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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*.

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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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influence of submerged fermentation, including the efficiency of the extract on *in vitro* antioxidative and anticancer activities. This report also tries to understand the link between antioxidant properties and cytotoxic effects.

Material and Methods

Sample collection and its isolation

I. amoenerosea Henn. growing on coleopteran insect (Fig. 1a) was collected from Darjeeling region situated between 87° 59′ – 88° 53′ East longitude and 26° 31′ – 27° 13′ North latitude in the Darjeeling hills (ca 2134 m AMSL) of Eastern Himalayas. The voucher specimen (No. Suh EF-01) was deposited at Herbarium, Department of Botany, Sikkim University, Sikkim. The conidiospores from the sample were discharged from fresh specimen over 2% water agar (WA) plates. Well separated conidiospores were selected from the plates using Ziess inverted microscope. The marked region was cut and transferred to potato dextrose agar PDA supplemented with 10 µg/mL streptomycin and the pure culture of *I. amoenerosea* (Fig. 1b) was maintained on PDA at 25 °C.

Inoculum preparation and mycelial growth

For seed culture, the agar was cut into plugs (6 mm in diameter with the help of cork borer) and three plugs from the growing tip were inoculated into 250 mL Erlenmeyer flasks containing 25 mL (n=3) of improved potato dextrose broth (PDB; potato starch 4.0 g, dextrose 20.0 g, 1.5 g of $MgSO_4.7H_2O$, 0.2 g of K_2HPO_4, and 0.01 g meat extracts per litre). The seed culture flask was incubated on a rotary shaker at 150 rpm and 25 ^oC for 5 days. Thereafter, for large scale mycelial growth each primary seed culture was transferred into a 2 L Erlenmeyer flask containing 300 mL of the seed culture medium and incubated at 25 °C for 30 days under static conditions. The experiment was carried out in three biological replicates.

Preparation of the fungal extract

After 24 days of submerged growth, the mycelium pellets were separated by filtration with Whatman No 1 filter paper and washed thoroughly with deionized H_2O. The mycelium pellet was dried at 50 ^oC for 36 hours, powdered, sieved (0.1 mm mesh) and stored at 4 °C until further use. The powdered mycelium was extracted with 10 vol. of 80 % methanol at room temperature for a period of 6 hours with continuous stirring. The extraction was repeated thrice using fresh solvents, and the extracts were pooled together and defatted by partitioning with hexanes, the MeOH layer was evaporated at low temperature. The residue thus obtained was dissolved in methanol or DMSO prior to use wherever mentioned. The yield of the extract was 7.5±0.3 % on dry- weight basis.

Determination of total phenolic content

Total soluble phenolics in the extracts were assessed using the method described by Singleton and Rossi[9] with slight modifications using Folin–Ciocalteau reagent. The blue color developed was read at 730 nm using UV-Vis spectrophotometer. Gallic acid (5-20 µg/mL) was used as the standard and the concentrations of total phenolic compounds were presented as gallic acid equivalents (GAE).

Determination of total flavonoid content

The total flavonoid content of the methanolic extract was quantified according to the method described by Zhishen *et al*[10]. The reaction mixture contained 250 µL of the methanolic extract, 1.25 mL

Fig. 1— Photographs showing *Isaria amoenerosea* in two different forms. a) Natural specimen growing on the body of an adult beetle and b) the fungus growing in broth media.

deionised water and 75 µL of 5% $NaNO_2$ which was incubated at room temperature for 5 minutes and added with 0.15 mL of 10% $AlCl_3$. The mixture was again incubated for 6 minutes at room temperature and 0.5 mL of 1 mM NaOH and 275 µL of deionized water were added. Finally, the absorbance was measured at 510 nm after 30 minutes of incubation. The flavonoid content was determined from a gallic acid standard curve (20-100 µg/mL) and expressed as GAE.

DPPH radical scavenging assay

The free radical scavenging activity of the methanolic extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was evaluated by the method reported by Brand- Williams *et al.*[11] with some modifications. In this assay, 1.0 mL of various concentrations of the fungal extract was mixed with 3.0 mL DPPH (Sigma 1898-66-4) solution in 95% methanol and with the absorbance adjusted to 1.0 (±0.02) at 517 nm. After 30 minutes of dark incubation, the absorbance was measured at 517 nm against 95%-methanol as a blank. Butylated Hydroxy Toluene (BHT) was used as a reference compound which was prepared in a similar manner. The percentage of free radical scavenging activity of the sample was calculated according to the following equation:

% Scavenging activity = [(Ac-As) / Ac] × 100

where Ac = absorbance of the control and As = absorbance of the sample.

