma*. *Insects,* 12, 970. https://doi.org/ 10.3390/insects12110970

Kalsbeek, V., Frandsen, F., & Steenberg, T. (1995). Entomopathogenic fungi associated with *Ixodes ricinus* ticks. *Exp Appl Acarol.,* 19, 45–51. doi:10.1007/BF00051936

Karthi, S., Vasantha-Srinivasan, P., Ganesan, R., Ramasamy, V., Senthil-Nathan, S., Khater, H.F. et al. (2020). Target Activity of *Isaria tenuipes* (Hypocreales: Clavicipitaceae) Fungal Strains against Dengue Vector *Aedes aegypti* (Linn.) and Its Non-Target Activity Against Aquatic Predators. *Journal of Fungi,* 29;6(4):196. doi: 10.3390/jof6040196.

Kavanagh, K. (2011). Fungi: Biology and Applications, 3rd Edition. Wiley-Blackwell, ISBN: 978-1-119-37432-9.

Kaushik, H., & Dutta, P. (2016). Establishment of Metarhizium anisopliae, an entomopathogen as endophyte for biological control in tea. *Res. on Crops,* 17, 375-387

Kaya, H.K. & Lacey, L.A. (2007). Introduction to microbial control. In *Field manual of techniques in invertebrate pathology: Application and evaluation of pathogens for control of insects and other invertebrate pests* (eds. L.A. Lacey & H.K Kaya) pp. 3-7. Springer: Dordrecht.

Keller, S, & Zimmermann, G. (1989). Mycopathogens of soil insects. In: Wilding N, Collins NM, Hammond PM, Webber JF, editors. *Insect-Fungus Interactions*, pp. 239- 270. Academic Press.

Keller, S., Kessler, P., & Schweizer, C. (2013). Distribution of insect pathogenic fungi in Switzerland with special reference to *Beauveria brongniartii* and *Metarhizium anisopliae*. *BioControl,* 48, 307–319.

Kendrick, B., & DiCosmo, F. (1979). Teleomorph-anamorph connections in Ascomycetes. In *The Whole Fungus*, (Ed Kendrick, B.), National Museum of Natural Sciences, National Museums of Canada and the Kananskis Foundation, Ottawa, Canada, pp. 283-410.

Kepler, R., Ban, S., Nakagiri, A., Bischoff, J., Hywel-Jones, N., Owensby, C.A., et al. (2013). The phylogenetic placement of hypocrealean insect pathogens in the genus *Polycephalomyces*: an application of one fungus one name. *Fungal Biology,* 117, 611–622. doi: 10.1016/j.funbio.2013.06.002.

Kepler, R.M., Luangsa-Ard, J.J., Hywel-Jones, N.L., Quandt, C.A., Sung, G.H., Rehner, S.A. et al. (2017). A phylogenetically-based nomenclature for *Cordycipitaceae* (*Hypocreales*). *IMA Fungus,* 8, 335-353. doi: 10.5598/imafungus.2017.08.02.08.

Khatri, B.O., & Kramer, J.F. (2011). Fingolimod for the treatment of multiple sclerosis: a review of the evidence from the FREEDOMS and TRANSFORMS studies. *Clin. Invest.,* 1, 575–578.

Khatri, B.O. (2016). Fingolimod in the treatment of relapsing-remitting multiple sclerosis: long-term experience and an update on the clinical evidence. *Therapeutic Advances in Neurological Disorders,* 9, 130-47. doi: 10.1177/1756285616628766.

Kiewnick, S. (2006). Effect of temperature on growth, germination, germ-tube extension and survival of *Paecilomyces lilacinus* strain 251. *Biocontrol Science Technology,* 16, 535–546.

Kim, S.W., Hwang, H.J., Xu, C.P., Sung, J.M., Choi, J.W., & Yun, J.W. (2003). Optimization of submerged culture process for the production of mycelial biomass and exo-polysaccharides by *Cordyceps militaris* C738. *Journal of Applied Microbiology,* 94, 120–126.

Kim, S-W., Park, S-S., Min, T-J & Yu, K-H. (1999). Antioxidant Activity ofErgosterol Peroxide (5,8-Epidioxy-5α,8α-ergosta-6,22E-dien-3β-ol) in *Armillaria mellea*. *Bull. Korean Chem. Soc.,* 20, 819-823.

Kim, S-Su., Lee, J.S., Cho, J.Y., Kim, Y.E., & Hong, E.K. (2010). Effects of C/N ratio and Trace Elements on Mycelial Growth and Exo-polysaccharide Production of *Tricholoma matsutake*. *Biotechnology and Bioprocess Engineering,* 15, 293-298. DOI 10.1007/s12257-008-0226-x.

Kim, Y.O., Kim, H.J., Abu-Taweel, G.M., Oh, J.S., & Sung, G.H. (2019). Neuroprotective and therapeutic effect of *Cordyceps militaris* on ischemia-induced neuronal death and cognitive impairments. *Saudi Journal of Biological Sciences,* 26, 1352–1357. https://doi.org/http:// doi/org/10.1016/j.sjbs.2018.08.011.

Kim, J., & Sung, G. H. (2018). Beauvericin synthetase contains a calmodulin binding motif in the entomopathogenic fungus *Beauveria bassiana. J. Gen. Appl. Microbiology,* 64, 145–147. doi: 10.2323/jgam.2017.09.003

Kirk, P.M., Cannon, P.F., Minter, D.W., & Stalpers, J.A. eds. (2008). Dictionary of the Fungi. 10 Edition. CAB International, Wallingford, UK.

Kiss, L. (2012). Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcodes for Fungi. *Proceedings of the National Academy of Sciences of the United States,* 109, E1811–E1811. https://doi.org/10.1073/pnas.1207143109.

Kikuchi, H., Takahashi, N., & Oshima, Y. (2004). Novel aromatics bearing 4-0-methylglucose unit isolated from the oriental crude drug *Bombyx batryticatus*. *Tetrahedron Letters,* 45, 367-370.

Kikuchi, H., Miyagawa, Y., Nakamura, K., Sahashi, Y., Inatomi, S., & Oshima, Y. (2004a). A novel carbon skeletal trichothecane, tenuipesine A, isolated from an entomopathogenic fungus, *Paecilomyces tenuipes*. *Organic Letters*, 6, 4531–4533.

Kikuchi, H., Miyagawa, Y., Sahashi, Y., Inatomi, S., Haganuma, A., Nakahata, N., & Oshima, Y. (2004b). Novel spirocyclic trichothecanes, spirotenuipesine A and B isolated from entomopathogenic fungus, *Paecilomyces tenuipes*. *Journal of Organic Chemistry*, 69, 352–356.

Kikuchi, H., Miyagawa, Y., Sahashi, Y., Inatomi, S., Haganuma, A., Nakahata, N., & Oshima, Y. (2004c). Novel trichothecanes, paecilomycine A, B, and C, isolated from entomopathogenic fungus, *Paecilomyces tenuipes. Tetrahedron Letters*, 45, 6225–6228.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution,*16:111-120.

King, C.B. (2006). Species diversity and production of antimicrobial compounds by Pacific North-western Clavicipitalean entomogenous fungi. MSc Thesis, The University of British Columbia.

Kobayasi, Y. (1939). On the genus *Cordyceps* and its allies on cicadae from Japan. *Bulletin of the Biogeographical Society of Japan,* 9, 145-176.

Kobayasi, Y. (1941). The genus *Cordyceps* and its allies. *Science Report of the Tokyo Bunrika Daigaku, Section B. No.* 845, 53-260.

Kobayasi, Y. (1982). Keys to the taxa of the genera *Cordyceps* and *Torrubiella. Transactions of the Mycological Society of Japan,* 23, 329-364.

Kobayasi, Y., & Shimizu, D. (1982). Monograph of the genus *Torrubiella. Bulletin of the National Science Museum, Tokyo, Series B,* 8, 43-78.

Koh, J.H., Kim, J.M., Chang, U.J., & Suh, H.J. (2003). Hypocholesterolemic effect of hot-water extract from mycelia of *Cordyceps sinensis*. Biol Pharm Bull. 26, 84-7. doi: 10.1248/bpb.26.84.

Komatsu, N., Okubo, S., Kikumoto, S., Kimura, K., Saito, G., & Sakai, S. (1969). Host-mediated antitumor action of schizophyllan, a glucan produced by *Schizophyllum commune. Gann*, 60, 137–144.

Kontogiannatos, D., Koutrotsios, G., Xekalaki, S., & Zervakis, G.I. (2021). Biomass and Cordycepin Production by the Medicinal Mushroom *Cordyceps militaris*—A Review of Various Aspects and Recent Trends towards the Exploitation of a Valuable Fungus. *Journal of Fungi*, 7, 986. https://doi.org/10.3390/jof7110986

Kopp, F., & Marahiel, M.A. (2007). Macrocyclization strategies in polyketide and nonribosomal peptide biosynthesis. *Nat. Prod. Rep.,* 24, 735–749. doi: 10.1039/b613652b.

Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., van Oers, M.H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*, 1, 1415-20.

Kumar, C.S., Jacob, T.K., Devasahayam, S., D'Silva, S., & Kumar, N.K. (2015). Isolation and characterization of a Lecanicillium psalliotae isolate infecting cardamom thrips (Sciothrips cardamomi) in India. *BioControl*, 60, 263–373.

Kurosaka, K., Takahashi, M., Watanabe, N., & Kobayashi, Y. (2003). Silent clean-up of very early apoptotic cells by macrophages. *J Immunol.,*171, 4672–9.

Kuzma, M., Jegorov, A., Kacer, P., & Havlicek, V. (2001). Sequencing of new beauverolides by high-performance liquid chromatography and mass spectrometry. *Journal of Mass Spectrometry*, *36*, 1108-1115.

Lacey, L.A., Fransen, J.J., & Carruthers, R. (1996). Global distribution of naturally occurring fungi of Bemisia, their biologies and use as biological control agents. In: Gerling D, Mayer R, editors. Bemisia: 1995. *Taxonomy, biology, damage, control and management*, Andover: Intercept. pp. 401-433.

Lacey, L.A., & Mercadier, G. (1998), 'The Effect of Selected Allelochemicals on Germination of Conidia and Blastospores and Mycelial Growth of the Entomopathogenic Fungus, *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes)', *Mycopathologia*, 142, 17-25.

Lacey, L.A., Frutos, R., Kaya, H.K., & Vail, P. (2001). Insect pathogens as biological control agents: do they have a future? *Biological Control,* 21, 230–48. doi:10.1006/bcon.2001.0938.

Lacey, L.A. (2012). Manual of Techniques in Invertebrate Pathology. 2nd Edition, Elsevier, ISBN 978-0-12-386899-2.

Lama, D. 2004. Taxonomical, Distributional and Ecological Studies of *Acer* L. *(*Aceraceae*)* in the Darjiling- Sikkim Himalayas. Ph.D.Thesis, NBU.

Lamarck, J.B. (1809). Philosophie Zoologique, ou Exposition des Considerations Relatives à l'histoire Naturelle des Animaux. Dentu, Paris.

Lazzarini, G.M.J., Rocha, L.F.N., & Luz, C. (2006). Impact of moisture on *in vitro* germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity in *Triatoma infestans*. *Mycological Research,*110, 485–92. doi:10.1016/j.mycres.2005.12.001.

Lee, S.M., Park, N.S., Jin, B.R., Kang, H.S., Jung, J.H., & Park, E. (2006). Effects of *Paecilomyces tenuipes* cultivated in egg yolk on lipid metabolism in rats on a high fat-cholesterol diet. *J Med Food.,* 9, 214-22. doi: 10.1089/jmf.2006.9.214.

Lee, H. H., Lee, S., Lee, K., Shin, Y. S., Kang, H., & Cho, H. (2015). Anti-cancer effect of *Cordyceps militaris* in human colorectal carcinoma RKO cells via cell cycle arrest and mitochondrial apoptosis. *Daru: journal of Faculty of Pharmacy, Tehran University of Medical Sciences*, *23*(1), 35. https://doi.org/10.1186/s40199-015-0117-6

Lei, K., Ke, Y., & Mao, N. (2011). "The optimization of the culture media for high production of cordycepin by *Cordyceps militaris* Fjnu-01," *Pharmaceutical Biotechnology*, 18, 504–508.

Lezama-Gutiérrez, Roberto., Molina-Ochoa, Jaime., Chávez-Flores, Omar., Ángel-Sahagún, César Andrés., Skoda, Steven R. et al. (2012). Use of the entomopathogenic fungi *Metarhizium anisopliae*, *Cordyceps bassiana* and *Isaria fumosorosea* to control *Diaphorina citri* (Hemiptera: Psyllidae) in

Persian lime under field conditions. *International Journal of Tropical Insect Science,* 32, 39–44. doi:10.1017/S1742758412000069

Li, Z. (1988). List on the insect hosts of *Beauveria bassiana.* In *Study and Application of Entomogenous Fungi in China, Vol.* 1 (eds. Y. Li & Z. Li *et al.)* pp. 241-255. Academic Periodical Press: Beijing.

Li, S.P., Li, P., Dong, T.T.X., & Tsim, K.W.K. (2001). Anti-oxidation activity of different types of natural *Cordyceps sinensis* and cultured *Cordyceps* mycelia. *Phytomedicine,* 8, 207–212.

Li, Y., Wang, X., Zhang, X., Lai, D., & Zhou, L. (2017). Structural diversity and biological activities of the cyclodipeptides from fungi. *Molecules*, 22(12), 2026.

