Research Article

Antioxidant and Antimicrobial Potentiality of Quantitatively Analysed Ethanol Extract from *Macrocybe crassa*

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ABSTRACT

Macrocybe crassa, a wild mushroom growing mainly in coastal areas of West Bengal, has been widely used as food for its flavour and taste. The present study was conducted to emphasize on antioxidant and antimicrobial activity as well as phenolic profile of crude ethanolic extract of this mushroom (EfraMac). Mycochemical analysis showed presence of bioactive components in the following order: phenol> flavonoid> ascorbic acid> β-carotene> lycopene. Further analysis with HPLC revealed that the dominant phenolic in EfraMac was pyrogallol, whereas vanillic acid, cinnamic acid and *p*-coumaric acid were present in less amounts. Antioxidant activity was determined by using superoxide radical scavenging, $DPPH^{\cdot}$ radical inhibition, chelating ability of ferrous ion and total antioxidant activity assays. Promising results were obtained as evident by low EC_{50} values (ranging from 0.53 mg/ml to 1.66 mg/ml) and high ascorbic acid equivalent antioxidant capacity data. In addition, EfraMac exhibited antimicrobial effect against three Gram positive (*Listeria monocytogenes*, *Staphylococcus aureus, Bacillus subtilis*) and two Gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria, whereas S*almonella typhimurium* was resistant to the fraction. Overall results indicated that the mushroom can serve as a good source of polyphenols in the human diet due to high concentrations of total phenolic compounds as well as their strong bioactivity. Moreover, the present study reports antioxidant and antimicrobial activity in relation with phenolic profile of *Macrocybe crassa* for the first time.

Keywords: Antimicrobial effect, free radical scavenging, HPLC, phenolic extract, wild edible mushroom

INTRODUCTION

Since ancient times, mushrooms are very appreciated to people not only for their texture and flavour but also for therapeutic value which was explored in traditional medicines. In the twentieth century, researchers have found that mushrooms are an important source of chemically diverse novel secondary metabolites which possess a wide spectrum of biological activities.[1] In nature polyphenols are among the most widespread class of metabolites and currently, there are an increasing number of reports regarding action of phenolic compounds in different mushroom species. Phenolic compounds consist of an aromatic ring bearing 1/more hydroxyl groups comprising simple phenolic acid and complicated polyphenols. The most common phenolic acids are benzoic acid derivatives (gallic acid, vanillic acid, syringic acid and protocatechuic acid) and cinnamic acid derivatives (*p*-coumaric acid, *o*-coumaric acid, caffeic acid, ferulic acid and chlorogenic acid).[2] Presence of carboxylic acid (COOH), two hydroxyl (OH) groups in para or ortho positions of the benzene ring and also a methoxy (OCH_3) group in meta position seems to be important for bio-activity of phenolic acids. These hydroxyl groups on benzene ring can act as good hydrogen donors and chelate metals involved in production of free radicals, hence are responsible for antioxidant property of phenolic acids.[3,4] Besides this, phenols are also able to exhibit antimicrobial activity. Alves examined antimicrobial activity of phenolic compounds present in carboxylic group in structure.[5] Their results suggested that only phenolic acids (benzoic acid and cinnamic acid derivatives) exhibited potentiality highlighting importance of the carboxylic group in structure. Although there are many commercial drugs in market to fight against microbe and free radical induced diseases but they are proved to be ineffective gradually.[6] To overlap disadvantages of synthetic antioxidants and antimicrobial drugs growing attention has been focused on natural products in an attempt to search for novel effective agents.

West Bengal, India has varied agro-climate suitable for abundance of different mushrooms. Recent investigations suggest that a number of wild edible mushrooms collected from West Bengal have diverse biological activities including anti-diabetic,[7] anti-cancer,[8] antimicrobial,[9] hepato-protective,[10] cardio-protective,[11] anti-parasitic,[12] anti-ulcer,[13] immune-stimulant[14] etc. However, diminutive is known about *M. crassa*, a comestible mushroom, growing abundantly in West Bengal during rainy season. In this work, we describe, for the first time, analysis of the phenolic content occurring in ethanol extract from *Macrocybe crassa* and its antimicrobial and antioxidant activity.