An equal amount of methanol and DPPH without sample served as a control.

ABTS radical scavenging assay

The 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 discolouration test as per Re *et al.*[12]. Specifically, 7 mM of ABTS stock solution was mixed with 2.45 mM potassium persulfate solutions and the mixture was kept in the dark at room temperature for 16 hours. The dark coloured $ABTS^{\bullet+}$ radical cation containing solution was suitably diluted with ethanol to yield an absorbance of 0.70 (±0.2) at 734 nm to be used for antioxidant assay. The assay was performed by adding 1 mL methanolic extracts to be tested to 3 mL 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 by antioxidants was measured by the change of absorbance of $ABTS^{\bullet+}$ radical at 734 nm. A standard solution of ascorbic acid was also prepared and tested at a range of 2 to 10 mg/mL in methanol.

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 1.0 mL of methanol in place of the sample.

Evaluation of metal-chelating activity

The Fe^{2+}-chelating ability by the extracts was estimated by the method of Jiang *et al.*[13] with slight modifications. Exactly 1.0 mL aliquot of a methanolic extract (2–10 mg/mL) was reacted with 0.1 mL of 2.0 mM aqueous ferrous chloride and 3.7 mL deionized water. After incubation for 5 minutes, the reaction was initiated by adding 0.2 mL of 5.0 mM ferrozine (Sigma 63451-29-6). The mixture was vortexed and left at room temperature for 10 minutes to equilibrate. After incubation, the absorbance of the resulting Fe^{2+}-Ferrozine complex was determined spectrophotometrically at 562 nm. A lower absorbance indicates a stronger Fe^{2+}-chelating ability and the percentage chelating capacity was calculated as:

Chelating activity (%) = (Ac - As)/Ac × 100,

where Ac = absorbance of the control in the reaction system and As = absorbance of the sample.

Reducing activity assay

The reductive ability of the extract was determined using the method of Oyaizu[14] with some modification. Exactly1.0 mL aliquot of various concentration of the methanolic extract (2–10 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide (HiMedia RM1034) solution. The mixture was placed in a water bath at 50 ℃, for 20 minutes. Then, 2.5 mL 10 % trichloroacetic acid was added followed by centrifugation at 1200 g for 10 minutes. 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 and incubated at room temperature for 10 minutes. The absorbance of the reaction mixture was measured at 700 nm. The higher absorbance of

the reaction mixture indicated a greater reducing power. The reference solution was prepared as above but contained water in place of samples. Ascorbic acid was used as standard and the reducing power was expressed as ascorbic acid equivalents.

Cell viability assay

The human carcinoma cells, HeLa (cervical cancer), PC3 (prostate cancer), and HepG2 (hepato carcinoma) were used to investigate the cytotoxic activity evaluation of the methanolic extract of the sample. 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.

Under the influence of a methanolic extract, cell viability was assessed by the MTT assay as described by Mosmann[15]. For the experiment, 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 hours adherence, the cells were treated with various concentration of fungal extract for 72 hours. After the incubation, 1:10 volume of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours 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 minutes on a plateshaker. The absorbance was measured at 570 nm using a microplate reader. 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). All experiments were performed in four replicates.

FT-IR spectroscopy

The crude extract was subjected to functional groups analysis by FTIR (Perkin Elmer Spectrum 1: FT-IR) having a resolution of 1.0 cm^{-1}. Potassium bromide (KBr) pellets were used to obtain the infrared spectra of crude extract powders[16] covering scan range from an entire region of around 4000-450 cm^{-1}.

Statistical analysis

Each experiment was performed in triplicates. The statistical values were calculated by using the statistical package of MS-Excel, v-2007. The graphs were plotted using Origin 8.0 and the data were analysed by a t-test to evaluate the significant difference at $p < 0.05$.