Li, L., Zhang, T., Li, C., Xie, L., Li, N., Hou, T., et al. (2019). Potential therapeutic effects of *Cordyceps cicadae* and *Paecilomyces cicadae* on adenine-induced chronic renal failure in rats and their phytochemical analysis. *Drug Des. Devel. Ther*., 13, 103–117. doi: 10.2147/dddt.S180543.

Li, X.Q., Xu, K., Liu, X.M., & Zhang, P. (2020). A Systematic Review on Secondary Metabolites of *Paecilomyces* Species: Chemical Diversity and Biological Activity. *Planta Med*., 86, 805-821. doi: 10.1055/a-1196-1906.

Lin, R., Liu, H., Wu, S., Pang, L., Jia, M., Fan, K., Jia, S., Jia, L. (2012). Production and in vitro antioxidant activity of exopolysaccharide by a mutant, *Cordyceps militaris* SU5-08. *Int J Biol Macromol*., 51, 153-7. doi: 10.1016/j.ijbiomac.2012.04.011.

Lin S, Liu ZQ, Xue YP, Baker PJ, Wu H, Xu F, Teng Y, Brathwaite ME, Zheng YG. (2016). Biosynthetic Pathway Analysis for Improving the Cordycepin and Cordycepic Acid Production in *Hirsutella sinensis*. *Appl Biochem Biotechnol.,* 179, 633-49. doi: 10.1007/s12010-016-2020-0.

Liang, Z. (1981). Two new species of *Paecilomyces* from insects. *Acta Microbiologica Sinica,* 21,31-34.

Lilly, V. G. & Barnett, H.L. (1953). The utilization of sugars by fungi. *W. Va. Univ. Agr. Exp. Sta. Bull.* 362 T, 5—66

Lingappa, S., Saxena, H., Devi, & Vimala, P.S. (2005). Role of biocontrol agents in management of *Helicoverpa armigera* (Hubner).In: Saxena H, Rai AB, Ahmad R, Gupta S (eds) Recent advances in *Helicoverpa armigera* management. Indian Society of Pulses Research and development, IIPR, Kanpur, pp 159–184.

Liu, L., Zhang, J., Chen, C., Teng, J., Wang, C., & Luo, D. (2015a). Structure and biosynthesis of fumosorinone, a new protein tyrosine phosphatase 1B inhibitor firstly isolated from the entomogenous fungus *Isaria fumosorosea*. *Fungal Genet Biol.,* 81:191-200. doi: 10.1016/j.fgb.2015.03.009.

Liu, Z.Q., Liu, T., Chen, C., Li, M.Y., Wang, Z.Y., Chen, R.S. et al. (2015b). Fumosorinone, a novel PTP1B inhibitor, activates insulin signaling in insulin-resistance HepG2 cells and shows anti-diabetic effect in diabetic KKAy mice. *Toxicol Appl Pharmacol*., 15, 61-70. doi: 10.1016/j.taap.2015.03.011.

Liu, F., Wu, X.L., He, Z.Y., Xiang, M.C., He, Y.C., Liu, X.Z. et al. (2016). Nutritional effects on the mycelial growth and enzymatic activity of *Isaria farinosa*, and *Hepialus* larvae growth. J*ournal of Applied Microbiology*, 120, 1605—1615.

Liu, F., Xiang, M., Guo, Y., Wu, X., Lu, G., Yang, Y. et al. (2018). Culture conditions and nutrition requirements for the mycelial growth of *Isaria farinosa* (Hypocreales: Cordycipitaceae) and the altitude effect on its growth and metabolome. *Scientific Reports,* 23, 15623. doi: 10.1038/s41598-018-33965-z.

Liu, K., Wang, F., Liu, G., & Dong, C. (2019). Effect of Environmental Conditions on Synnema Formation and Nucleoside Production in Cicada Flower, *Isaria cicadae* (Ascomycetes). *International Journal of Medicinal Mushrooms,* 21, 59-69. doi: 10.1615/IntJMedMushrooms.2018029506.

Liu, W., Zhang, Z., Zhang, T., Qiao, Q., & Hou, X. (2022). Phenolic profiles and antioxidant activity in different organs of *Sinopodophyllum hexandrum*. *Front. Plant Sci.,* 13, 1037582. doi: 10.3389/fpls.2022.1037582

Linnaeus, C. (1758). Systema Naturae. 10th ed. Stockholm.

Linnaeus, C. (1767). Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis, 12th edition, Vol. 2: *Regnum vegetabile*. (Laurentius Salvius: Stockholm).

Lloyd, C. (1919). Mycological notes, no. 61. *Mycological Writings,* 5, 877-903.

Luangsa-ard, J.J., Hywel-Jones, N.L., & Samson, R.A. (2004). The polyphyletic nature of *Paecilomyces* sensu lato based on 18Sgenerated rDNA phylogeny. *Mycologia,* 96, 773-780.

Luangsa-ard, J.J., Hywel-Jones, N.L., Manoch, L., & Samson, R.A. (2005). On the relationships of *Paecilomyces* sect. Isarioidea species. *Mycological Research,* 109, 581-589.

Luangsa-ard, J.J., Tasanathai, K., Mongkolsamrit, S., & Hywel-Jones, N. (2007). Atlas of Invertebrates-Pathogenic Fungi of Thailand Volume 1 | NSTDA Bookstore, n.d. https://bookstore.nstda.or.th/shop/product/web-400219-atlas-of-invertebrate pathogenic-fungi-of-thailand-volumn-1-4617.

Luangsa-ard, J.J., Berkaew, P., Ridkaew, R., Hywel-Jones, N.L. (2009). A beauvericin hot spot in the genus *Isaria*. *Mycological Research,* 113, 1389–1395

Luangsa-ard, J.J., Houbraken, J., van Doorn, T., Hong, S-B., Borman, A.M, *et al*. (2011). *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. *FEMS Microbiology Letters,* 321, 141–149.

Lu, H-E., Jian, C-H., Chen, S-F., Chen, T-M., Lee, S-T., Chang, C-S., & Weng, C-F. (2010). Hypoglycaemic effects of fermented mycelium of *Paecilomyces farinosus* (G30801) on high-fat fed rats with streptozotocin-induced diabetes. *Indian J Med Res.,* 131, 696-701.

Lu, Y., Luo, F., Cen, K. *et al.* (2017). Omics data reveal the unusual asexual-fruiting nature and secondary metabolic potentials of the medicinal fungus *Cordyceps cicadae*. *BMC Genomics,* 18, 668. https://doi.org/10.1186/s12864-017-4060-4.

Lücking, R., Aime, M.C., Robbertse, B. *et al.* (2020). Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding?. *IMA Fungus,* 11**,** 14. https://doi.org/10.1186/s43008-020-00033-z.

Lutzoni, F., Kauff, F., Cox, C.J., McLaughlin, D., Celio, G., Dentinger, B. et al. (2004). Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *American Journal of Botany,* 91, 1446-1480.

Luz, C., & Fargues, J. (1997). Temperature and moisture requirements for conidial germination of an isolate of the hyphomycete *Beauveria bassiana* (Bals.) Vuill., pathogenic to *Rhodnius prolixus* (Stahl) (Hem., Reduviidae). *Mycopathologia,* 138, 117-125.

Ma, C., Li, Y., Niu, S., Zhang, H., Liu, X., & Che, Y. (2011). *N*-Hydroxypyridones, Phenylhydrazones, and a Quinazolinone from *Isaria farinosa***.** *Journal of Natural Products,* 4, 32–37doi:10.1021/np100568w

Mapook, A., Hyde, K.D., Hassan, K. *et al.* (2022). Ten decadal advances in fungal biology leading towards human well-being. *Fungal Diversity,* 116, 547–614 https://doi.org/10.1007/s13225-022-00510-3

Madariaga-Mazón, A., González-Andradeb, M., Toriello, C., Navarro-Barranco, H., & Mata, R. (2015). Potent anti-calmodulin activity of cyclotetradepsipeptides isolated from Isaria fumosorosea using a newly designed biosensor. *Natural Product Commun*incations, 10, 113-6.

Magnuson, J. K. and Lasure, L. L. (2004). Organic acid production by filamentous fungi. In Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine (Tkacz, J. S. and Lange, L., eds.). pp. 307-340, Springer. U.S.

Mains, E. (1940). Species of *Cordyceps. Mycologia,* 32, 310-320.

Mains, E. (1947). New and interesting species of *Cordyceps. Mycologia,* 39,535-545

Mains, E. (1949). New species of *Torrubiella, Hirsutella* and *Gibellula. Mycologia,* 41, 304 310.

Mains, E. (1950). Entomogenous species of *Akanthomyces, Hymenostilbe* and *Insecticola* in North America. *Mycologia,* 42, 567-589.

Mains, E. (1955). Some entomogenous species of *Isaria. Papers of the Michigan Academy of Science, Arts, and Letters,* 40, 23-32.

Major, R. (1944). Agostino Bassi and the parasitic history of disease. *Bulletin of the History of Medicine,* 16, 97-107.

Mallebrera, B., Juan-Garcia, A., Font, G., & Ruiz, M. J. (2016). Mechanisms of beauvericin toxicity and antioxidant cellular defense. *Toxicol. Lett.,* 246, 28–34. doi: 10.1016/j.toxlet.2016.01.013

Manjula, B. (1983). A revised list of the agaricoid and boletoid basidiomycetes from India and Nepal. Proc. *Indian Acad. Sci.,* (*Plant Sci*.), 92, 81 --213.

Mannino, M.C., Huarte-Bonnet, C., Davyt-Colo, B., & Pedrini, N. (2019). Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of entomopathogenic fungi. *Journal of Fungi,* 5, 33.

Mau, J.-L., Lin, H.-C., & Chen, C.-C. (2002a). Antioxidant properties of several medicinal mushrooms. *Journal of Agricultural and Food Chemistry*, 50, 6072–6077.

Mau, J.-L., Lin, H.-C., & Song, S.-F. (2002b). Antioxidant properties of several specialty mushrooms. *Food Research International*, 35, 519–526

Manzi, P., Gambelli, L., Marconi, S., Vivanti, V., & Pizzoferrato, L. (1999). Nutrients in edible mushroom, an inter-species comparative study. *Food Chemistry,* 65, 477-482.

Manzi, P., Aguzzi, A., & Pizzoferrato, L. (2001). Nutritional value of mushrooms widely consumed in Italy. *Food Chemistry*, 73, 321-325.

Mascarin, G.M., Batista, A.S., & Biaggioni, L.R. (2010). Culture media selection for mass production of *Isaria fumosorosea* and *Isaria farinosa. Brazilian Archives of Biology and Technology,* 53, *753–761.* doi:10.1590/S1516-89132010000400002

Massee, G. (1895). A revison of the genus *Cordyceps. Annals of Botany,* 9, 1-44.

Masse G. (1906). A text book of fungi.

Martin, M.M. (1992). The Evolution of Insect-Fungus Associations: From Contact to Stable Symbiosis. *American Zoologist, 32(4), 593–605.* doi:10.2307/3883704.

Masoudi, A., Wang, M., Zhang, X., Wang, C., Qiu, Z., Wang, W. et al. (2020). Meta-Analysis and evaluation by insect-mediated baiting reveal different patterns of Hypocrealean entomopathogenic fungi in the soils from two regions of China. *Front. Microbiol.* 11:1133. doi: 10.3389/fmicb.2020.01133.

Matthew, B., & Andrew, F. (2007). Can fungal biopesticide control malaria? *Nature reviews Microbiology,* 5, 377-383.

Menyhárd, D.K., Keserú, G.M., & Náray-Szabó, G. (2009). Calmodulin in complex with proteins and small molecule ligands: operating with the element of surprise. Implications for structure-based drug design. *Current Computer-Aided Drug Design*, 5, 264-279.

Mayr, E. (1983). The Growth of Biological Thought: Diversity, Evolution, and Inheritance. Harvard University Press, Cambridge, Massachusetts.

McCoy, C.W., & Albrigo, L.G. (1975). Feeding injury to the orange caused by the citrus rust mite, *Phyllocoptruta oleivora* (Prostigmata: Eriophyoidea). *Annals of the Entomological Society of America,* 68, 289–297.

McLaughlin, D.J., Hibbett, D.S., Lutzoni, F., Spatafora, J.W., & Vilgalys, R. (2009). The search for the fungal tree of life. *Trends Microbiol*ogy, 17, 488-97. doi: 10.1016/j.tim.2009.08.001.

McPherson, J.E., Packauskas, R.J., Taylor S.J. & O'brien M.F. (1990). Eastern range extension of *Leptoglossus occidentalis* with a key to *Leptoglossus* species of America North of Mexico (Heteroptera: *Coreidae*). – *Great Lakes Entomologist,* 23, 99–104.

Medo, J., & Cagáň, L. (2011). Factors affecting the occurrence of entomopathogenic fungi in soils of Slovakia as revealed using two methods. *Biological Control,* 59, 200–208.

Meyling, N.V., & Eilenberg, J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agro ecosystems: Potential for conservation biological control. *Biological Control,* 43, 145- 155.

Meyer, J.M., Hoy, M.A., & Boucias, D.G. (2008), 'Isolation and Characterization of an *Isaria fumosorosea* Isolate Infecting the Asian Citrus Psyllid in Florida', *Journal of Invertebrate Pathology,* 99, 96-102.

Meyling, N.V., & Eilenberg, J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biological Control*, 43, 145-155.