Analysis will be valuable for potential uses of the mushroom for food enhancement and pharmaceutical industry.

Moreover, increased study on *M. crassa* could improve economic value of the species and further work on its cultivation.

MATERIALS AND METHODS

Collection and authentication

Basidiocarps of *M. crassa* were collected from coastal area of West Bengal, India in the month of August, 2014 and identified using standard literature.[15] A voucher specimen has been deposited in the same department under the number of AMF 1069. The fruiting bodies of *M. crassa* were cleaned to remove residual compost and dried to eliminate moisture content.

Preparation of extract

Ethanol fraction was prepared from dried mushroom powder according to the method of Giri.[16] 5 gm of mushroom powder was soaked in 200 ml ethanol and stirred at 25°C for 1 day at 150 rpm. Subsequently the solvent was separated through Whatman No. 1 filter paper and the entire extraction process was repeated on the residue. After filtration, the combined solvent was rotary evaporated at 40°C under vacuum (Butchi, Switzerland) to acquire ethanolic fraction of *M. crassa* (EfraMac). EfraMac was stored in amber coated bottle at -20°C until further analysis. The yield percentage of extract was calculated based on dry weight as:

$$Yield\ (\%) = \frac{(W1 \times 100)}{W2}$$

Where W1 = Weight of extract after solvent evaporation; W2 = Weight of the minced mushroom

Mycochemical analysis

The content of total phenolic compounds in EfraMac was estimated using Folin-ciocalteu reagent and gallic acid (10 – 40 µg) as standard.[17] The results were expressed as µg of gallic acid equivalents per mg of dry extract. Total flavonoid content was determined using aluminium nitrate and potassium acetate. Quercetin (5 – 20 µg) was used to calculate the standard curve.[18] The results were expressed as µg of quercetin equivalents per mg of dry extract. β-carotene and lycopene were estimated by measuring absorbance at 453, 505 and 663 nm.[19] Ascorbic acid was determined by titration against 2, 6-dichlorophenol indophenol dye.[20]

Determination of phenolic profile by HPLC

Eleven standards, gallic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, ferulic acid, myricetin, salicylic acid, quercetin, cinnamic acid, pyrogallol and kaempferol were purchased from Sigma Aldrich (MO, USA). The standard stock solutions (10 - 50 µg/ml) were prepared with HPLC grade methanol and water (1:1 v/v). All standard calibration curves showed high degrees of linearity (r^2 > 0.99).

Sample compounds were identified on the basis of the retention times and absorption spectra of standard materials. Components were quantified by comparing their peak areas with those of standard curves.

10 mg EfraMac was dissolved with 1 ml of HPLC grade methanol and filtered through 0.2 µm filter paper. 20 µl filtrate was loaded on the HPLC system (Agilent, USA). Separation was achieved on a Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) using a flow rate of 0.8 ml/min at 25°C. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). A gradient program was used for elution: 0-2 min, 5% A; 2-5 min, 15% A; 5-10 min, 40% A; 10-15 min, 60% A; 15-18 min, 90% A. The absorbance of standard and sample solution was measured at 280 nm.

Superoxide radical scavenging assay

The scavenging potential of EfraMac for superoxide radical was analysed as described by Martinez.[21] 3 ml reaction mixture sequentially contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, various concentrations (0.3 – 0.7 mg/ml) of EfraMac, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. Sample was illuminated with light to start reaction and the increased absorbance was measured at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in dark and served as blank. Butylated hydroxyl anisole (BHA) was used as a positive control. Degree of scavenging was calculated by the following equation:

$$Scavenging\ effect\ (\%) = \frac{(A0 - A1)}{A0} \times 100$$

Where A0 was the absorbance of control and A1 was absorbance in presence of sample