Results and Discussion

Total phenolics and flavonoid content

The Folin–Ciocalteau reagent is reduced by a phenolate ion which is produced when a phenol loses H^+ ion under the basic reaction conditions. The change in redox assay is monitored spectro-photometrically and it is not limited to just polyphenols but to any other substances that could be oxidized by the Folin–Ciocalteu reagent[17,18]. The total phenolic content was 11.58±0.12 µg/mg of gallic acid equivalent per mg of methanolic extract. The total flavonoid content was 9±2.1 µg/mg GAE per mg fungal extract. All values are expressed in mean±SEM (n = 3). Methanol has been considered as an effective solvent to extract phenolic compounds[19]. Here, the result of total phenol and flavonoid content in methanolic extract was found to be 11.58±0.12 and 9.0±2.1 µg/mg GAE respectively. This result is consistent with the findings of other investigators during some methanolic extraction using the mycelial culture of *Isaria*[20]. The content of both total phenol as well as flavonoids content was also comparable to those found in *Macrocybe lobayensis* showing 12.58±0.57 µg/mg GAE of total phenols and 10.95±0.0.89 µg/mg quercetin equivalents of flavonoids respectively[21]. However, the contents of these molecules in *Paris polyphylla* a medicinal plant obtained from the higher reaches of the Himalaya were much higher[22].

DPPH radical scavenging activity

DPPH is a stable nitrogen-centered, lipophilic free radical that is generally used as a substrate in evaluating the antioxidative capacities of antioxidants[14].

At all tested concentrations, the DPPH radical was inhibited ($p < 0.05$) by the fungal extract. Thus the DPPH radical scavenging activity of fungal extract increased from 3.37 to 56.17% with the increase in concentrations from 2 to 10 mg/mL (Fig. 2). However, no significant radical scavenging activity was observed at a lower concentration of the sample tested. The radical scavenging activity of a known synthetic antioxidant butylated hydroxy toluene (BHT) was stronger than that of the fungal extract. Antioxidant activity is a common feature of metabolite extracted from an entomopathogenic fungus like *Isaria*[13,23]. The DPPH scavenging activity of *I. amoenerosea* extract was in consonance to that

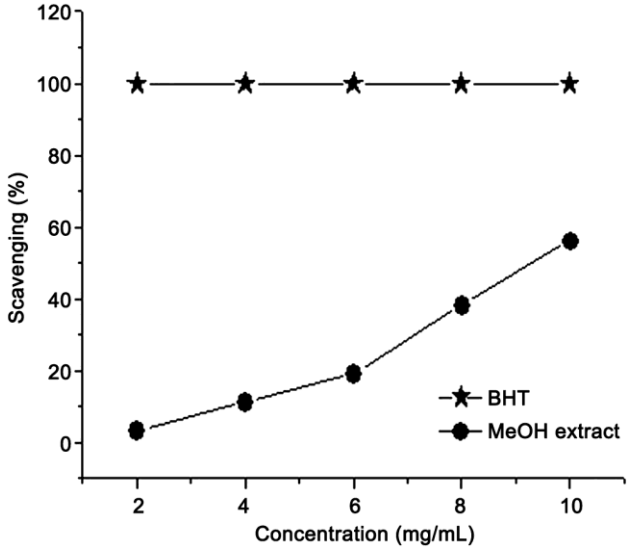

Fig. 2 — DPPH scavenging activities of different concentrations of methanolic extract of cultured mycelium of *Isaria amoenerosea*. BHT was used as a reference compound.

of another fungus, *Termitomyces microcarpus* R. Heim having a scavenging activity of upto 60%[24].

ABTS assay

$ABTS^{\bullet+}$ radical scavenging effects of the extract of *I. amoenerosea* correlated well with the increasing extract concentrations. The results indicated significant scavenging of ABTS radical which gradually increased from 1.49 to 83.35% (p <0.001) as the extract concentration increased from 2 mg/mL to 10 mg/mL (Fig. 3). However, ascorbic acid, a known antioxidant, showed a scavenging activity of up to 99% at a similar concentration. Nonetheless, the result shows similarity to the methanolic extract of the edible fungus, *Macrocybe lobayensis*[25]. Scavenging of ABTS radical cation by *Isaria amoenerosea* has not been reported so far.