Microsoft Corporation. (2018). *Microsoft Excel*. Retrieved from https://office.microsoft.com/excel

Mietkiewski, R., Tkaczuk, C., Zurek, M., &Van Der Geest, L.P. (2014). Temperature requirements of four ˙ entomopathogenic fungi. *Acta Mycologia,* 29, 109–120.

Millstein, J.A., Brown, G.C., & Nordin, G.L. (1982). Microclimatic humidity influence on conidial discharge in *Erynia* sp. (Entomophthorales:

Entomophthoraceae), and entomopathogenic fungus of the alfalfa weevil (Coleoptera: Curculionidae). *Environ Entomol.*, 11, 1166–1169.

Minter, D., Brady, B. & Hall, R. (1983). Five hyphomycetes isolated from eriophyid mites. *Transactions of the British Mycological Society,* 81, 455-471.

Mishra, S., Kumar, P., & Malik, A. (2015). Effect of temperature and humidity on pathogenicity of native Beauveria bassiana isolate against Musca domestica L. *Journal of parasitic diseases: official organ of the Indian Society for Parasitology*, *39*(4), 697–704. https://doi.org/10.1007/s12639-013-0408-0.

Molnár, I., Gibson, D.M., & Krasnoff, S.B. (2010). Secondary metabolites from entomopathogenic Hypocrealean fungi. *Natural Product Reports,* 27, 1241-75. doi: 10.1039/c001459c.

Mosmann T. (1983). Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods,* 65, 55–63.

Mongkolsamrit, S., Noisripoom, W., Thanakitpipattana, D., Wutikhun, T., Spatafora, J.W., & Luangsa-ard, J. (2018). Disentangling cryptic species with isaria-like morphs in Cordycipitaceae. *Mycologia,* 110, 230–257.

Mora, M.A., Castilho, A.M.C, & Fraga. M.E. (2017). Classification and infection mechanism of entomopathogenic fungi. *Arq. Inst. Biol.*, 84, 1–10. doi: 10.1590/1808-1657000552015.

Moraga, E.Q. (2020). Entomopathogenic fungi as endophytes: Their broader contribution to IPM and crop production. *Biocontrol Sci. Technology,* 1–14.

Moradali, M., Mostafavi, H., Ghods, S., & Hedjaroude, G. (2007). Immunomodulating and anticancer agents in the realm of macromycetes fungi (macrofungi). *International Immunopharmacology,* 7, 701–724.

Mori, S., Chang, J.T., Andrechek, E.R., Matsumura, N., Baba, T., Yao, G. et al. (2009). Anchorage-independent cell growth signature identifies tumors with

metastatic potential. *Oncogene*, 6;28(31):2796-805. doi: 10.1038/onc.2009.139.

Mohsin, M., Negi, P., & Ahmed, Z. (2011). Determination of the antioxidant activity and polyphenol contents of wild Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (W.Curt. Fr.) P. Karst. (Higher Basidiomycetes) from central Himalayan hills of India. *Int J Med Mushrooms,* 13, 535–544.

Money, N. P. (2016). Are mushrooms medicinal? *Fungal Biology*, 120, 449–453.

Moore, D., Bridge, P.D., Higgins, P.M., Bateman, R.P., & Prior, C. (1993). Ultra-violet radiation damage to *Metarhizium flavoviridae* conidia and protection given by vegetable and mineral oils and chemical sunscreens *Ann Appl Biol*., 122, 605-616.

Moritz, C., & Hillis, D.M. (1996). Molecular Systematics: Context andControversies. In: Molecular Systematics. pl, eds.D.M.Hillis, C. Moritz and K.B. Mable. Sinauer Associates, Inc., Sunderland, Massachusetts, USA.

Mudoi, A., & Das, P., Hazarika, L.K., Das, K., & Roy, S. (2019). Variations in hemocyte profile induced by Beauveria bassiana (Bals.) Vuill. in Periplaneta americana (L.) (Blattodea: Ectobiidae) *International Journal of Tropical Insect Science,* 1-11. DOI 10.1007/s42690-019-00054-1.

Mugnai, L., Bridge, P.D. & Evans, H.C. (1989). A chemotaxonomic evaluation of the genus *Beauveria. Mycological Research,* 92, 199-209.

Mundkur, B.B. (1938). Fungi of India--Supplement I. ICAR Sci. Monograph. 12 54.

Mukerji, K.G., & Juneja, R.C. (1974). Fungi of India. Supplement to the list of Indian Fungi 1962-1972; (Delhi: Emkay Publications) 223.

Nagata, M., & Yamashita, I. (1992). Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. *Nippon Shokuhin Kogyo Gakkaish,* 39, 925–928.

Nam, K.S., Jo, Y.S., Kim, Y.H., Hyun, J.W., & Kim, H.W. (2001). Cytotoxic activities of acetoxyscirpenediol and ergosterol peroxide from *Paecilomyces*

tenuipes. *Life Science,* 1;69(2), 229-37. doi: 10.1016/s0024-3205(01)01125-0.

Nascimento, É., Da Silva, S.H., Marques, E.D.R., Roberts, D.W., & Braga, G,Ú.L. (2010). Quantification of cyclobutane cyrimidine dimers induced by UVB radiation in conidia of the fungi *Aspergillus fumigatus, Aspergillus nidulans*, Metarhizium *acridum* and *Metarhizium robertsii. Photochem. Photobiol*., 86, 1259–1266.

Negi, C.S., Joshi, P., & Bohra, S. (2015). Rapid Vulnerability Assessment of Yartsa Gunbu (*Ophiocordyceps sinensis* [Berk.] G.H. Sung et al) in Pithoragarh District, Uttarakhand State, India. *Mountain Research and Development*, 35(4): 382-391.

Negi, V.S., Rana, S.K., Giri, L., & Rawal, R.S. (2020). Caterpillar Fungus in the Himalaya, Current Understanding and Future Possibilities. *G.B. Pant National Institute of Himalayan Environment,* Kosi-Katarmal, Almora , Uttarakhand, India

Nelson, R.H. (2013). Hyperlipidemia as a risk factor for cardiovascular disease. *Primary care*, *40*(1), 195–211. https://doi.org/10.1016/j.pop.2012.11.003.

Newman, D.J., & Cragg, G.M. (2010). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod.,*75, 311–335.

Newman, D.J., & Cragg, G.M. (2014). Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of Natural Products Review.* 10.1021/acs.jnatprod.5b01055.

Nilsson, R.H., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K.H., & Kõljalg, U. (2006). Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. *PloS One* 1(1):e59. https://doi.org/10.1371/journal.pone.0000059

Nwinuka N.M., Ibeh G.O., Ekeke G.I. (2005) Proximate composition and levels of some toxicants in four commonly consumed spices. *Journal of Applied Sciences and Environmental Management,* 9, 150–155.

Oboh, G., & Shodehinde, S.A. (2009). Distribution of nutrients, polyphenols and antioxidant activities in the pilei and stipes of some commonly consumed edible mushrooms in Nigeria. *Bull Chem Soc Ethiop.,* 23, 391–398.

Obornik, M., Jirku, M., & Dolezel, D. (2001). Phylogeny of mitosporic entomopathogenic fungi: is the genus *Paecilomyces* polyphyletic? *Canadian Journal of Microbiology,* 47, 813-819.

O'Donnell, K. (1993). Fusarium and its near relatives. Pp. 225-233. In: *The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics.* Eds., D. R. Reynolds, and J. W. Taylor. CAB International, Wallingford, United Kingdom.

Ogawa, Y., Yahagi, N., Yahagi, R., Kobayashi, H., (2014). Specific antiproliferative activity against human cancer cells with metabolites from several species related to the genus Cordyceps, *Int. J. Curr. Microbiol. App. Sci.,* 3, 607-617

Ojuri, O.T., Ezekiel, C.N., Sulyok, M., Ezeokoli, O.T., Oyedele, O.A., Ayeni, K.I, Eskola, M.K., Sarkanj, B., Hajslova, J., Adeleke, R.A. et al, (2018). Assessing the mycotoxicological risk from consumption of complementary foods by infants and young children in nigeria. *Food Chem. Toxicol.,* 121, 37–50.

Ouedraogo, A., Fargues, J., Goettel, M. S., & Lomer, C. J. (1997). Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviride*. *Mycopathologia* 137, 37–43. doi:10.1023/A:1006882621776

Oh, G.S., Hong, K.H., Oh, H., Pae, H.O., Kim, I.K., Kim, N.Y. et al. (2001). 4-Acetyl-12, 13-epoxyl-9-trichothecene-3, 15-diol isolated from the fruiting bodies of *Isaria japonica* Yasuda induces apoptosis of human leukemia cells (HL-60) *Biol. Pharm. Bull.,* 24, 785–789.

Oh, H., Kim, T., Oh, G.S., Pae, H.O., Hong, K.H., Chai, K.Y. et al. (2002). (3R,6R)-4-methyl-6-(1-methylethyl)-3-phenylmethyl-perhydro-1,4-oxazine-2,5-dione: an apoptosis-inducer from the fruiting bodies of *Isaria japonica. Planta Med.* 68, 345-8. doi: 10.1055/s-2002-26744.

latunji, O.J., Feng, Y., Olatunji, O.O., Tang, J., Ouyang, Z., & Su, Z. (2016). Cordycepin protects PC12 cells against 6-hydroxydopamine induced neurotoxicity via its antioxidant properties. *Biomed Pharmacother.*, 81, 7-14. doi: 10.1016/j.biopha.2016.03.009.

Oliveira, M., Reis, F. S., Sousa, D., Tavares, C., Lima, R. T., Ferreira, I. C. F. R. et al. (2014). A methanolic extract of *Ganoderma lucidum* fruiting body inhibits the growth of a gastric cancer cell line and affects cellular autophagy and cell cycle. *Food & Function*, 5, 1389–1394.

Omoto, C., Dennehy, T.J., McCoy, C.W., Crane, S.E., & Long, J.W. (1994). Detection and characterization of the interpopulation variation of citrus rust mite (Acari: Eriophyidae) resistance to dicofol in Florida citrus. *Journal of Economic Entomology*, 87, 566–572.

Ortiz-Urquiza, A., & Keyhani, N.O. (2013). Action on the surface: Entomopathogenic fungi versus the insect cuticle. *Insects,* 4, 357.

Oyaizu, M. (1986). Studies on products of browning reaction: Antioxidative activities of products of browning reaction prepared from glucoseamine. *Jap. J. Nutr.*, 44, 307–315.

Padmini, P.C.P., & Padmaja, V. (2010). Impact of different relative humidities on in vitro growth and sporulation of entomopathogenic fungal isolates of *Beauveria* species. *Int J Pharm Biol Sci Arch.,* 1, 355–359.

Panasiuk, L., Jedziniak, P., Pietruszka, K., Piatkowska, M., Bocian, L. (2019). Frequency and levels of regulated and emerging mycotoxins in silage in Poland. *Mycotoxin Research,* 35, 17-25, 2019. doi: 10.1007/s12550-018-0327-0.

Panda, A. K., & K. C. Swain. (2011). Traditional uses and medicinal potential of *Cordyceps sinensis* of Sikkim. *Journal of Ayurveda and Integrative Medicine,* 2, 9–13.

Pae, H.O., Oh, G.S., Choi, B.M., Seo, E.A., Oh, H., Shin, M.K. et al. (2003). Induction of apoptosis by 4-acetyl-12,13-epoxyl-9-trichothecene-3,15-diol from *Isaria japonica* Yasuda through intracellular reactive oxygen species formation and caspase-3 activation in human leukemia HL-60 cells. *Toxicology in Vitro,* 17, 49–57. doi:10.1016/s0887-2333(02)00097-8

Palmer, E. (2018). Gilenya–Novartis. Online report. Available from: https://www.fiercepharma.com/special-report/gilenya-novartis

Park, W. M., Kim, G.H., & Hyeon, J.W. (1995). New synthetic medium for growth of mycelium of *Pleurotus* species. *Korean Journal of Mycology,* 23, 275-283.

Park, Gi-B., Park, Gi-B., Shrestha, B & Sung, J-M. Sung (2004). Investigation on favourable conditions for mycelial growth of *Paecilomyces tenuipes. Journal of Mushroom Science and Production,* 2, No 1, p21-27.

Park, E., Park, N-S., Park, H-R., Jin, B-R., & Lee, S-M. (2006). Fruiting body extracts of *Paecilomyces tenuipes* ameliorate lipid and antioxidant metabolism in rats fed with high fat-cholesterol diet. *Food Science Biotechnology,* 15, 710-714.

Park, E., Jeon, G., & Jin, B. (2007). Antigenotoxic Effect of *Paecilomyces tenuipes* Cultivated on Soybeans in a Rat Model of 1,2-Dimethylhydrazine-induced Colon Carcinogenesis. *Food Science and Biotechnology,* 16, 1064-1068.

Park, S.E., Yoo, H.S., Jin, C.-Y., Hong, S.H., Lee, Y.-W., Kim, B.W. et al. (2009). Induction of Apoptosis and Inhibition of Telomerase Activity in Human Lung Carcinoma Cells by the Water Extract of *Cordyceps militaris*. *Food Chem. Toxicol*. 47, 1667–1675.

Park, J.H., Park, N.S., & Lee, S.M. (2011). Effect of *Dongchunghacho* rice on blood glucose level, lipid profile, and antioxidant metabolism in streptozotocin-

induced diabetic rats. *Food Science Biotechnology,* 20, 933. https://doi.org/10.1007/s10068-011-0129-z

Payne, *C.,* Hafez, M., Jones, K, Harpaz, 1., Akingbohungbe, A, Paterson, R. & Hag Amed, S. (1988). Pathogens for the control of insects: where next? [and discussion]. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 318, 225-248.