DPPH radical scavenging assay

The methodology for determination of radical scavenging activity of EfraMac was adapted from Shimada.[22] Briefly, EfraMac was mixed with methanol (2 ml) and 0.101 mM DPPH to give final concentration of 0.1, 0.5 and 1 mg/ml. After 30 min at room temperature in dark, absorbance was measured at 517 nm against blank. EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison. Radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation:

$$Scavenging\ effect\ (\%) = \frac{(A0 - A1)}{A0} \times 100$$

Where A0 was the absorbance of control and A1 was absorbance in presence of sample

Chelating ability of ferrous ions

The ability of investigated extract to chelate ferrous ion was determined with slight modification.[23] 2 ml Reaction mixture contained different concentrations of EfraMac (0.1 – 1 mg/ml) mixed with methanol and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the

addition of 0.2 ml of 5 mM ferrozine. After 10 min incubation at room temperature, the absorbance was determined at 562 nm against a blank. Ethylenediamine tetraacetic acid (EDTA) was used as positive control. EC_{50} value is the effective concentration at which ferrous ions were chelated by 50%. The percentage of inhibition of ferrozine- Fe^{2+} complex formation was calculated using following formula:

$$Scavenging\ effect\ (\%) = \frac{(A0 - A1)}{A0} \times 100$$

Where A0 was the absorbance of control and A1 was absorbance in presence of sample

Determination of total antioxidant capacity by phosphomolybdenum method

The assay was carried out as described by Prieto[24] with little modification by Mitra.[25] The reaction mixture consisted of 0.3 ml sample solution and 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium sulphate and 4 mM ammonium molybdate). Blank was prepared by adding 0.3 ml water and 3 ml reagent solution. Tubes were capped and incubated at 95°C for 90 min. Samples were cooled at room temperature and absorbance was measured at 695 nm against blank. Concentrations of ascorbic acid (1 – 30 µg) were used to obtain a standard curve. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Disk diffusion method

Antimicrobial activity of EfraMac was determined by the agar-disc diffusion method.[26] *Listeria monocytogenes* MTCC Code 657, *Salmonella typhimurium* MTCC Code 98, *Bacillus subtilis* MTCC Code 736, *Escherichia coli* MTCC Code 68, *Pseudomonas aeruginosa* MTCC Code 8158 and *Staphylococcus aureus* MTCC Code 96 were obtained from the culture collection of the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. Nutrient agar was poured into each sterilized petridish (90 mm diameter) after injecting cultures (100 µl) of bacteria and medium was distributed homogeneously. Paper discs (5 mm) were loaded with EfraMac (2 mg/disc). The impregnated discs were air dried before placing it on the petri dishes with the test microorganisms. The plates were incubated as per the bacterial requirement. Studies were performed in triplicate and the inhibition zones were compared with those of blank discs.

RESULTS AND DISCUSSION

Extractive yield and mycochemical analysis

The extract was brown in colour, sticky in nature and had extractive value of 7.8%. Total phenolic content was evaluated by the Folin-ciocalteau method and reported as gallic acid equivalent by reference to standard curve (y = 0.0124x - 0.0262, r^2 = 0.997). The assay is based on transfer of electrons from phenolic compounds and reducing agents present in fraction forming blue complexes that can be monitored spectrophotometrically.[27] EfraMac was found to contain phenol as much as 11.7 ± 0.8 µg gallic acid equivalent /mg of extract. Total flavonoid content was determined by using quercetin as standard (y = 0.0094x - 0.0106, r^2 = 0.984). EfraMac also contained flavonoid as 7.59 ± 1.24 µg quercetin equivalent /mg of extract. This assay is highly sensitive for flavonoids as only flavonoids can form coloured complexes with aluminium chloride in alkaline medium.[28] Very negligible amount of β-carotene and lycopene were found such as 0.46 ± 0.09 µg/mg and 0.41 ± 0.06 µg /mg of the extract respectively. Ascorbic acid was also found (1.74 ± 0.35 µg /mg of extract) in minor quantities.