Ferrous ion chelating capacity

To assess the iron chelating properties of the crude methanolic extract, the disruption of Fe^{2+} ferrozine complex was assayed. In the present study, the chelation power of the methanolic extract was significant since the extract reduced absorbance of Fe^{2+} ferrozine complex at all the tested concentrations. The decrease in purple colour formed due to ferrozine-Fe^{2+} complex formation displayed a dose-dependent relationship with 73.82% chelation measured at 10 mg/mL (Fig. 4). The chelating ability of the extract increased with an increase in concentration. However, EDTA showed a greater chelating activity as compared to our fungal extract.

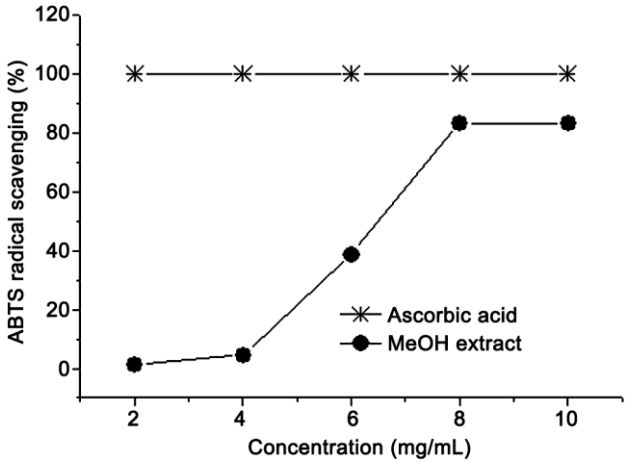

Fig. 3 — ABTS radical scavenging activities of methanolic extract of cultured mycelium of *Isaria amoenerosea*. Ascorbic acid was used as a reference compound.

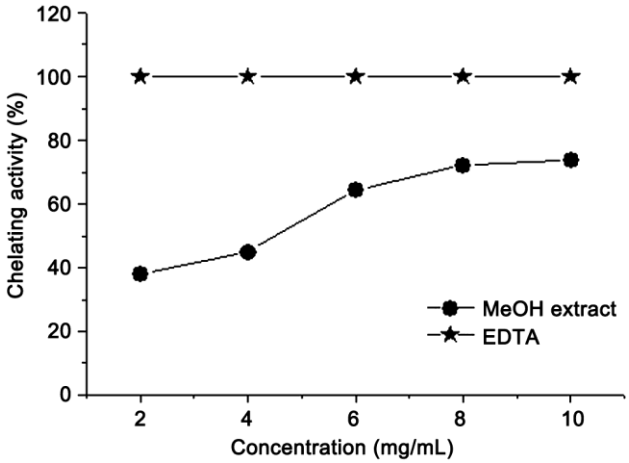

Fig. 4 — Metal chelating activities (% inhibition of Fe2+-ferrozine complex formation) of lyophilized extracts of *Isaria amoenerosea* in methanol.

The finding regarding the Fe^{2+}-chelating activity of methanolic extract of *I. amoenerosea* was in sync with the results obtained for low molecular weight exo-polysaccharide and intra-polysaccharides[13,23.]

Reducing capacity

The reductive capacity of the sample extract was based on the property of the sample to reduce yellow ferric form to blue ferrous form. Reducing powers of methanolic extracts was excellent (p <0.001) and increased steadily from 0.33 to 0.90 with the increased concentrations from 2 to 10 mg/mL (Fig. 5). Similar findings of the enhanced reducing power of water soluble polysaccharides have been previously reported from the genus *Isaria*[13]. The increased reducing ability observed may be due to the

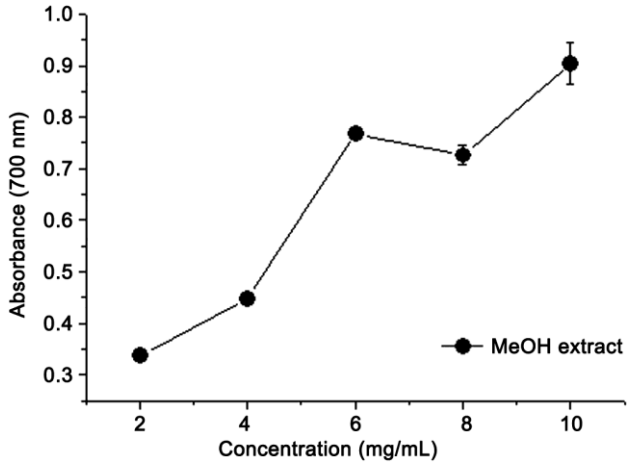

Fig. 5 — Reductive ability of Methanolic extract of cultured mycelium of *Isaria amoenerosea*. The reducing power is expressed as ascorbic acid equivalents (AAE).