Pedrini, N., Crespo, R., & Juárez, M.P. (2007). Biochemistry of insect epicuticle degradation by entomopathogenic fungi. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.,* 146, 124–137.

Pedigo, L.P., & Rice, M.E. (2014). Entomology and Pest Management: Sixth Edition. PHI Learning Private Limited, Delhi, India

Pell, L.K., Eilenberg, L., Hajek, A., & Steinkraus, D. (2001). Biology, ecology and pest management potential of Entomophthorales. In *Fungi as biocontrol agents: progress, problems and potential* (eds. T.M. Butt, C. Jackson & N. Magan) pp. 71-167. CAB International: Wallingford.

Pemberton, R.W. (1999). Insects and other arthropods used as drugs in Korean traditional medicine. *Journal of Ethnopharmacololgy,* 65, 207-16. doi: 10.1016/s0378-8741(98)00209-8.

Penton-Arias, E., & Haines, D.D. (2016). Chapter 11 - Natural Products: Immuno-Rebalancing Therapeutic Approaches, Editor(s): Diana Boraschi, Giselle Penton-Rol, *Immune Rebalancing*, Academic Press, 2016, Pages 229-249, ISBN 9780128033029, https://doi.org/10.1016/B978-0-12-803302-9.00011-7.

Petit R.H. (1895). Studies in artificial cultures. Cornell University Agricultural Experiment station, Botanical and Entomological Divisions.Bulletin 97.

Peeters, H., Zocher, R., Madry, N., & Kleinkauf, H. (1983). Incorporation of radioactive precursors into beauvericin produced by *Paecilomyces fumosoroseus*. *Phytochemistry*, 22, 1719–1720.

Peeters, H., Zocher, R., & Kleinkauf, H. (1988). Synthesis beauvericin by a multifunctional enzyme. *Journal of Antibiotics,* 12, 352–359.

Petch, T. (1934). *Isaria. Transactions of the British Mycological Society,* 19, 34-38.

Pham, T.M.N., Ha, T.N., Nguyen, V.K.D., Nguyen, T.H., Nguyen, C.D., Dinh, M.H. & Ngo, K.S. (2020). The cytotoxic activity of extracts from the biomass and fruit bodies of *Isaria tenuipes* VHI-2 fungus on MCF-7 cancer cell line. *International Journal of Agricultural Technology,* 16(4): 907-916.

Polar, P. (2007). Studies on the use of entomopathogenic fungi for the control of cattle ticks. Ph.D. thesis, The University of the West Indies, Trinidad.

Pradhan, B.K. (2016). Caterpillar Mushroom, *Ophiocordyceps sinensis* (Ascomycetes): A Potential Bioresource for Commercialization in Sikkim Himalaya, India. *International Journal of Medicinal Mushrooms*, 18, 337–346

Pradhan, P., Dutta, A.K., Paloi, S., Roy, A., & Acharya, K. (2016). Diversity and distribution of macrofungi in the Eastern Himalayan ecosystem. *Eurasia J Biosciences,* 10, 1-12.

Pradhan, B.K., Sharma, G., Subba, B., Chettri, S., Chettri, A., Chettri, D.R., & Pradhan, A. (2020). Distribution, harvesting and trade of Yartsa Gunbu (*Ohiocordyceps sinensis*) in the Sikkim Himalaya, India. *Mt Res Dev.,* 40, 41–49.

Prommaban, A., Sriyab, S., Marsup, P., Neimkhum, W., Sirithunyalug, J., Anuchapreeda, S. et al. (2022). Comparison of chemical profiles, antioxidation, inhibition of skin extracellular matrix degradation, and anti-tyrosinase activity between mycelium and fruiting body of *Cordyceps militaris* and *Isaria tenuipes*. *Pharmaceutical Biology,* 60, 225-234. doi: 10.1080/13880209.2021.2025255.

Prosser, J.I. (1995). Kinetics of Filamentous Growth and Branching. In: Gow N.A.R., Gadd G.M. (eds) The Growing Fungus. Springer, Dordrecht. https://doi.org/10.1007/978-0-585-27576-5_14

Pupin, A.M., Messias, C.L., Piedrabuena, A.E., & Roberts, D.W. (2000). Total lipids and fatty acids of strains of *Metarhizium anisopliae*. *Braz J Microbiol*., 31, 121–128.

Qian, Y., Sun, X., Wang, X., Yang,X., Fan, M., Zhong, J. et al. (2021). Mechanism of *Cordyceps cicadae* in Treating Diabetic Nephropathy Based on Network Pharmacology and Molecular Docking Analysis. *Journal of Diabetes Research*, 1-10.

Rajula, J., Rahman, A., & Krutmuang, P. (2020). Entomopathogenic fungi in southeast Asia and Africa and their possible adoption in biological control. *Biological Control.* doi: https://doi.org/10.1016/j.biocontrol.2020.104399.

Ramakrishnan, K., & Subramanian, C.V. (1952) The fungi of India--a second supplement. J. Madras University. B21 303-305

Rangel, D.E.N., Braga, G.U.L., Anderson, A.J., & Roberts, D.W. (2005). Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographical origins. *J Invertebr Pathol*., 88, 116-125.

Rangel, D.E.N., Anderson. A.J., & Roberts, D.W. (2006). Growth of *Metarhizium anisopliae* on non-preferred carbon sources yields conidia with increased UV-B tolerance. *J Invertebr Pathol*., 93, 127-134.

Rangel, D.E.N., Fernandes, E.K.K., Dettenmaier, S. J., & Roberts, D. W. (2010). Thermotolerance of germlings and mycelium of the insect-pathogenic fungus *Metarhizium* spp. and mycelial recovery after heat stress. *Journal of Basic Microbiology,* 50, 344–350. doi:10.1002/jobm.200900430.

Rao, Y.K., Fang, S.-H., Wu, W.-S., & Tzeng, Y.-M. (2010). Constituents Isolated from *Cordyceps militaris* Suppress Enhanced Inflammatory Mediator's Production and Human Cancer Cell Proliferation. *Journal of Ethnopharmacology,* 131, 363–367.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay'. *Free Rad Biol Med J.,* 26, 1231–7.

Rehner, S.A., & Samuels, G.J. (1994). Taxonomy and phylogeny of *Gliocladium* analyzed by large subunit rDNA sequences. *Mycological Research* 98, 625-634.

Rehner, S.A. (2005). Phylogenetics of the insect pathogenic genus *Beauveria.* In *Insect-fungal associations: ecology and evolution* (eds. F.E. Vega & M. Blackwell) pp. 3-27. Oxford University Press: New York.

Rehner, S.A., & Buckley, E. (2005). A *Beauveria* phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97, 84-98.

Rehner, S. A. (2009). Molecular systematics of entomopathogenic fungi. In S. P. Stock, J. Vandenberg, I. Glazer & N. Boemare (Eds.), *Insect Pathogens: Molecular Approaches and Techniques* (pp. 145-165). Wallingford: CABI.

Rehner, S.A., Minnis, A.M., Sung, G.H., Luangsa-ard, J.J. et al., (2011). Phylogeny systematics of the anamorphic, entomopathogenic genus Beauveria. *Mycologia,* 103, 1055–1073.

Reis, F.S., Pereira, E., Barros, L., Sousa, M.J., Martins, A., & Ferreira, I.C.F.R. (2011). Biomolecule profiles in inedible wild mushrooms with antioxidant value. *Molecules,* 16:4328–4338.

Reis, F.S., Barros, L., Calhelha, R.C., Ciric, A., Van Griensven, L.J., Sokovic, M., & Ferreira, I.C. (2013). The methanolic extract of *Cordyceps militaris* (L.) Link fruiting body shows antioxidant, antibacterial, antifungal and antihuman tumor cell lines properties. *Food and Chemical Toxicology,* 62, 91–98. https://doi.org/10.1016/j.fct.2013.08.033

Reis, F.S., Martins, A., Vasconcelos, M.H., Morales, P., & Ferreira, I.C.F.R. (2017). Functional foods based on extracts or compounds derived from mushrooms. *Trends in Food Science and Technology*, 66, 48–62.

Ren, X.Y., He, L., Cheng, J.W., & Chang, J.M. (2014). Optimization of the solid-state fermentation and properties of a polysaccharide from *Paecilomyces cicadae*

(Miquel) Samson and its antioxidant activities in vitro. *PLoS One,* 9(2), 87578.

Reeslev, M. & Jensen, B. (1995). Influence of Zn^{2+} and Fe^{3+} on polysaccharide production and mycelium yeast dimorphism of *Aureobasidium pullulans* in batch cultivations. *Appl. Microbiol. Biotechnol*., 42: 910-915.

Reynolds, D. (1993). The fungal holomorph: an overview. In *The fungal holomorph: mitotic, meiotic, and pleomorphic speciation in fungal systematics* (eds. D. Reynolds & J. Taylor) pp. 15-25. CAB International: Wallingford.

Roberts, D.W., & Campbell, A.S. (1977). Stability of entomopathogenic fungi. *Misc. Publ. Entomol. Soc. Am.,* 10, 19-76.

Robel, S., Berninger, B., & Gotz, M. (2011). The stem cell potential of glia: lessons from reactive gliosis. *Nat Rev Neuroscience*, 12, 88–104. https://doi.org/10.1038/nrn2978 PMID: 21248788.

Robles-Acosta, I.N., Chacón-Hernández J.C., Torres-Acosta., Landeros-ores J., Vanoye-Eligo V., & Arredondo-Valdes R. (2019). Entomopathogenic fungi as biological control agents of *Phyllocoptruta oleivora* (Prostigmata: Eriophyidae) under greenhouse conditions. *Florida Entomologist,* 102, 303-308

Roberts, D.W. & Humber, R.H. (1984). Entomopathogenic fungi. In: *Infection processes by fungi.* (Edited by Aist, J.R. and Roberts, D.W), Rockefeller Foundation Press, New York. pp. 1-12.

Rossman A.Y. (1996). Morphological and Molecular Perspectives on Systematics of the Hypocreales. *Mycologia,* 88,. 1-19.

Roy, A., & Acharya, K. (2019). Studies on Diversity and Ecology of macro-fungi in Terai and Dooars regions of West Bengal. Project report Submitted to West Bengal Biodiversity Board, Government of West Bengal; 1-189.

Ruch, R.J., Cheng, S.J., & Klaunig, J.E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 10, 1003-1008.

RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Shafi, G., Munshi, A., Hasan, T.N.,. Alshatwi, A.A., Jyothy, A., & Lei,D.K.Y. (2009). "Induction of apoptosis in HeLa cells by chloroform fraction of seed extracts of Nigella sativa," *Cancer Cell International,* 9, 1–8.

Sánchez-Peña, S.R., Lara-J., S.J., & Medina, R.F. (2011). Occurrence of entomopathogenic fungi from agricultural and natural ecosystems in Saltillo, Mexico, and their virulence towards thrips and whiteflies. *Journal of Insect Science,* 11:1 available online: insectscience.org/11.1.

Sanjuan, T., Henao, L.G., & Amat, G. (2001). Spatial distribution of *Cordyceps* spp. (Ascomycotina: Clavicipitaceae) and its impact on the ants in forests of the Amazonia Colombian foothill. *Rev. Biol. Tropic*., 49, 945–955.

Santos, T.S., Passos, E.Md,, Seabra, M.GdJ., Souto, E.B., Severino, P., & Mendonça, M.Dc. (2021). Entomopathogenic Fungi Biomass Production and Extracellular Biosynthesis of Silver Nanoparticles for Bioinsecticide Action. *Appl. Sci.,* 11, 2465,. https://doi.org/ 10.3390/app11062465

Saccardo, P. A. (1882-1931, 1972). Sylloge fungorum. 26 vols. P. A. Saccardo, Pavia, Italy.

Sadananda, T.S., Govindappa, M., Ramachandra, Y.L., Chandrappa, C.P., & T. Umashankar, T. (2016). "In vitro apoptotic activity of endophytic fungal lectin isolated from endophyte, *Aspergillus flavus* of *Viscum album* on human breast adenocarcinoma cell line (MCF-7)," *Metabolomics*, vol. 6.

Savill, J., & Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature,* 407, 784–8.

St Leger, R.J. (1991). Integuement as a barrier to microbial infections. In *Physiology of the insect epidermis* (eds. K. Binnington & A. Retnakaran) pp. 285-306. CSIRO: Canberra.

St. Leger, R.J., & Wang, C. (2010). Genetic engineering of fungal biocontrol agents to achieve efficacy against insect pests. *Applied Microbiology and Biotechnology,* 85:901-907.

Sakakura, A., Suzuki, K., Katsuzaki, H., Komiya, T., Imamura, T., Aizono, Y., & Imai, K. (2005). Hanasanagin: a new antioxidative pseudo-di-peptide, 3,4-diguanidinobutanoyl-DOPA, from the mushroom, *Isaria japonica. Tetrahedron Letetrs,* 46, 9057–9059. doi:10.1016/j.tetlet.2005.10.100

Samish, M., Gindin, G., Alekseev, E., & Glazer, I. (2001). Pathogenicity of entomopathogenic fungi to different developmental stages of *Rhipicephalus sanguineus* (Acari: Ixodidae). *J Parasitol* 87, 1355–1339.

Samson, R.A., & Evans, H.C. (1973). Notes on entomogenous fungi from Ghana: I. the genera *Gibellula* and *Pseudogibellula. Acta Botanica Neerlandica,* 522-528.