In HPLC eleven standard phenolic components were analysed (Figure 1) of which four constituents were detected as vanillic acid, *p*-coumaric acid, cinnamic acid and pyrogallol (Figure 2). The analysis showed that pyrogallol was the major component in EfraMac while *p*-coumaric acid was present in trace amount. Vanillic acid and cinnamic acid were present in moderate quantities (Table 1). There were 6 unidentified peaks at RT 7.554 min, 16.142 min and 17.625 min with 3 strong peaks at RT 5.893 min, 7.033 min and 7.803 min in the HPLC chromatogram (Figure 2). Sun have conducted an excellent work on phenolics and reported spectral information of 39 phenolic compounds.[29] UV absorption is a characteristic of phenolics as it is dependent on chemical structure; thus unknown peaks in EfraMac could be assumed by comparing UV bands with that of reported phenolic compounds (Figure 3). In EfraMac, UV spectrum of all 6 unknown compounds were analysed and found to be similar with *p*-hydroxybenzoic acid (RT 5.893 min), syringic acid (RT 7.033 min) and caffeic acid (RT 7.554 min) respectively, whereas rest two components (RT 16.142 and 17.625 min) could not be recognized. Thus it can be assumed that EfraMac was mainly composed of benzoic acid derivatives besides pyrogallol.

Superoxide radical scavenging assay

Superoxide anion ($O_2^{\cdot-}$), a free radical, can be generated either through metabolic process or oxygen activation by physical irradiation. Addition of one electron to molecular oxygen forms this primary reactive oxygen species (ROS), which again can generate other harmful ROS such as peroxynitrate (ONOO·), peroxyl radicle (LOO·), singlet oxygen, hydroxyl radical (OH·) and hydrogen peroxide.[30] Presence of these radicals can magnify cellular damage; therefore, it is important to characterize scavenging ability of superoxide radical by antioxidants. The superoxide radical scavenging activities of EfraMac and BHA are shown in Figure 4. At the concentration of 0.3 mg/ml, scavenging activities of EfraMac and BHA were 13.97% and 53% respectively. EfraMac was potential in superoxide radical scavenging activity as evident by its low EC_{50} value (0.64 ± 0.01 mg/ml). Whereas EC_{50} value of ethanol extract of *Agaricus bisporus* was 8.08 mg/ml indicating higher activity of EfraMac.[30]

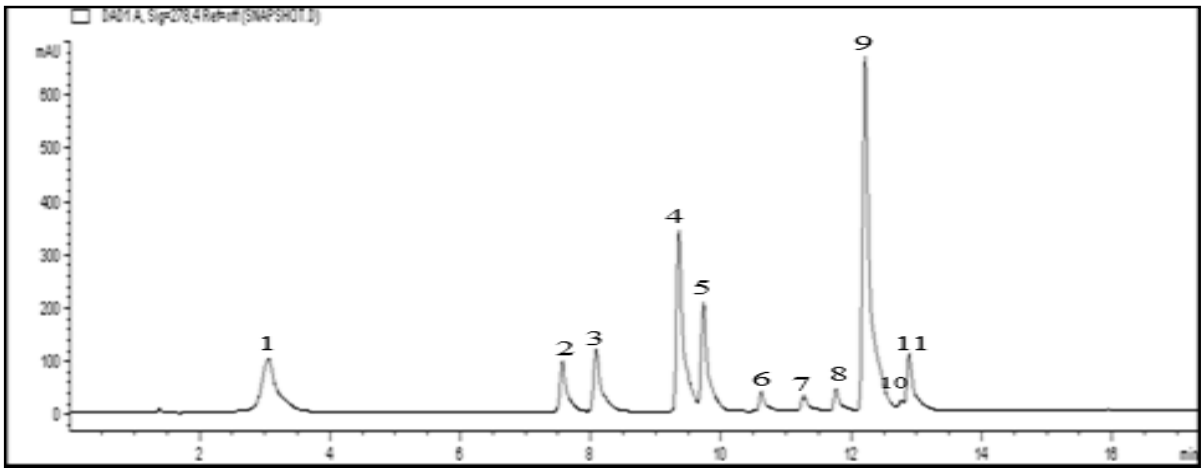

Figure 1: HPLC chromatogram of standards (50 µg/ml each): (peaks: 1: gallic acid, 2: chlorogenic acid, 3: vanillic acid, 4: *p*-coumaric acid, 5: ferulic acid, 6: myricetin, 7: salicylic acid, 8: quercetin, 9: cinnamic acid, 10: pyrogallol, 11: kaempferol