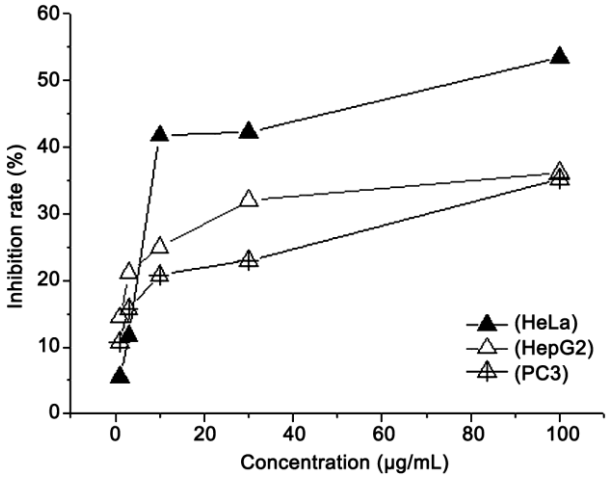

Fig. 6 — Effect of different concentrations of methanolic extracts of cultured mycelium of *Isaria amoenerosea* on HeLa, HepG2 and PC3 cell lines.

hydrogen-donating capabilities of the reductones which could react with free radicals to stabilize and terminate radical chain reactions during submerged fermentation, converting them to more stable products[26]. Reductones work by breaking the chain of free radicals which could be monitored by monitoring the intensity of the blue colour formed at 700 nm. Though the reducing ability of the sample does not have any bearing on radical scavenging activity, it is considered as a strong antioxidant parameter[18]. The methanolic extract of *I. amoenerosea* followed the general trend of increasing reducing power with increasing extract concentration as in the case of edible fungi like *Amanita vaginata*[27] and *Russula albonigra*[28].

Cytotoxic activity

The effect of methanolic mycelium extract of *I. amoenerosea* on the inhibition of cell growth was investigated by the MTT assay. Among the three cancer cell lines studied, the extract caused significant inhibition of HeLa cell growth within 72 hours of treatment. However, the extract had a moderate effect on the inhibition of PC3 and HepG2 cell growth. The graphically determined half inhibitory concentration was 82.50 µg/mL for HeLa cells. The same cells treated with 100 µg/mL extract caused 53.48% inhibition of growth. Similarly, inhibition of HepG2 and PC-3 cells at 100 µg/mL sample extract was 36.29 and 35.26% respectively. Thus, the extract was not much effective against HepG2 and PC-3 cell growth (Fig. 6). Fungal anticancer substances may be either high molecular weight components comprising of polysaccharides and protein-bound polysaccharides or low molecular weight compounds consisting of phenolics, steroids, sesquiterpenes, triterpenes, sterols and polyketides. These low molecular weight substances are capable to penetrate cell membrane and function on specific signal transduction cascade[25,29]. It has been reported that the genus *Isaria* is immensely rich in secondary metabolites whereby more than seventy secondary metabolites have been chacterized[30]. Furthermore, the consumption of medicinal fungus is believed to boost the immune response and anticancer functions[31]. Altogether, the results obtained for antioxidant properties and cytotoxic effects of methanolic mycellial extract of *I. amoenerosea* are in accordance with the similar observations[5,13].

FT-IR study

The application of infrared spectroscopy mainly the Fourier transforms infrared spectroscopy and near infrared spectroscopy to food, science has been widely used for qualitative analysis of bioactive components and to a lesser extent for quantitative analysis of macronutrient components[32]. Moreover, the methods also provide with very useful qualitative and quantitative information about the biochemistry and chemistry of antioxidants[33]. The spectrum analysis (Fig. 7) of the lyophilized extract of *I. amoenerosea* 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} indicate γ C=O stretching and γ C-(C=O)-C stretching at 1638 cm^{-1} whereas peak at