Samson, R.A. (1974). *Paecilomyces* and some allied Hyphomycetes. *Studies in Mycology,* 6, 1-119.

Samson, R.A., & Evans, H.C. (1975). Notes on entomogenous fungi from Ghana III. The genus *Hymenostilbe. Proceedings of the Koninklijke Nederlandse Akademie Van Wetenschappen Series C-Biological and Medical Sciences,* 78, 73-80.

Samson, R & Evans, H. (1977). Notes on entomogenous fungi from Ghana IV. The genera *Paecilomyces* and *Nomuraea. Proceedings of the Koninklijke Nederlandse Akademie Van Wetenschappen Series C-Biological and Medical Sciences* 80, 127-134.

Samson, R.A., McCoy, C., & O'Donnell, K. (1980). Taxonomy of the acarine parasite *Hirsutella thompsonii. Mycologia,* 72, 359-377.

Samson, RA., Evans, H.C., & Latge, J.P. (1988). *Atlas of entomopathogenic fimgi.* SpringerVerlag: Berlin.

Samson, R.A. (1995). Constraints associated with taxonomy of biocontrol fungi. *Canadian Journal of Botany,* 73, 83-88.

Sandhu, S. S., Sharma, A. K., Beniwal, V., Goel, G. B. P., Kumar, A., Jaglan, S., Sharma, A. K., & Malhotra, S. (2012). Myco-biocontrol of insect pests: Factors involved, mechanism, and regulation. *Journal of Pathogens*, 1-11. https://doi.org/10.1155/2012/126819.

Schmidt, K., Gunther, W., Stoyanova, S., Schubert, B., Li, Z.Z., & Hamburger, M. (2002). Militarinone a, a neurotrophic pyridone alkaloid from *Paecilomyces militaris*. *Organic Letters,* 4, 197–199. doi: 10.1021/ol016920j.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque et al. (2012). Fungal Barcoding Consortium; Fungal Barcoding Consortium Author List. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Science,* U S A. 17;109(16):6241-6. doi: 10.1073/pnas.1117018109.

Seong, K.E., Young, S.K., Chong, K.L., & Seong, S.H. (2000). Possible mode of antiviral activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes simplex viruses. *Journal of Ethnopharmacol*ogy, 72, 475–481.

Service, R.F. (1995). Antibiotics that resist resistance. *Science*, 270, 724–727.

Sharma, S. (2004). Trade of Cordyceps sinensis from high altitudes of the Indian Himalaya: conservation and biotechnological priorities. *Current Science,* 86, 1–12.

Sharma, S. K., Gautam, N., & Atri, N. S. (2015). Optimized extraction, composition, antioxidant and antimicrobial activities of exo and intracellular polysaccharides from submerged culture of *Cordyceps cicadae*. *BMC complementary and alternative medicine*, 15, 446.

https://doi.org/10.1186/s12906-015-0967-y (Retraction published BMC Complement Altern Med. 2018 Oct 16;18(1):276)

Sharma, S.K. (2015). Optimized Extraction and Antioxidant Activities of Polysaccharides from Two Entomogenous Fungi. *Journal of Bioanalysis and Biomedicine,* 180-187. doi:10.4172/1948-593X.1000141.

Sharma, V.P., Kumari, B., Barh, A., Kamal, S., Kashyap, R., & Annepu, S.K. (2021). Biochemical Profiling and Cultivation of Medicinal Fungus *Isaria cicadae* (Ascomycetes) from India. *Int J Med Mushrooms,* 23, 91-101. doi: 10.1615/IntJMedMushrooms.2021037856.

Shin, K.H., Lim, S.S., Lee, S.H., Lee, Y.S., & Cho, S.Y. (2001). Antioxidant and immunostimulating activities of the fruiting bodies of *Paecilomyces japonica*, a new type of *Cordyceps* sp. *Ann. N. Y. Acad. Sci.,* 928, 261–273. doi: 10.1111/j.1749-6632.2001.tb05655.x.

Shin, K. H., Lim, S. S., Lee, S., Lee, Y. S., Jung, S. H., & Cho, S. Y. (2003). Anti-tumour and immuno-stimulating activities of the fruiting bodies of *Paecilomyces japonica*, a new type of *Cordyceps* spp. *Phytotherapy Research*, 17, 830–833. doi:10.1002/ptr.1253.

Shim, J-S., Chang, H.R., Min, E-G., Kim, Y-H., Y., & Han, Y.H. (2001). Cytotoxicity of *Paecilomyces tenuipes* Against Human Carcinoma Cells, HepG2 and MCF-7 *In Vitro*, Mycobiology, 29, 170-172. DOI: 10.1080/12298093.2001.12015781

Shim, S. M., Lee, K. L., Im, K. H., Lee, U. Y., Lee, M. W. & Lee, T. S. (2003). The characteristics of cultural conditions for the mycelial growth and fruiting body formation of *Paecilomyces sinclairii*. *Korean Journal of Mycology*, 31, 8-13

Shim, J-S., Chang, H.R., Min, E-G., Kim, Y-H., Y., & Han, Y.H. (2001). Cytotoxicity of *Paecilomyces tenuipes* Against Human Carcinoma Cells, HepG2 and MCF-7 *In Vitro*, Mycobiology, 29, 170-172. DOI: 10.1080/12298093.2001.12015781

Shin, K. H., Lim, S. S., Lee, S., Lee, Y. S., Jung, S. H., & Cho, S. Y. (2003). Anti-tumour and immuno-stimulating activities of the fruiting bodies of *Paecilomyces japonica*, a new type of *Cordyceps* spp. *Phytotherapy Research*, 17, 830–833. doi:10.1002/ptr.1253.

Shin, J-C., Shrestha, B., Lee, W-H., Park, Y-J., Kim, S-Y., Jeong, G-R. et al. (2004). Distribution and favourable conditions for mycelial growth of *Cordyceps pruinosa* in Korea. *The Korean Journal of Mycology*, 32 No. 02. P. 79-88.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry*, 40, 945–948.

Shimazu, M. (2001). *Paecilomyces cateniannulatus,* a commonly found, but an unrecorded entomogenous fungus in Japan. *Applied Entomology and Zoology* 36, 283-288.

Shimazu, M., & Takatsuka, J. (2010). *Isaria javanica* (anamorphic Cordycipitaceae) isolated from gypsy mothlarvae, *Lymantria dispar* (Lepidoptera: Lymantriidae), in Japan. *Appl. Entomol. Zool.,* 45, 497–504.

Shrestha, B., Lee, W-H., Han, S-K., & Sung, J-M. (2006). Observations on Some of the Mycelial Growth and Pigmentation Characteristics of *Cordyceps militaris* Isolates. *Mycobiology*, 34, 83-91.

Shrestha, B., Zhang, W.M., Zhang, Y.J., et al, (2010). What is the Chinese caterpillar fungus *Ophiocordyceps sinensis* (Ophiocordycipitaceae)? *Mycology,* 1, 228–236.

Shrestha, B. (2011). Diversity of Cordyceps Fungi in Nepal. *Nepal Journal of Science and Technology*, *12*, 103-110. https://doi.org/10.3126/njst.v12i0.6487

Shrestha, B., Tanaka, E., Hyun, M.W., Han, J.G., Kim, C.S., Jo, J.W. et al. (2016). Coleopteran and Lepidopteran hosts of the entomopathogenic genus *Cordyceps* sensu lato. *Journal of Mycology,* e7648219. https://doi.org/10.1155/2016/7648219

Sieber, T.N., Petrini, O., & Greenacre, M.J. (1998). Correspondence analysis as a tool in fungal taxonomy. *Systematic and Applied Microbiology,* 21, 433-441. https://doi.org/10.1016/S0723-2020(98)80053-2

Seifert, K.A., & Samuels, G.J. (2000). 'How should we look at anamorphs?' Studies of *Mycology*, 45, 5-18.

Seifert, K. A., & Gams, W. (2011). The genera of Hyphomycetes - 2011 update. *Persoonia*, *27*, 119–129. https://doi.org/10.3767/003158511X617435.

Senanayake, I.C., Rathnayaka, A.R., Marasinghe, D.S., Calabon, M.S., Gentekaki, E., Lee, H.B. et al. (2020). Morphological approaches in studying fungi: collection, examination, isolation, sporulation and preservation. *Mycosphere,* 11, 2678–2754, www.mycosphere.org

Silva, Dilani D., Rapior, S., Sudarman, Enge; Stadler, M., Xu, J. et al. (2013). Bioactive metabolites from macrofungi: ethnopharmacology, biological activities and chemistry. *Fungal Diversity,* 62, 1–40. doi:10.1007/s13225-013-0265-2.

Singleton, V.L., & Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J EnolVitic.,* 16, 144–58.

Slawik M, &Vidal-Puig AJ. (2006). Lipotoxicity, overnutrition and energy metabolism in aging. *Ageing Res Rev*., 5, 144-64. doi: 10.1016/j.arr.2006.03.004.

Smith, D., & Onions, A. H. S. (1983). "The Preservation and Maintenance of Living Fungi." Commonwealth Mycological Institute, Kew, UK.

Smith, P. (1993). Control of *Bemisia tabaci* and the Potential of *Paecilomyces fumosoroseus* as a Biopesticide. *Biocontrol News and Information*, 14, 71-78.

Smith, D, & Kolkowski, J. (1996). Chapter 6 - Fungi, Editor(s): Jennie C. Hunter-Cevera, Angela Belt, Maintaining Cultures for Biotechnology and Industry,

Academic Press, 101-132, ISBN 9780123619464, https://doi.org/10.1016/B978-012361946-4/50007-7.

Snelson, J.T. (1975). Animal ectoparasites and disease vector causing major reduction in world food supplies. *FAO Plant Prot. Bull*., 13, 103–114.

Song, T.Y., & Yen, G.C. (2002). Antioxidant properties of *Antrodia camphorata* in submerged culture. *J Agric Food Chem.,* 22;50(11):3322-7. doi: 10.1021/jf011671z.

Sosa-Gómez, D.R., & Alves, S.B. (2000). Temperature and Relative Humidity Requirements for Conidiogenesis of *Beauveria bassiana* (Deuteromycetes: Moniliaceae). *An. Soc. Entomol. Brasil,* 29(3).

Sosa-Gómez, D.R., Humber, R.A., Hodge, K.T., Binneck, E. et al., (2009). Variability of the mitochondrial SSU rDNA of *Nomuraea* species and other entomopathogenic fungi from Hypocreales. *Mycopathologia,* 167, 145–154.

Sofowara, A. (1993). Medicinal Plants and Traditional Medicine in Africa, Spectrum Books, Ibadan, Nigeria.

Souza, R.K.F., Azevedo, R.F.F., Lobo, A.O., & Rangel, D.E.N. (2014). Conidial water affinity is an important characteristic for thermotolerance in entomopathogenic fungi. *Biocontrol Science and Technology,* 24, 448-461, DOI: 10.1080/09583157.2013.871223

Spatafora, J.W., & Blackwell. M. (1993). Molecular systematics of unitunicate perithecial ascomycetes: the Clavicipitales-Hypocreales connection. *Mycologia,* 85, 912- 922.

Strader, C.R., Pearce, C.J., Oberlies, N.H. (2011). Fingolimod (FTY720): a recently approved multiple sclerosis drug based on a fungal secondary metabolite. *J Nat Prod.*, 74, 900–907.

Stauderman, K., Avery, P., Aristizábal, L., & Arthurs, S. (2012). Evaluation of *Isaria fumosorosea* (Hypocreales: Cordycipitaceae) for control of the Asian citrus

psyllid, *Diaphorina citri* (Hemiptera: Psyllidae). *Biocontrol Science and Technology*, 22, 747-761.

Steinhaus, E.A. (1956). Microbial control—The emergence of an idea: A brief history of insect pathology through the nineteenth century. *Hilgardia,* 26, 107–160.

Steinhaus, E. (1975). *Disease in a minor chord*. Ohio State University Press: Columbus.

Steiniger, C., Hoffmann, S., Mainz, A., Kaiser, M., Voigt, K., Meyer, V., et al. (2017). Harnessing fungal nonribosomal cyclodepsipeptide synthetases for mechanistic insights and tailored engineering. *Chem. Sci.,* 8, 7834–7843. doi: 10.1039/C7SC03093B.

Styers, D., Sheehan, D.J., Hogan, P., & Sahm, D.F. (2006). Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. *Ann Clin Microb Antimicrob.,* 5:2.

Subramanian, C.V., & Ramakrishnan, K. (1958). List of Indian Fungi 1952-56. Madras University. B26 327-421

Subramanian, C.V., & Tyagi, P.D.(1964). List of Indian fungi 1956-1960. J. Madras Univ. B24 1-134.

Subramanian, C.V. (1971). Hyphomycetes, ICAR, New Delhi. pp 930

Subramanian, C.V. (1992). Tropical mycology and biotechnology. *Current Science,* 63, 167–172.