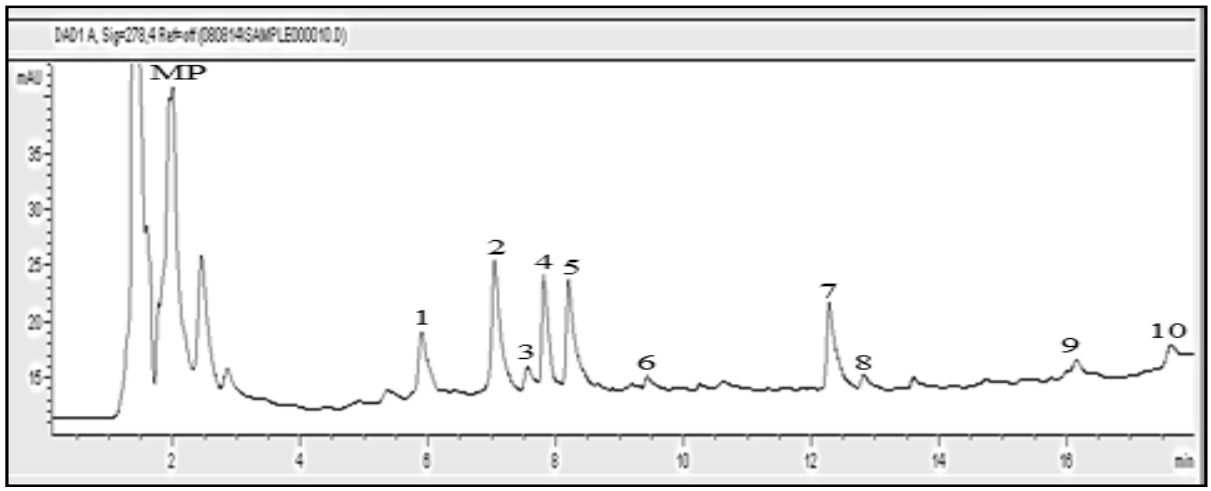

Figure 2: Enlarged HPLC chromatogram of ethanol extract of *Macrocybe crassa* (EfraMac) (MP: mobile phase, peaks: 1: probably *p*-hydroxybenzoic acid, 2: probably syringic acid, 3: probably caffeic acid, 4: unknown, 5: vanillic acid, 6: *p*-coumaric acid, 7: cinnamic acid, 8: pyrogallol, 9: unknown, 10: unknown)