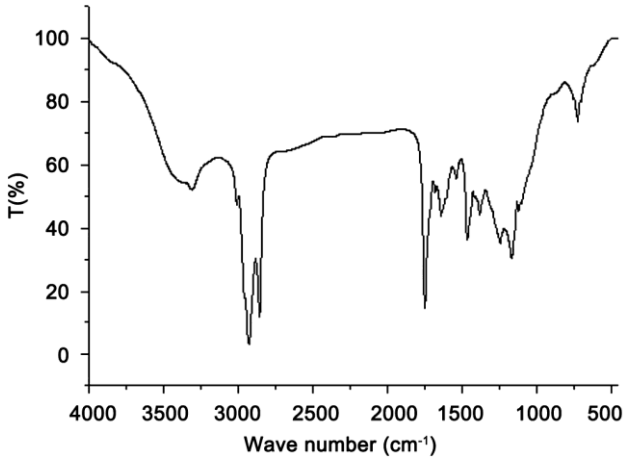

Fig. 7 — Fourier transform infrared spectra of lyophilized extract of *Isaria amoenerosea* prepared as KBr pellet and scanned in the range 4000 to 450 cm^{-1}.

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.

Conclusion

The current investigation with the methanolic extracts of the mycelium of *Isaria amoenerosea* exhibited both antioxidant and cytotoxic attributes and this work represents the first report of this kind from Darjeeling Hills. In the present report, the results vindicated a positive correlation between antioxidant properties and anticancer effects. The fungus contained an appreciable amount of phenols and flavonoids which is related to its antioxidant properties and that in turn may be responsible for its cytotoxic abilities. However, cytotoxic activity of plant extracts may vary with the varying cell types and this was evident in this case also. The characterization of the antioxidant and anticancer compounds, their structure-activity relationship, mechanistic aspects of their biological activity etc. still remain to be investigated, which may be taken up in near future.

Acknowledgment

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References

1 Zhang J J, Li Y, Zhou T, Xu DP, Zhang P, *et al*., Bioactivities and health benefits of mushrooms mainly from china, *Molecules*, 2016, **21**(7), 938.

2 Liu F, Xiang M, Guo Y, Wu X, Lu G, *et al*., Culture conditions and nutrition requirements for the mycelial growth of *Isaria farinosa* (Hypocreales: Cordycipitaceae) and the altitude effect on its growth and metabolome, *Sci Rep*, 2018, **8**(1), 15623.

3 Zhu J S, Halpern G M and Jones K, The scientific rediscovery of a precious ancient Chinese herbal regimen: *Cordyceps sinensis*-Part-I, *J Alt Compl Med*, 1998a, **4**(3), 289-303.

4 Zhu J S, Halpern G M and Jones K, The scientific rediscovery of a precious ancient Chinese herbal regimen: *Cordyceps sinensis*-Part-II, *J Alt Compl Med*, 1998b, **4**(4), 429-457.

5 Jiang Y H, Jiang X L, Wang P, Mou H J, Hu X K, *et al.*, The antitumor and antioxidative activities of polysaccharides isolated from *Isaria farinosa* B05, *Microbiol Res*, 2008, **163**(4), 424-430.

6 Thakur S S, Ranganayaki R S, Gupta K and Balaram P, Identification of α- and β-hydroxy acid containing cyclodepsipeptides in natural peptide mixtures using negative ion mass spectrometry, *J Am Soc Mass Spectrom*, 2009, **20**(12), 2221–2228.

7 Luangsa-ard J J, Tasanathai K, Mongkolsamrit S and Hywel-Jones N, *Atlas of Invertebrate-Pathogenic Fungi of Thailand (Vol-I)*, (National Center for Genetic Engineering and Biotechnology, Pathum Thani), 2007, 1-82

8 Botterweck A A, Verhagen H, Goldbohm R A, Kleinjans J and van den Brandt P A, Intake of butylated hydroxyanisole and stomach cancer risk: Results from analyses in the Netherlands Cohort study, *Food Chem Toxicol*, 2000, **38**(7), 599-605.

9 Singleton V L and Rossi J A, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, *Am J Enol Viticult*, 1965, **16**(3), 144-158.

10 Zhishen J, Mengcheng T and Jianming W, The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals, *Food Chem*, 1999, **64**(4), 555–559.

11 Brand-Williams W, Cuvelier M E and Berset C, Use of a free radical method to evaluate antioxidant activity, *LWT-Food Sci Technol*, 1995, **28**(1), 25-30.

12 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, *et al.*, Antioxidant activity applying an improved ABTS radical cation decolorization assay, *Free Radic Biol Med*, 1999, **26**(9-10), 1231-1237.