Sun, Y-F., Sun, Y., Wang, Z., Han, R., Lu, H., Zhang, J. et al. (2017). *Isaria cicadae* conidia possess antiproliferative and inducing apoptosis properties in gynaecological carcinoma cells, *Mycology*, DOI: 10.1080/21501203.2017.1386243

Sung, G. H., Hywel-Jones, N. L., Sung, J. M., Luangsa-Ard, J. J., Shrestha, B., & Spatafora, J. W. (2007). Phylogenetic classification of *Cordyceps* and the

clavicipitaceous fungi. *Studies in mycology*, 57, 5–59. https://doi.org/10.3114/sim.2007.57.01

Sung, G-H., Shrestha, B., Han, S-K Kim, S-Y & Sung, J.M. (2010). Growth and Cultural Characteristics of *Cordyceps cardinalis* Collected from Korea. *Mycobiology,* 38, 274-281 DOI:10.4489/MYCO.2010.38.4.274

Sung, Gi-Ho., Shrestha, B., Han, S-K., & Sung, J-M. (2011). Growth and Cultural Characteristics of *Ophiocordyceps longissima* Collected in Korea, *Mycobiology*, 39, 85-91.

Suganya, T., & Selvanarayanan, V. (2010). In vitro study on the effect of bhendi varieties on the infectivity of *Beauveria bassiana* (Bals.) Vuill to *Spodoptera litura* Fab. J Biopest 3, 369–372.

Sutton, B. (1996). Conidiogenesis, classification and correlation. In a *century of mycology* (ed.B. Sutton) pp. 137-160. Cambridge University Press: Port Chester.

Suzuki, K., Tsushima, M., & Goryo, M., et al., (2017). Neuro-protective properties of the fungus Isaria japonica: Evidence from a mouse model of aged-related degeneration. In: Au Rahman, editor. *Frontiers in Clinical Drug Research–Alzheimer Disorders*, Bentham Science Publishers, 6, 154–186. https://doi.org/10.2174/ 9781681083391117060009.

Taber, W.A. & Vining, L.C. (1939). Studies on *Isaria cretacea*: Nutritional and morphological characteristics of two strains and morphogenesis of the synnema. pp. 513-539.

Talib, W.H., & Mahasheh, A.M. (2010). "Antiproliferative activity of plant extracts used against cancer in traditional medicine," *Scientia Pharmaceutica*,. 78, 33–45.

Takano, F., Kikuchi, Y., Fushiya, S., Hojo, N., Nozoe, S., & Yahagi, N. (1996). The culture fluid of *Isaria japonica* Yasuda augments anti-sheep red blood cell antibody response in mice. *Biol Pharm Bull.,* 19, 641–643.

Takano, F., Yahagi, N., Yahagi, R., Takada, S., & Yamaguchi, M., et al., (2005), The liquid culture filtrates of *Paecilomyces tenuipes* (Peck) Samson (= *Isaria japonica* Yasuda) and *Paecilomyces cicadae* (Miquel) Samson (=*Isaria sinclairii* (Berk.) Llond) regulate Th1 and Th2 cytokine response in murine Peyer's patch cells *in vitro* and *ex vivo*. *Int Immunopharmacol.,* 5, 903–916.

Tam, P.C.F. (1995). Heavy metal tolerance by ectomycorrhizal fungi and metal amelioration by *Pisolithus tinctorius*. *Mycorrhiza,* 5, 181–187.

Tandon, R.N., & Chandra, S. (1964). Second supplement to the list of Indian fungi 1957-1962; University of Allahabad Studies, Allahabad

Tang, J., Qian, Z., & Wu, H. (2018). Enhancing cordycepin production in liquid static cultivation of *Cordyceps militaris* by adding vegetable oils as the secondary carbon source. *Bioresour Technol*., 268, 60-67. doi: 10.1016/j.biortech.2018.07.128.

Tao, Y. W., Lin, Y. C., She, Z. G., Lin, M. T., Chen, P. X., & Zhang, J. Y. (2015). Anticancer activity and mechanism investigation of beauvericin isolated from secondary metabolites of the mangrove endophytic fungi. *Anticancer Agents Med. Chem.,* 15, 258–266. doi: 10.2174/1871520614666140825112255.

Tandon, R.N., & Bilgrami, K.S. (1957). Assimilation of disaccharides by some fungi causing "leaf spot" diseases. *Proceedings of the Indian Academy of Sciences - Section B* 46, 274–284. https://doi.org/10.1007/BF03052455.

Terayama, Y., Ohtsuka, C., & Suzuki, K. (2016). The effect *of Isaria japonica* powder produced by the silkworm on higher cortical function among patients with Alzheimer's dementia (Japanese with English abstract*). Journal of Iwate Medical Association*, 68, 223–227.

Tescari, G. (2001). *Leptoglossus occidentalis*, coreide neartico rinvenuto in Italia – (Heteroptera, Coreidae) [*Leptoglossus occidentalis*, nearctic coreid found in Italy - (Heteroptera, Coreidae)]. *Soc. veneziana Sci. natur., Lavori*, 26, 3–5.

Tevini, M. (1993). Moecular biological effects of ultraviolet radiation. In: M. Tevini (Ed.), UV-B radiation and Ozone Depletion (pp.1—15). Lewis Publishers, Boca Raton, FL.

Thakre, M., Thakur, M, Malik, N., & Ganger, S. (2011). Mass scale cultivation of entomopathogenic fungus *Nomuraea rileyi* using agricultural products and agro wastes. *Journal of Biopesticides*, 4, 176-179.

Thakur, R., & Sandhu, S. S. (2010). Distribution, occurrence and natural invertebrate hosts of indigenous entomopathogenic fungi of Central India. *Indian journal of microbiology*, 50, 89–96. https://doi.org/10.1007/s12088-010-0007-z.

Thatoi, H., & Singdevsachan, S.K. (2014). Diversity, nutritional composition and medicinal potential of Indian mushrooms: A review. *African Journal of Biotechnology,* 13, 523-545. http://dx.doi.org/10.5897/AJB2013.13446.

Tilak, T., & Rao, R. (1968). Second supplement to the fungi of India (1962-67).

Tigano-Milani, M.S., Honeycutt, R.J., Lacey, L.A., Assis, R. et al., (1995). Genetic variability of *Paecilomyces fumosoroseus* isolates revealed by molecular markers. J. Invert. Pathol., 65, 274–282.

Toman, E., Makrlík, E., & Vanura, P. (2011). On the complexation of the sodium cation with beauvericin: experimental and theoretical study. *Monatsh. Chem.* 142, 779–782. doi: 10.1007/s00706-011-0499-1.

Trease, G.E., & Evans, W.C, (1989).Pharmacognsy(13th ed) Baillere.Tindall London pp176-180.

Tsai, S.Y. (2002). Antioxidant properties and their cytotoxic activities on tumour cells of *Ganoderma tsugae* and *Agrocybe cylindracea* and antimutagenic properties of *Agrocybe cylindracea* [MSc thesis]. [Taichung (Taiwan)]: National ChungHsing University.

Tsushima, M., Yamamoto, K., Goryo, M., Suzuki, F., & Suzuki, K. (2010). Hot-water extract of *Paecilomyces tenuipes* from the silkworm pupae improves D-

galactose-induced brain aging in mice. *J Insect Biotechnol Sericology*, 79, 45–51. https://doi.org/10.11416/jibs.79.2_045

Tuan, N.N., Ngan, N.T., Kuo, P.C., Hung, H.Y., Lan, T.N., Thanh, N.T. et al. (2017). Characterization of Cyclodepsipeptides from the Mycelium of *Isaria japonica* from Vietnam. *Nat Prod Commun.,* 12, 377-378.

Tulloch, M. (1976). The genus *Metarhizium. Transactions of the British Mycological Society,* 66, 407-411.

Ueda, H., Takahara, S., Itoh, S., Nomi, H., Shibahara, N. & Katsuoka, Y. (2005). Preoperative administration of FTY720 prolonged renal allograft survival. *Transplant Immunology*, 14, 1–8.

Um, M., Galadima, I.B., Gambo, F.M., & Zakaria, D. (2018). "A Review on the Use of Entomopathogenic Fungi in the Management of Insect Pests of Field Crops." *Journal of Entomology and Zoology Studies,* 6, 27–32.

van Engeland, M., Nieland, LJ., Ramaekers, F.C., Schutte, B., & Reutelingsperger, C.P. (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry*, 1, 1-9.

Vamanu, E. (2012). Determination of antioxidant and antimicrobial properties of *Agaricus bisporus* from Romanian market. *Ovidius University Annals of Chemistry,* 23, 47–52.

Vasudeva, R.S. (1960). The Fungi of India, Revised ICAR New Delhi 9 552.

Vega, F.F., Meyling, N., Luangsa-ard, J., & Blackwell, M. (2012). Fungal Entomopathogens. *Insect Pathology,* 171-220.

Venkatachalam, P, & Nadumane, V.K. (2021). Modulation of Bax and Bcl-2 genes by secondary metabolites produced by *Penicillium rubens* JGIPR9 causes the apoptosis of cancer cell lines, *Mycology*, 12, 69-81, DOI: 10.1080/21501203.2019.1707315.

Vermes, I., Haanen, C., & Reutelingsperger, C. (2000). Flow cytometry of apoptotic cell death. *J Immunol Methods*, 21, 167-90. doi: 10.1016/s0022-1759(00)00233-7.

Vidal, C., Fargues, J., & Lacey, A. (1997). Intraspecific variability of Paecilomyces fumosoroseus: effect of temperature on vegetative growth. *J Invertebr Pathol.,* 70:18–26. doi: 10.1006/jipa.1997.4658.

Vilcinskas, A., Jegorov, A., Landa, Z., Götz, P., & Matha, V. (1999). Effects of beauverolide L and cyclosporin A on humoral and cellular immune response of the greater wax moth, Galleria mellonella. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.,* 122, 83-92. doi: 10.1016/s0742-8413(98)10082-8.

Vishwakarma, P., Singh, P & Tripathi, N.N. (2017). *In-vitro* antioxidant activity and nutritional value of four wild oyster mushroom collected from North-Eastern Part of Uttar Pradesh. *Mycosphere*, 8, 592–602. Doi 10.5943/mycosphere/8/4/8

Wang, Y. (1987). Mycology in ancient China. *The Mycologist,* 1, 59-61.

Wang, H.X., Liu, W.K., Ng, T.B., Ooi, V.E.C., & Chang, S.T. (1995). Immunomodulatory and antitumor activities of a polysaccharide-peptide complex from a mycelial culture of *Tricholoma* sp., a local edible mushroom. *Life Sci.*, *57,* 269–281.

Wang, P., Jiang, X., Jiang, Y., Hu, X & Hwang, H. (2008). Optimization of Fermentation Medium and Conditions for Mycelial Growth and Water-soluble Exopolysaccharides Production by *Isaria farinosa* B05. *Preparative Biochemistry and Biotechnology*, 38, 294-307, DOI: 10.1080/10826060802165121

Wang, Q & Xu, L. (2012). Beauvericin, a Bioactive Compound Produced by Fungi: A Short Review. *Molecules,* 17, 2367-2377; doi:10.3390/molecules17032367

Wang, Y., Wang, P., Ma, H., & Zhu, W. (2013). Developments around the bioactive diketopiperazines: a patent review. *Expert Opin Ther Pat.,* 23, 1415–1433.

Wang, J., Zhang, D.M., Jia, J.F., Peng, Q.L., Tian, H.Y., Wang, L., & Ye, W.C. (2014). Cyclodepsipeptides from the ascocarps and insect-body portions of fungus *Cordyceps cicadae*. *Fitoterapia,* 97, 23-7. doi: 10.1016/j.fitote.2014.05.010.

Wang, J.B., St. Leger, R.J., & Wang, C. (2016). Chapter Three - Advances in Genomics of Entomopathogenic Fungi, Editor(s): Brian Lovett, Raymond J. St. Leger, *Advances in Genetics,* Academic Press, Volume 94, Pages 67-105, ISSN 0065-2660, ISBN 9780128046944, https://doi.org/10.1016/bs.adgen.2016.01.002.

Wang, Z., Wu, X., Liang, Y.N., Wang, L., Song, Z.X., Liu, J.L., Tang, Z.S. (2016). Cordycepin Induces Apoptosis and Inhibits Proliferation of Human Lung Cancer Cell Line H1975 via Inhibiting the Phosphorylation of EGFR. *Molecules*, 27, 1267. doi: 10.3390/molecules21101267.

Wang, X., Gong, X., Li, P., Lai, D., & Zhou, L. (2018). Structural Diversity and Biological Activities of Cyclic Depsipeptides from Fungi. *Molecules,* 15, 169. doi: 10.3390/molecules23010169.

Wang, H., Yang, Z., Chen, K., Yu, R., Xu, L., & Lv G. (2022). *Isaria cicadae* Miquel prevents intestinal fibrosis by activating transforming growth factor-β1 signaling to regulate the balance between matrix metalloproteinases and tissue inhibitors of metalloproteinase 1 in mice with Crohn's disease. *Journal of Functional Foods,* 88, 1-10.

Wasser, S. P., & Weis, A. L. (1999). Medicinal properties of substances occurring in higher basidiomycetes mushroom: current perspective. *International Journal of Medicinal Mushrooms,* 1, 31–51.

Wasser, S.P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology,* 60, 258–274.

Wattel, J. (1990). Taxonomy as a critical science. *Bijdr. Dierk*. 60, 335-339.

Wätjen, W., Debbab, A., Hohlfeld, A., Chovolou, Y., & Proksch, P. (2014). The mycotoxin beauvericin induces apoptotic cell death in H4IIE hepatoma cells accompanied by an inhibition of NF-kB-activity and modulation of MAPkinases. *Toxicol. Lett.* 231, 9–16. doi: 10.1016/j.toxlet.2014.08.021

Wen, T.C., Li, G.R., Kang, J.C., Kang, C., & Hyde, K.D. (2014). Optimization of Solid-state Fermentation for Fruiting Body Growth and Cordycepin Production by *Cordyceps militaris*. *Chiang Mai J. Sci*., 41, 858-872.