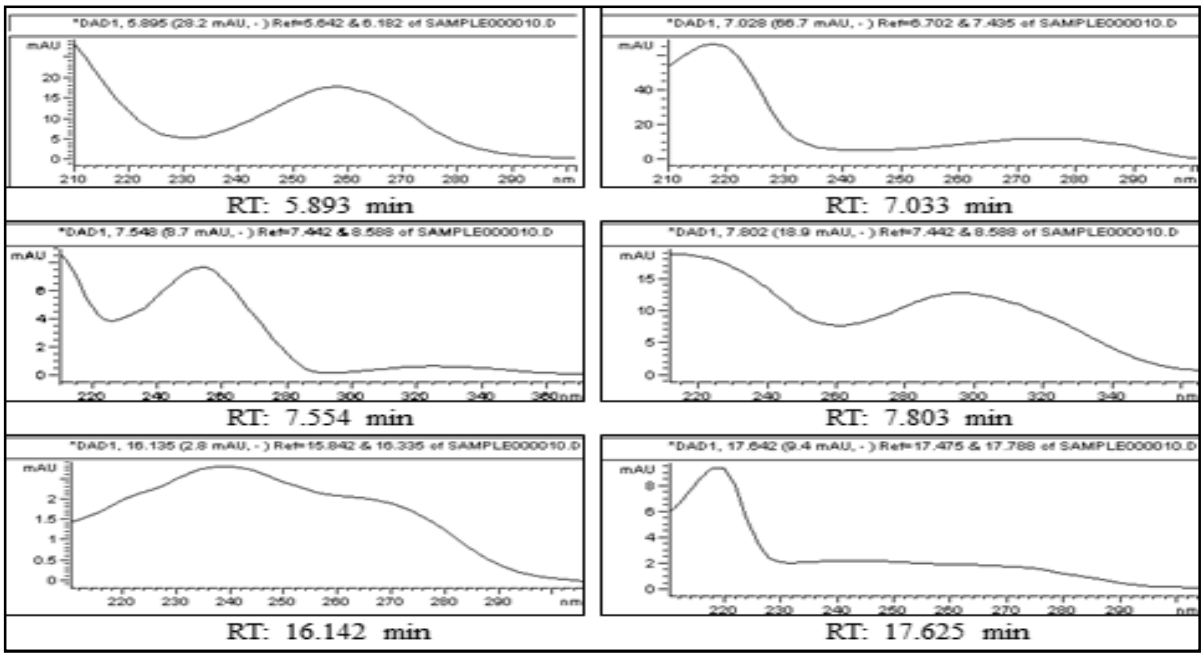

Figure 3: UV spectra of 6 unidentified peaks shown in the HPLC chromatogram of ethanol extract of *Macrocybe crassa* (EfraMac)

Table 1: Phenolic profile of ethanol extract of *Macrocybe crassa* (EfraMac) as determined by HPLC.

Compound	Retention time (min)	UV band (nm)	Area	Concentration (µg /mg)
Gallic acid / 3,4,5-trihydroxybenzoic acid	3.07	272	Nd	Nd
Probably *p*-hydroxybenzoic acid[29]	5.89	258	57.2	Nd
Probably syringic acid[29]	7.03	275	116.4	Nd
Probably caffeic acid[29]	7.55	330	18.1	Nd
Chlorogenic acid	7.58	210, 325	Nd	Nd
Unknown	7.8	295	74.1	Nd
Vanillic acid / 4-hydroxy-3-methoxybenzoic acid	8.1	260, 295	87.2	0.41
p-Coumaric acid / trans-4-hydroxycinnamic acid	9.37	310	8.8	0.04
Ferulic acid / 3-methoxy-4-hydroxycinnamic acid	9.74	290, 325	Nd	Nd
Myricetin	10.63	250, 373	Nd	Nd
Salicylic acid / 2-hydroxybenzoic acid	11.28	303	Nd	Nd
Quercetin	11.77	255, 372	Nd	Nd
Cinnamic acid	12.22	277	78.5	0.16
Pyrogallol	12.77	276	13.6	0.68
Kaempferol	12.89	265, 365	Nd	Nd
Unknown	16.142	239, 270	16.4	Nd
Unknown	17.625	240	13.4	Nd

Nd: not determined.

DPPH radical scavenging assay

In methanol solution $DPPH^{\cdot}$, a stable organic N_2-centered free radical, produces violet colour with absorption maximum at 517 nm. Antioxidant molecules can quench $DPPH^{\cdot}$ by providing hydrogen atoms or by electron donation. Thus $DPPH^{\cdot}$ is converted to a colourless /bleached product i.e. 2, 2-diphenyl-1-hydrazine and as a result absorbance is decreased at 517 nm. Hence, the more rapidly absorbance decreases, the more effective antioxidant activity of extract.[27] This widespread test can be used to depict extent of quenching ability of free radicals by antioxidants across a short time scale.

