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REGULAR ARTICLE

Comparison of antioxidant properties in cap and stipe of *Lentinula edodes* –A medicinal mushroom

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ABSTRACT

Total phenolics, condensed tannins, ascorbic acid, total antioxidant activity, reducing power, ferric reducing antioxidant power, radical scavenging activity (RSA) on DPPH & ABTS and metal chelating activity of methanolic and aqueous extract from cap and stipe of *Lentinula edodes* have been evaluated. Different extracts contained 2.40-5.60 mg gallic acid equivalent of phenolics, 1.23-3.26 mg catechins equivalent of condensed tannins, and 2.31–11.96 mg ascorbic acid per gram of extract. Aqueous extract from cap contained higher phenolics, condensed tannins, ascorbic acid, RSA on DPPH and ABTS, reducing power, and metal chelating activity. We found that this mushroom species present antioxidant potential especially higher for cap indicating that the cap is the material that most contributes to the antioxidant activity.

Keywords: Antioxidant properties; *Lentinula edodes*; Cap; Stipe; Methanolic extract; Aqueous extract

INTRODUCTION

Oxidation is an important process through which energy is produced in biological systems. However, there are many reactive oxygen species and free radicals that are associated or formed as a result of the oxidation process. These reactive species often cause cell death and are involved in other degenerative processes associated with ageing. Reactive oxygen species (ROS) along with free radicals are found to play an important role in functional changes associated with diseases like cancer, rheumatoid arthritis, cirrhosis. Antioxidant containing natural foods can, however, be used to reduce the oxidative damage. Mushrooms have long been used as food because of their unique taste and subtle flavor. Experimental evidence indicates that mushrooms contain many biologically active compounds that offer health benefits and protection against degenerative diseases (Barros et al., 2008). They are rich sources for compounds like polyphenols, ascorbic acid, tocopherols, lycopene, β-carotene, carboxylic acids and various dietary fibers. There are various mushroom species, which are sources of physiological agents for medicinal applications, *viz.*, antiviral, anti-tumor, cardiovascular and antibacterial (Barros et al., 2007). Antioxidant properties of several specialty and edible mushrooms have been studied earlier (Babu and Rao, 2013; Mishra et al., 2013 and 2014). *Lentinula edodes* is the second most important edible mushroom in the world accounting 25% of the world mushroom production. It is capable of generating stamina, curing colds, improving the blood circulation, lowering serum cholesterol level and possessing anti-tumor and anti-viral activities (Mishra and Kushwaha, 2011). There has been no report on antioxidant activities of *Lentinula edodes* grown under North-Western Indian Himalayan conditions. The objective of the present investigation is to evaluate the antioxidant properties from cap and stipe of *L. edodes* including total phenolics, total antioxidant activity, reducing power, scavenging effects on radicals, metal chelating activity etc.

MATERIALS AND METHODS

Culture maintenance and cultivation

The mother cultures of *Lentinula edodes* obtained from Mushroom Research and Training Centre, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India were propagated using 2.0% Malt-Extract Agar (MEA) medium. Spawn of each mushroom

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species was prepared in 500 ml clean glass bottles using a mixture of wheat grains (200g), $CaCO_3$ (1.5 g) and $CaSO_4$ (6 g) as per standard methodology. Bottles were filled with the wheat grains, plugged with non-absorbent cotton and autoclaved at 121ºC for 90 min. After cooling, five mycelial-agar plugs (5 mm dia) were placed into the grain mixture and then incubated for one week at 24º C. Spawn was then shaken in order to evenly distribute mycelia on the grains. Spawn was incubated for an additional week, shaken again and then stored at 4ºC until used. The mushroom was cultivated as per standard methodology (Mishra and Kushwaha, 2011).

Sample preparation

Freshly harvested mushroom fruiting bodies were shade dried and grinded in a super mill grinder 1500 series (Newport Scientific Pvt. Ltd.). Mushroom powder (2g) was extracted with 100mL methanol and water separately, using semiautomatic soxhlet apparatus (pelican, socsplus, 2AS, Chennai). The methanolic and aqueous extracts from cap and stipe of *L. edodes* were evaporated at 40ºC to dryness, re-dissolved in methanol at a concentration of 10 mg/ml and stored at 4°C to measure antioxidant activities and related parameters. Estimation of different antioxidant activities and bioactive compounds were carried out in triplicate.

Determination of total polyphenol

Total polyphenol (TPP) in the mushroom samples was estimated by a colorimetric assay, based on standard procedures with some modifications (Singleton and Rossi, 1965). Briefly, 1.0 ml of mushroom extract (10mg/ml) was mixed with Folin and Ciocalteu's phenol reagent (1.0 ml). After 3 min, 1.0 ml of saturated sodium carbonate solution was added to the mixture and volume was adjusted to 10 ml with distilled water. The reaction was kept in dark for 90 min, after which the absorbance was read at 725 nm (Thermo scientific chemito spectrascan UV 2600 spectrophotometer). Gallic acid was used to calculate the standard curve (1-80 µg/ml). The results were mean values + standard deviations and expressed as mg of gallic acid equivalent (GAE)/g of extract.