Weng, S.C., Chou, C.J., Lin, L.C., Tsai, W.J., & Kuo, Y.C. (2002). Immunomodulatory functions of extracts from the Chinese medicinal fungus *Cordyceps cicadae*. *Journal of Ethnopharmacology*, 83, 79-85. doi: 10.1016/s0378-8741(02)00212-x.

Weng, Q., Zhang, X., Chen, W., & Hu, Q. (2019). Secondary Metabolites and the Risks of *Isaria fumosorosea* and *Isaria farinosa*. *Molecules, 24*(4), 664. https://doi.org/10.3390/molecules24040664

Wheeler, A.G. (1992). *Leptoglossus occidentalis*, a new conifer pest and household nuisance in Pennsylvania. *Penn. Dep. Agric. Bull.,* 18, 29–30.

White, T.J., Bruns, T., Lee, S.J., Taylor, J.L. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protoc.,* 18, 315–22.

Wong, J.H., Ng, T.B., Chan, H.H.L., Liu, Q., Man, G.C.W., Zhang, C.Z. et al., (2020). Mush room extracts and compounds with suppressive action on breast cancer: Evidence from studies using cultured cancer cells, tumor bearing animals, and clinical trials. *Appl. Microbiol. Biotechnology,* 104, 4675–4703, doi:10.1007/s00253 020 10476 4.

Wosten, H.A.B., & de Vocht, M.L. (2000). Hydrophobins, the fungal coat unravelled. *Biochimica et Biophysica Acta,* 1469, 79–86.

Wu, Y. L., Sun, C. R., & Pan, Y. J. (2005). Structural analysis of a neutral (1 - 3), (1 - 4)-β-D-glucan from the mycelia of *Cordyceps sinensis*. *Journal of Natural Products,* 68, 812–814.

Wu, Q., Patocka, J., Nepovimova, E., & Kuca, K. (2018). A Review on the Synthesis and Bioactivity Aspects of Beauvericin, a *Fusarium* Mycotoxin. *Front. Pharmacol*., 9, 1338. doi: 10.3389/fphar.2018.01338

Wu, S., Toews, M.D., Oliveira-Hofman, C., Behle, R.W., Simmons, A.M., & Shapiro-Ilan, D.I. (2020). Environmental Tolerance of Entomopathogenic Fungi: A New Strain of *Cordyceps javanica* Isolated from a Whitefly Epizootic Versus Commercial Fungal Strains. *Insects,* 11, 711. doi:10.3390/insects11100711.

Wyllie, A.H. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol.,* 68:461–63.

Xiao, D-M., Yu, S., & Xiao, J-H. (2016). Antioxidant activities of alkali-soluble polysaccharides from medicinal mushroom *Cordyceps taii* and its chemical characteristics. *Biomedical Research*, 27, 199-206.

Xie, J-W., L-F, Huang W., Hu, Y-B., He, K.P., & Wong. (2010). Analysis of the Main Nucleosides in *Cordyceps sinensis* by LC/ ESI-MS. *Molecules*, 15, 305–314.

Xu, Y., Orozco, R.,Wijeratne, E. M., Gunatilaka, A. A., Stock, S. P., & Molnár, I. (2008). Biosynthesis of the cyclooligomer depsipeptide beauvericin, a virulence factor of the entomopathogenic fungus *Beauveria bassiana. Chem. Biol*. 15, 898–907. doi: 10.1016/j.chembiol.2008.07.011

Xu, L., Liu, Y., Zhou, L., & Wu, J. (2011). Modeling of *Fusarium redolens* Dzf2 mycelial growth kinetics and optimal fed-batch fermentation for beauvericin production. *J. Ind. Microbiol. Biot.*, 38, 1187–192.

Xuewei Ye, Weiyun Chai, Xiao-Yuan Lian & Zhizhen Zhang (2016): Novel propanamide analogue and antiproliferative diketopiperazines from mangrove *Streptomyces* sp. Q24, *Natural Product Research,* 1-7. DOI: 10.1080/14786419.2016.1253079

Yahagi, N., Komatsu, M., Hiramatsu, M., Shi, H., Yahagi, R., & Kobayashi, H. (1999). Radical scavenging activities of condensed culture medium of *Isaria*

japonica Yasuda and hot water extract of *Fomes fomentarius* (L.: Fr) Kicrx., *Nat. Med. J.,* 53, 319-323.

Yahagi, H., Yahagi, T., Furukawa, M., & Matsuzaki, K. (2020). Antiproliferative and Antimigration Activities of Beauvericin Isolated from *Isaria* sp. on Pancreatic Cancer Cells. *Molecules,* 8, 4586. doi: 10.3390/molecules25194586.

Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J.(1998). HPLC Method for Evaluation of the Free Radical-scavenging Activity of Foods by Using 1,1-Diphenyl-2-picrylhydrazyl. *Biosci. Biotechnol. Biochem*., 62, 1201–1204.

Yamanaka, K., Inatomi, S., & Hanaoka, M. (1998). Cultivation characteristics of *Isaria japonica*. *Mycoscience,* 39, 43-48, DOI: 10.1007/BF02461577.

Yamanaka, K. & Inatomi, S. (1997). Cultivation of *Isaria japonica* fruit bodies on mixed plant/insect media. *Food Rev. Int*., 13, 455-460.

Yahr, R., Schoch, C. L., & Dentinger, B. T. (2016). Scaling up discovery of hidden diversity in fungi: impacts of barcoding approaches. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *371*(1702), 20150336. https://doi.org/10.1098/rstb.2015.0336

Yan, J.K., Wang, W.Q., & Wu, J.Y. (2014). Recent advances in *Cordyceps sinensis* polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. *J Funct Foods,* 6, 33-47. doi: 10.1016/j.jff.2013.11.024.

Yang, J. Y., Zhang, W. Y., Shi, P. H., Chen, J. P., Han, X. D., &Wang, Y. (2005). Effects of exopolysaccharide fraction (EPSF) from a cultivated *Cordyceps sinensis* fungus on c-Myc, c-Fos, and VEGF expression in B16 melanoma-bearing mice. *Pathology, Research and Practice,* 201, 745–750.

Yang, S.,Jin, L., Ren, X., Lu, J., & Meng, Q. (2014). Optimization of fermentation process of *Cordyceps militaris* and antitumor activities of polysaccharides *in vitro*. *Journal of Food and Drug Analysis,* 22, 468–476. doi:10.1016/j.jfda.2014.01.028.

Yang, J., Dong, H., Wang, Y., Jiang, Y., Zhang, W., Lu, Y. et al. (2020). *Cordyceps cicadae* polysaccharides ameliorated renal interstitial fibrosis in diabetic nephropathy rats by repressing inflammation and modulating gut microbiota dysbiosis. *International Journal of Biological Macromolecules,* 163, 442–456. https:// doi.org/10.1016/j.ijbiomac.2020.06.153.

Yang, R.H., Wang, X.L., Wang, K., Hsiang, T., Wang, W.J., Wu, H.J. et al. (2021). Deep Sequencing, Nested PCR, and Denaturing Gradient Gel Electrophoresis Reveal a Wider Distribution of Chinese Caterpillar Mushroom, *Ophiocordyceps sinensis* (Ascomycetes), in Native Soil Types. *Int J Med Mushrooms,* 23, 93-104. doi: 10.1615/IntJMedMushrooms.2021037759. PMID: 33822511.

Yang, X., Chen, L., Zhao, L., Yang, Y., Wang, J., Yan, L., & Zhang, H. (2021). *Cordyceps sinensis*-derived fungus *Isaria felina* ameliorates experimental autoimmune thyroiditis in mice. *Biomedicine & Pharmacotherapy,* 140, 111733. doi:10.1016/j.biopha.2021.111733.

Yanagita, I., & Kogane, F. (1963). Cytochemical and physiological differentiation of mould pellets. *Journal of General and Applied Microbiology,* 9, 171-87.

Yokoyama, E., Arakawa, M., Yamagishi, K., & Hara, A. (2006). Phylogenetic and structural analyses of the mating-type loci in Clavicipitaceae. *FEMS Microbiol. Lett.,* 264, 182– 191.

Young, A.M. (2005). A Field Guide to the Fungi of Australia. UNSW Press, Sydney.

Yue, K., Ye, M., Zhou, Z., Sun, W., & Lin, X. (2013). The genus *Cordyceps*: a chemical and pharmacological review. *J Pharm Pharmacology*, 65, 474-93. doi: 10.1111/j.2042-7158.2012.01601.x.

Zeng, Z., Mou, D., Luo, L., Zhong, W., Duan, L., & Zou. X. (2021). Different Cultivation Environments Affect the Yield, Bacterial Community and Metabolites of *Cordyceps cicadae*. *Front. Microbiol.,* 12, 669785. doi: 10.3389/fmicb.2021.669785

Zha, L-S., Xiao, Y-P., & Jeewon, R., et al., (2019). Notes on the Medicinal Mushroom Chanhua (*Cordyceps cicadae* (Miq.) Massee). *Chiang Mai Journal of Science,* 46, 1023-1035 http://epg.science.cmu.ac.th/ejournal/.

Zhan Y., Dong C.-H., & Yao Y.-J. (2006). Antioxidant activities of aqueous extract from cultivated fruit-bodies of *Cordyceps militaris in vitro*. *Journal of Integrative Biology,* 48, 1–5.

Zhan, J., Burns, A.M., Liu,M. X., Faeth, S. H., & Gunatilaka, A. A. (2007). Search for cell motility and angiogenesis inhibitors with potential anticancer activity: Beauvericin and other constituents of two endophytic strains of *Fusarium oxysporum*. *J. Nat. Prod.,* 70, 227–232. doi: 10.1021/np060394t

Zhang, M., Cui, S., Cheung, P., & Wang, Q. (2007). Antitumour polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumour activity. *Trends in Food Science & Technology*, 18, 4–19.

Zhang, N., Wang, J., Hu, F., Wang, X., Wang, X., Li, Z., & Fan, M. (2009). Isolation and purification of peroxy-ergosterol from *Paecilomyces fumosoroseus* by high-speed-counter-current chromatography and identification by ESI-MS. *Food Ferment. Ind.,* 35, 14–17.

Zhang, Y., Liu, S., Liu, H., Liu, X., & Che, Y. (2009). Cycloaspeptides F and G, Cyclic Pentapeptides from a *Cordyceps*-colonizing isolate of *Isaria farinosa*. *J Nat Prod*., 72, 1364-7. doi: 10.1021/np900205m.

Zhang, Y., Kong, H., Fang, Y., Nishinari, K, & Phillips, Glyn O. (2013). Schizophyllan: A review on its structure, properties, bioactivities and recent developments. *Bioactive Carbohydrates and Dietary Fibre,* 1, 53–71. doi:10.1016/j.bcdf.2013.01.002

Zhang, Y., Zhang, S., Li, Y., Ma, S., Wang, C., & Xiang, M., *et al.,* (2014). Phylogeography and evolution of a fungal-insect association on the Tibetan plateau. *Mol Ecol*., 23, 5337–55. https://doi.org/10.1111/mec.12940.

Zhang Y, Li Q, Shu Y, et al. (2015). Induction of apoptosis in S180 tumour bearing mice by polysaccharide from *Lentinus edodes* via mitochondria apoptotic pathway. *J Funct Foods,* 15:151–159.

Zhang, Q.Q., Liu, Y., Di, Z., Han, C.C., & Liu, Z. (2016). The Strategies for Increasing Cordycepin Production of *Cordyceps Militaris* by Liquid Fermentation. *Fungal Genomic and Biology*, 6, 1-5. DOI: 10.4172/2165-8056.1000134

Zhang, J., Meng, L.L., Wei, J.J., Fan, P., Liu, S.S., Yuan, W.Y. et al. (2017). PTP1B Inhibitors from the Entomogenous Fungi *Isaria fumosorosea*. *Molecules,* 24, 2058. doi: 10.3390/molecules22122058.

Zhang, X., Hu, Q., & Weng. Q (2019). Secondary metabolites (SMs) of *Isaria cicadae* and *Isaria tenuipes*. *RSC Adv*., 1, 172–184.

Zhang, J., Wen, C., Duan, Y., Zhang, H., & Ma, H. (2019). Advance in Cordyceps militaris (Linn) Link polysaccharides: Isolation, structure, and bioactivities: A review. *International Journal of Biological Macromolecules,* 132, 906–914. https://doi.org/10.1016/j.ijbiomac.2019.04.020.

Zhen, X., Lundborg C.S., Sun X., Hu X., & Dong H. (2019). Economic burden of antibiotic resistance in ESKAPE organisms: a systematic review. *Antimicrob. Resist. Infect. Contr.,* 8:137.

Zheng, L.P., Gao, L.W., Zhou, J.Q., Sima, Y.H., & Wang, J.W. (2008). Antioxidant activity of aqueous extract of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. *African Journal of Biotechnology,* 7, 3004-3010.

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food chemistry*, 64, 555–9.

Zhong, S., Pan, H.J., Fan, L.F., Lv, G., Wu, Y., Parmeswaran, B. et al. (2009). Advances in research of polysaccharides in *Cordyceps* species. *Food Technology and Biotechnology,* 47:304–312.

Zhong, X.K., Jin, X., Lai, F.Y., Lin, Q.S., & Jiang, J.G. (2010). Chemical analysis and antioxidant activities *in vitro* of polysaccharide extracted from *Ficus indica* Mill cultivated in China. *Carbohydrate Polymer,* 82, 722–7.