The radical scavenging activity values of EfraMac were examined and compared with ascorbic acid, used as a positive control. Figure 5 showed that the potentiality of EfraMac for inhibiting free radical amplified with increasing concentration. The activity ranged from 11% to 62.59% at concentrations from 0.5 to 2 mg /ml. EC_{50} value of EfraMac (1.66 ± 0.06 mg/ml) was much higher than ascorbic acid (EC_{50} 4.3 ± 0.3 µg/ml). Compared with ethanol extract of *Pleurotus flabellatus* (EC_{50} 1.8 mg/ml), the results suggested that EfraMac showed well scavenging activity.[31]

Chelating ability of ferrous ions

Transition metals have been reported as catalyst to initiate radical formation. Ferrous iron, a transition metal, has capacity to reduce oxygen to superoxide radical and it can also catalyze decomposition of peroxide. It helps in generation of hydroxyl radical from hydrogen peroxide by Fenton's reaction. Chelating agents may stabilize transition metals in living systems and inhibit the generation of radicals; consequently reducing free radical induced damage.[32] Development of potential chelating agents from natural agents thus provides an effective way to protect human beings from free radical damage.

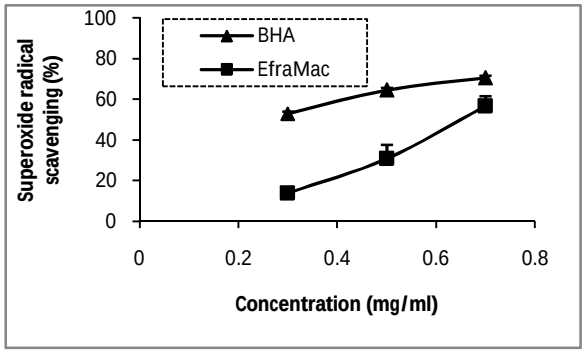

Figure 4: Superoxide radical scavenging activity of ethanol fraction from *Macrocybe crassa* (EfraMac). Results are mean ± SD of three separate experiments, each in triplicate.

Figure 6 shows that formation of the Fe^{2+} -ferrozine complex was prevented by EfraMac. Absorbance of the Fe^{2+} -ferrozine complex was decreased in a linear dose-dependent manner. Chelating effect was 12.96% at a concentration of 0.1 mg /ml which increased to 73.75% at 1 mg /ml concentration. At 0.53 ± 0.02 mg/ml concentration EfraMac revealed good chelating potencies

of 50%. EfraMac showed better activity than ethanol extract of *Grifola frondosa* as the fraction could chelate 51.7–75.4% at 5-20 mg /ml concentrations.[33] However, EDTA showed the strongest chelating ability.

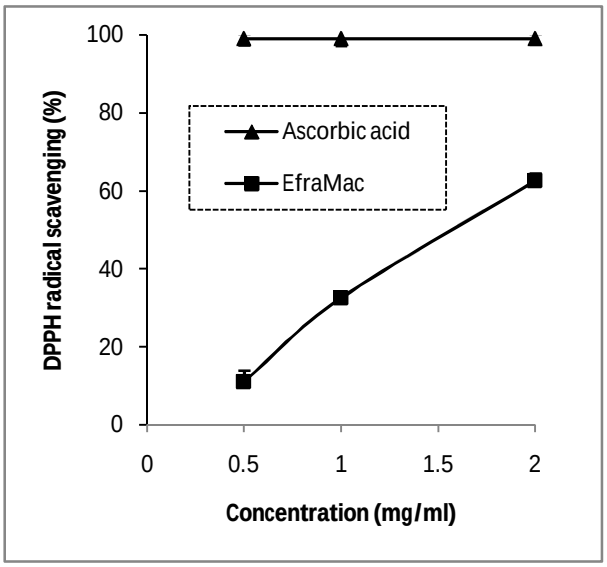

Figure 5: DPPH radical scavenging activity of ethanol fraction from *Macrocybe crassa* (EfraMac). Results are mean ± SD of three separate experiments, each in triplicate.