13 Jiang Y, Jiang X, Wang P and Hu X, *In vitro* antioxidant activities of water-soluble polysaccharides extracted from *Isaria farinosa* B05, *J Food Biochem*, 2005, **29**(3), 323-335.

14 Oyaizu M, Studies on product of browning reaction antioxidative activities of product of browning reaction prepared from glucosamine, *Jpn J Nutr*, 1986, **44** (6), 307–315.

15 Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J Immunol Methods*, 1983, **65**(1-2), 55–63.

16 Devi N N and Prabakaran J J, Bioactive metabolites from an endophytic fungus *Penicillium* sp. isolated from *Centella asiatica*, *Curr Res Environ Appl Mycol*, 2014, **4**(1), 34–43.

17 Singleton V L, Orthofer R and Lamuela-Raventos R M, Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, *Method Enzymol*, 1999, **229**, 152–178.
18 Huang D, Ou B and Prior R L, The chemistry behind antioxidant capacity assays, *J Agric Food Chem*, 2005, **53**(6), 1841–1856.
19 Siddhuraju P and Becker K, Studies on antioxidant activities of *Mucuna* seed (*Mucuna pruriens* var *utilis*) extracts and various non-protein amino/imino acids through *in-vitro* models, *J Agric Food Chem*, 2003, **83**(14), 1517-1524.
20 Cheung L M and Cheung P C K, Mushroom extracts with antioxidant activity against lipid peroxidation, *Food Chem*, 2005, **89**(3), 403–409.
21 Khatua S and Acharya K, Functional ingredients and medicinal prospects of ethanol extract from *Macrocybe lobayensis*, *Pharmacogn J*, 2018, **10** (6), 1154-1158.
22 Lepcha D L, Chhetri A and Chhetri D R, Antioxidant and cytotoxic attributes of *Paris polyphylla* smith from Sikkim Himalaya, *Pharmacogn J*, 2019, **11**(4), 705-711.
23 Sharma S K, Optimized extraction and antioxidant activities of polysaccharides from two entomogenous fungi, *J Bioanal Biomed*, 2015, **7**(6), 180-187.
24 Mitra P, Mandal N C and Acharya K, Phytochemical characteristics and free radical scavenging activity of ethanolic extract of *Termitomyces microcarpus* R. Heim, *Der Pharma Lett*, 2014, **6** (5), 92-98.
25 Khatua S, Ghosh S and Acharya K, Chemical composition and biological activities of methanol extract from *Macrocybe lobayensis*, *J App Pharm Sci*, 2017, **7**(10), 144-151.
26 Shimada K, Fujikawa K, Yahara K and Nakamura T, Anti-oxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion, *J Agric Food Chem*, 1992, **40** (6), 945–948.
27 Paloi S and Acharya K, Evaluation of antioxidative activity and chemical composition of ethanolic extract from *Amanita vaginata* (Bull.) Lam.: An *in vitro* study, *Asian J Pharm Clin Res*, 2014, **7**(2), 88-92.
28 Dasgupta A, Ray D, Chatterjee A, Roy A and Acharya K, *In-vitro* antioxidative behaviour of ethanolic extract of *Russula albonigra*, *J Chem Pharm Res*, 2014, **6**(3), 1366-1372.
29 Liu K, Wang J, Zhao L and Wang Q, Anticancer, antioxidant and antibiotic activities of mushroom *Ramaria flava*, *Food Chem Toxicol*, 2013, **58**, 375–380.
30 Weng Q, Zhang X, Chen W and Hu Q, Secondary metabolites and the Risks of *Isaria fumosorosea* and *Isaria farinosa*, *Molecules*, 2019, **24**(4), 664.
31 Cheung P C K, Mini-review on edible mushrooms as source of dietary fiber: Preparation and health benefits, *Food Sci Hum Wellness*, 2013, **2**(3-4), 162-166.
32 Lu X and Rasco B A, Determination of antioxidant content and antioxidant activity in foods using infrared spectroscopy and chemometrics: A Review, *Crit Rev Food Sci Nutr*, 2012, **52**(10), 853–875.
33 Cozzolino D, Infrared spectroscopy as a versatile analytical tool for the quantitative determination of antioxidants in agricultural products, foods and plants, *Antioxidants*, 2015, **4**(3), 482-497.