Zhou, K., Zhang, X., Zhang, F., & Li, Z. (2011). Phylogenetically diverse cultivable fungal community and polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS) genes associated with the South China Sea sponges. *Microbial Ecology, 62,* 644-54. doi: 10.1007/s00248-011-9859-y.

Zhou, Y., Mei, X., Li, Y., Yang, W., Su, X., & Hu, H. (2022). Cordycepin inhibits the proliferation and progression of NPC by targeting the MAPK/ERK and β-catenin pathways. *Oncology letters*, *23*(1), 20. https://doi.org/10.3892/ol.2021.13138

Zhu, J.S., Halpern, G.M., & Jones, K. (1998). The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis* Part I. *J Altern Complement Med.,* 4, 289–303.

Zimmermann, G. (1982). Effect of high temperature and artificial sunlight on the viability of conidia of *Metarhizium anisopliae*. *J Invertebr Pathol.,* 40, 36-40.

Zimmermann, G. (2007a). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii. Biocontrol Science and Technology,* 17, 553-596.

Zimmermann, G. (2007b). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae. Biocontrol Science and Technology,* 17, 879-920.

Zimmermann, G. (2008). The entomopathogenic fungi *Isaria farinosa* (formerly *Paecilomyces farinosus*) and the *Isaria fumosorosea* species complex (formerly *Paecilomyces fumosoroseus*): biology, ecology and use in biological control. *Biocontrol Science and Technology*, 18, 865-901, DOI: 10.1080/09583150802471812

Zobel, S., Boecker, S., Kulke, D., Heimbach, D., Meyer, V., & Süssmuth, R. D. (2016). Reprogramming the biosynthesis of cyclodepsipeptide synthetases to

obtain new enniatins and beauvericins. *Chembiochem.,* 17, 283–287. doi: 10.1002/cbic.201500649.

APPENDIX-A

List of publications

Chhetri, A., Pokhrel, Y.R., Shahi, N., Lama, D., Chhetri, D.R. (2019). Antioxidant and cytotoxic activities of *Isaria amoenerosea* Henn.: An entomopathogenic fungus from Darjeeling Hills, Eastern Himalaya. *Indian J Nat Prod Resource,* 10:111–118.

Chhetri, D.R., Chhetri, A., Shahi, N., Tiwari, S., Karna, S., Lama, D., & Pokharel, Y.R. (2020). *Isaria tenuipes* Peck, an entomopathogenic fungus from Darjeeling Himalaya: Evaluation of in-vitro antiproliferative and antioxidant potential of its mycelium extract. *BMC complementary medicine and therapies*, *20*(1), 185. https://doi.org/10.1186/s12906-020-02973-w.

Chhetri, D.R., & Chhetri, A. The genus *Isaria*: A review on its mycomedicinal potential and bioactive secondary metabolites. (Communicated, 2023).

Chhetri, D.R., & Chhetri, A. Antioxidant and antiproliferative properties of mycelia of entomopathogenic fungi *Isaria farinosa* and *Isaria fumosorosea* from Darjeeling hills. (Communicated, 2023).

APPENDIX-B:

List of conferences/seminars attended

a. National conference on biodiversity, environmental challenges and wildlife conservation (January, 24th and 25th, 2018). Organized by Government P.G. College Thana Gazi (Alwar), under the Auspicious of International Society for Life Sciences. Title of research abstract for oral presentation was: An *in vitro* study on antioxidant activities of *Isaria* SU01.

b. National conference on Recent Trends in Environmental Sustainability and Green Practices (RTESGP)-2019) November -15-16,2019, Organized by Dept. of Botany, Government College, Bundi Rajasthan in collaboration with The Society of Life Sciences. Title of research abstract for oral presentation was: An *invitro* antioxidant and cytotoxic activities of liquid static culture of *Isaria fumosorosea*.

National Conference on

Biodiversity, Environmental Challenges and Wildlife Conservation

January 24th & 25th, 2018

Organized by :

Govt. P.G. College, Thanagazi (Alwar)

under the auspices of :

International Society for Life Sciences (ISLS)

Certificate

This is to certify that Prof./ Dr./ Mr. /Ms. Abhijit Chhetri Research Scholar (Botany) Sikkim Uni, Tadong, Sikkim, India *has participated in the National Conference on 'Biodiversity, Environmental Challenges and Wildlife Conservation' as Chairperson / Invited Speaker / Oral Presenter / Poster Presenter / Delegate. The title of his/her research paper was* "An in Vitro study on antioxidant activities of Isaria SU01" *His/Her participation was highly appreciated.*

Dr. Sharwan Kumar	Dr. Subhash Yadav	Dr. Rajani Mathur	Dr. Jitendra Kumar Chawla & Dr. Punar Dutt Meena
Chairman	Co- Chairman	Convener	Organizing Secretaries

National Conference
on
Recent Trends in Environmental Sustainability and Green Practices
(RTESGP-2019)
November 15-16, 2019
CERTIFICATE
This is to certify that Prof. / Dr. / Ms. Abhijit Chhetri
of Sikkim University, Tadong, Sikkim
has participated in the National Conference organized by Department of Botany, Government College, Bundi (Rajasthan)
in collaboration with The Society of Life Sciences Satna (M.P.) as a Keynote Speaker / Chairperson / Invited Speaker / Oral
Paper Presenter / Poster Presenter / Delegate. The title of his / her research paper was An InVitro Antioxidant
And Cytotoxic Activities of Liquid Static Culture of Isaria
Fumosoroseа His / her particpation was highly appreciated.
Dilip K. Rathore
Organizing Secretary
S.M. Meena
Co-Ordinator
O.P. Sharma
Convener
Prahlad Dubey
Chairman
J.K. Jain
Patron
2004

Photo plate 1: Spontaneous specimens of *Isaria* spp., collected from different ecological region of Darjeeling hills.

Photo plate 2: Morphology of obverse mycelium of nine isolates of *Isaria* spp., on Glucose Peptone Agar after 14 days of incubation.

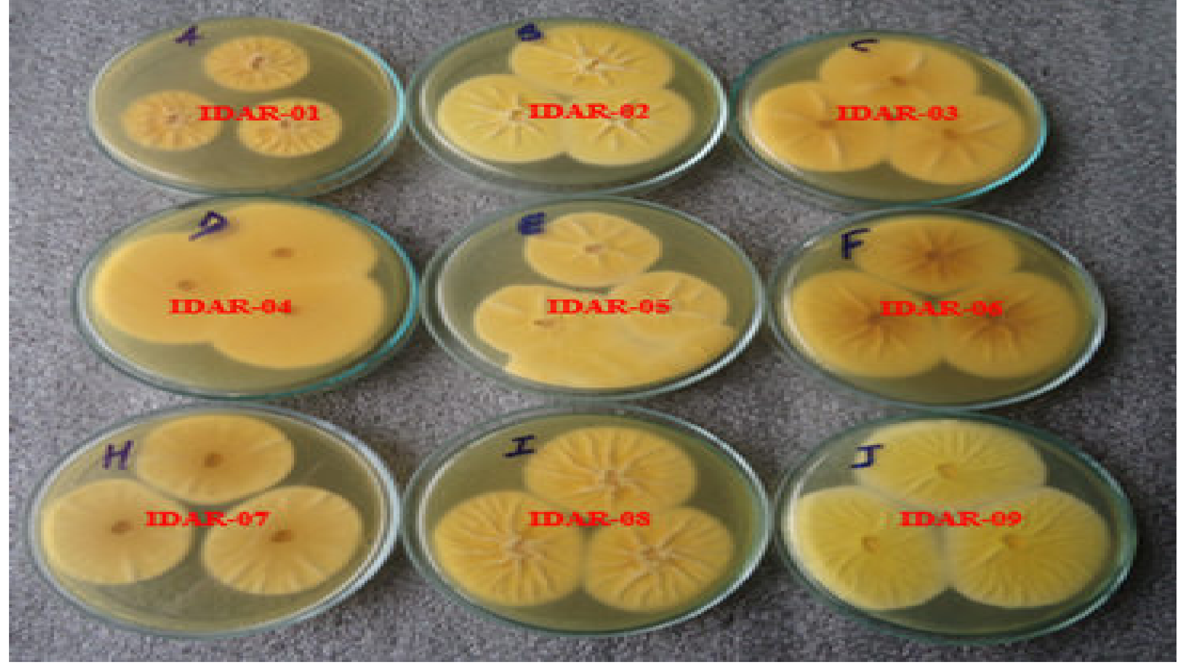
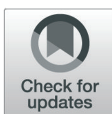


Photo plate 3: Morphology of reverse mycelium of nine isolates of *Isaria* spp., on Glucose Peptone Agar after 14 days of incubation.

Chhetri *et al. BMC Complementary Medicine and Therapies* (2020) 20:185
https://doi.org/10.1186/s12906-020-02973-w

BMC Complementary Medicine and Therapies

RESEARCH ARTICLE Open Access



Isaria tenuipes Peck, an entomopathogenic fungus from Darjeeling Himalaya: Evaluation of in-vitro antiproliferative and antioxidant potential of its mycelium extract

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Abstract

Background: *Isaria tenuipes* is one of the potent species in the members of the genus *Isaria*, which is well reported to possess multiple bioactive substances of therapeutic importance. Therefore, an in vitro experimental study was carried to evaluate the bioactivities of the crude methanolic extract from the mycelium of this fungus.

Methods: The fungus was authenticated through morphological characters and the species discrepancy was resolved using the nuclear rDNA ITS sequence. The methanolic extract was fingerprinted by FTIR. The antioxidant components in terms of total phenols and flavonoids were determined as gallic acid and quercetin equivalents respectively. Antioxidant activities of the methanolic extract was assessed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) radical cation ($ABTS^{0+}$), Fe^{2+}chelating activity, and hydroxyl radical scavenging assays. Cytotoxicity of the extract was determined by [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) assay on three cancer cell lines: HeLa, HepG2, and PC3. Apoptosis was further studied by propidium iodide (PI) and Annexin-V/PI staining flow cytometric analysis. Anti-proliferation capacity was studied by colony-forming assay.

(Continued on next page)

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Antioxidant and cytotoxic activities of *Isaria amoenerosea* Henn.: An entomopathogenic fungus from Darjeeling Hills, Eastern Himalaya

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Isaria amoenerosea Henn., an entomopathogenic fungus parasitizing on the beetle adult was collected from the subtropical forest of Darjeeling, India. Axenic culture of the mycelia for metabolite extraction was carried out through submerged fermentation. The methanolic mycellial extract was evaluated for its antioxidant as well as cytotoxic activities. Through a series of the *in-vitro* antioxidant assay, it was found that the methanolic extract scavenged DPPH and ABTS radicals up to the extent of 56.17 and 83.35% respectively at the tested concentrations. The 10 mg/mL extract chelated 73.82% of ferrous ions and it also showed an appreciable capacity to reduce ferric ions to ferrous ions. Cell viability was assessed by the MTT assay on the three human carcinoma cells: HeLa (cervical cancer), PC3 (prostate cancer) and HepG2 (hepato carcinoma). Among them, HeLa was recorded to be most susceptible exhibiting 53.48% inhibition at 100 µg/mL. The total phenol and flavonoid content of the extract was 11.58±0.12 and 9±2.1 µg/mg of gallic acid equivalent respectively. The evidence presented herein suggests that the mycelial methanolic extract indicates a correlation between the presence of varied functional groups (FTIR spectrum) and antioxidative as well as cytotoxic activities. No direct test of the hypothesis has been made which demands further investigations.

Keywords: Antioxidant activity, Cell viability, Entomopathogenic fungus, FTIR spectrum, *Isaria amoenerosea*.

IPC Code; Int.cl. (2015.01)- A61K 35/66, A61K 36/00, A61P, A61P 39/00

Introduction

Entomopathogenic fungi (EF) are widely available in the soil and many of them have the potential to be developed into functional foods for the prevention and treatment of several chronic diseases[1]. Recently, exploration of natural products having health benefits has become an important field of research. It has been reported that there are more than 1,200 entomopathogenic fungi in the world which are of immense pharmacological importance[2]. EF have traditionally been used as health foods in Japan, Korea and China[3,4]. The biologically active compounds extracted from many species of entomopathogenic genera have been reported to possess numerous biological and pharmacological activities. These include effective antibacterial, antifungal, insecticidal, anti-oxidative activity, anti-tumor activity, antidiabetic activity, powerful immunosuppressant activity, enhancing the apoptosis of cytotoxic T-cell, and inhibitors of serine palmitoyltransferase[5]. Extracts of fungal hyphae of *Isaria* has been reported to produce diverse metabolites[6] and submerged fermentation is an essential step for enhancement of metabolite production and extraction of the bioactive substances. Fungal species in the present study was identified according to taxonomic keys and monographs following the standard protocol described by Luangsa-sard *et al.*[7]. Based on a microscopic description and cultural features, the isolate was positioned in the genus *Isaria*. *Isaria amoenerosea* Henn. [syn: *Paecilomyces amoeneroseus* (Henn.) Samson] is an entomogenous fungus identified as a parasite on beetle adults (Coleoptera) which forms characteristic orange or reddish synnemata. At present, there is a great demand for natural antioxidants since synthetic ones are carcinogenic, damages the human liver and are linked to stomach cancer[8].

To the best of the author's knowledge, there are no reports on the antioxidative and cytotoxic activities of mycelial methanolic extract from *I. amoenerosea*. Therefore, the objective of this work is to assay the

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