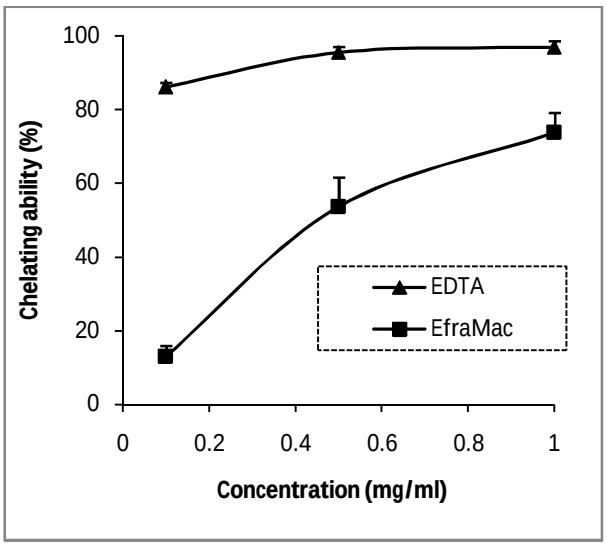

Figure 6: Ferrous ion chelating ability of ethanol fraction of *Macrocybe crassa* (EfraMac). Results are mean ± SD of three separate experiments, each in triplicate.

Determination of total antioxidant capacity (TAC)

Phosphomolybdenum method is a good system for evaluation of total antioxidant capacity. The assay is based on reduction of Mo (VI) to Mo (V) by the antioxidant compound and formation of green phosphate /Mo (V) complex at acidic pH.

Total antioxidant capacity of EfraMac was investigated and compared against ascorbic acid. EfraMac showed 12.71 ± 0.73 µg ascorbic acid equivalent /mg of dry extract antioxidant capacity.

Disk diffusion method

Recently, treatment of bacteria, fungi and protozoa with available antibiotics are becoming more difficult. A number of micro-organisms are becoming resistant to the existent drugs which are an emerging problem all over the world. On the other hand, mushrooms need various antifungal and antibacterial agents to survive in natural environment. As a result, now most of the commercial antimicrobial drugs are sourced from fungi.[34] Thus when commercial drugs are proved to be unproductive, search for new medicines from nature is a necessary alternative.

EfraMac was found to be active against all the tested Gram positive and Gram negative species (Table 2) except *Salmonella typhimurium*. This agrees with previous reports that, in general, Gram positive bacteria are considered more sensitive than Gram negative to different antimicrobial compounds because of the variances in structure of their cell walls.[35] Alves have reported that vanillic acid and *p*-coumaric acid have antibacterial effect against *E. coli*. *p*-coumaric acid can inhibit *E. coli* cell function by disrupting permeability of cell membrane and binding to DNA. There are also some reports regarding inhibition of *Staphylococcus aureus* by *p*-coumaric acid and caffeic acid.[5] Vanillic acid and *p*-coumaric acid and probably caffeic acid were present in EfraMac which might have played a key role in exhibiting antimicrobial effect of the fraction.

Table 2: Antibacterial effect of ethanol extract from *Macrocybe crassa* (MacPre). – is implied by <6 mm inhibition zone and + is implied by 6-8 mm inhibition zone.

Microorganism		EfraMac (2 mg /disc)
Gram (+)ve bacteria	*Listeria monocytogenes*	+
	Staphylococcus aureus	+
	Bacillus subtilis	+
Gram (-)ve bacteria	*Escherichia coli*	+
	Pseudomonas aeruginosa	+
	Salmonella typhimurium	-

CONCLUSION

In this study, for the first time, phenolic contents, antioxidant and antimicrobial activity of ethanol extract obtained from *Macrocybe crassa* (EfraMac) have been examined. Profiling of phenolic compounds exposed the abundance of pyrogallol. Other phenolics were present in the order of vanillic acid> cinnamic acid> *p*-coumaric acid. There were six unidentified compounds of which two might be benzoic acid derivatives and one could be caffeic acid as revealed by UV-absorption maxima. Besides these, EfraMac also contained flavonoid, ascorbic acid, β-carotene and lycopene. The extract was a good scavenger of free radicals, strong chelator of ferrous ion with high ascorbic acid equivalent antioxidant capacity. Furthermore, EfraMac exhibited antimicrobial effect

against Gram positive and Gram negative pathogenic bacteria which might be correlated with the presence of pyrogallol and benzoic acid derivatives in the fraction. Thus this study suggests that this wild edible mushroom, *Macrocybe crassa*, could be utilized as a potential source of natural antioxidant and antibiotics.

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