Determination of Condensed Tannins and ascorbic acid

Condensed tannins (CT) were estimated using the standard method with some modification (Sun et al., 1998). To the freshly prepared extracts (0.10 ml), methanol (0.90 ml) was added followed by 1% vanillin reagent (2.50 ml) and 9 M HCl (2.50 ml). The solution was mixed thoroughly and absorbance was recorded at 500 nm after 20 minutes incubation at 30ºC. Condensed tannins content was calculated from the standard calibration curve based on catechins (0-10mg/mL). Ascorbic acid (AA) was determined according to the volumetric method (Thimmaiah, 1999). Ten milliliter of 4% oxalic acid was added to standard solution of vitamin C (100 µM, purity 99%) and the resulting solution was titrated against 2, 6-dichloroindophenol dye until a pink colour end point was obtained (titer value V_1). Again, dried methanolic extract of each sample (100 mg) was extracted with 4% oxalic acid and volume was made to 100 ml. The filtered extract (5 ml) was mixed with 10 ml of 4% oxalic acid and titrated against 2, 6-dichloroindophenol dye until a pink colour end point was obtained (titer value V_2). Ascorbic acid content was calculated based on the following equation:

$$\text{Amount of ascorbic acid (mg/100 g sample)} = [(0.5 \text{ mg} \times V_2 \times 100 \text{ ml})/(V_1 \times 15 \text{ ml} \times \text{Wt. of samples})] \times 100$$

Where, V_1 and V_2 were the volume of the dye used to titrate vitamin C and sample extract, respectively. The result was expressed as mg ascorbic acid/g extract.

Determination of radical scavenging activity on DPPH and ABTS

Radical scavenging activity (RSA) on DPPH (2, 2-diphenyl-1-picrylhydrazyl) was determined by measuring the decrease in absorbance of methanolic DPPH solution at 515 nm in the presence of the extract (Brand-Williams et al., 1995) with some modifications. The stock solution prepared by dissolving 24 mg of DPPH in 100 mL methanol was stored at -20C° and working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to get an absorbance of 1.17 ± 0.02 units at 515 nm. Mushroom samples (150 µL) of different concentrations (5 - 25 mg/ml) were allowed to react with 2850 µL of DPPH working solution for 24 h in dark and absorbance was read at 515 nm. Radical scavenging activity was calculated as a percentage of DPPH discoloration by the equation:

$$\text{RSA on DPPH (\%)} = [(A_{control} - A_{sample})/(A_{control})] \times 100$$

Where, A_{sample} is the absorbance of the solution recorded during addition of extract/reference at a particular level, and $A_{control}$ is the absorbance of the DPPH solution without addition of extract.

The radical scavenging activity (RSA) on ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] was determined by measuring the decrease in absorbance of methanolic ABTS solution at 745 nm in the presence of the extract (Arnao et al., 2001). The stock solutions; 7.0 mM ABTS and 2.3 mM ammonium persulfate, were prepared. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The working solution was diluted by mixing 1.0 mL ABTS solution with 3.0 mL

methanol to obtain an absorbance of 0.9 ± 0.02 units at 745 nm. Mushroom sample extracts (200 µL) of different concentrations (5 - 25 mg/ml) were allowed to react with 2000 µL of the freshly prepared ABTS solution for 30 min in dark condition and absorbance was taken at 745 nm. The percentage inhibition was calculated by the equation:

$$\text{RSA on ABTS (\%)} = [(A_{control} - A_{sample})/(A_{control})] \times 100$$

Where, A_{sample} is the absorbance of the solution recorded during addition of extract/reference at a particular level and $A_{control}$ is the absorbance of the ABTS solution without addition of extract.

Determination of ferric reducing antioxidant power (FRAP)

The FRAP assay was done according to the method of Benzie and Strain (1996) with some modifications. The stock solutions 300mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution were prepared. The fresh working FRAP solution was prepared by mixing 25mL acetate buffer, 2.5mL TPTZ solution and 2.5mL $FeCl_3.6H_2O$ solution and warmed at 37^{0}C before use. Mushroom extracts (150 µL) of different concentrations (5-25 mg/ml) were allowed to react with 2850 µL of the FRAP solution for 30 min in dark condition and absorbance of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The FRAP value was determined by plotting a standard curve obtained by addition of ferrous sulfate heptahydrate (Merck, Darmstadt, Germany) to the FRAP reagent. The results were expressed in µM equivalent to $FeSO_4.7H_2O$ as FRAP value.

Determination of total antioxidant activity

The total antioxidant activity of mushroom extracts was estimated using the phosphomolybdenum method (Prieto et al., 1999) based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of specific green phosphate/Mo (V) compounds. A 0.3 ml aliquot of extract solution (5-25 mg/ml) combined with 2.7 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was capped and incubated in a boiling water bath at 95°C for 90 min. Samples were allowed to cool at room temperature and absorbance was measured at 695 nm. For the blank, 0.3 ml methanol/double distilled water was mixed with 2.7 ml of the reagent. A standard curve of trolox (10-100µM) was prepared and total antioxidant activity was expressed as µM trolox equivalent.

Reducing power assay

The reducing power (RP) was determined according to the standard methodology (Huda-Faujan et al., 2009). Various concentrations (5-25 mg/ml) of methanolic/aqueous extracts (200 µl) were taken and volume was made to 1.0 ml by adding distilled water, followed by addition of 2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The resulting mixture was incubated at 50^{o}C for 20 min. Furthermore, 2.5 ml of 10% trichloroacetic acid (w/v) were added and centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of de-ionized water and 0.5 ml of 0.1% of ferric chloride. The absorbance was measured at 700 nm; higher absorbance indicates higher reducing power.

Determination of metal chelating activity

The chelating of ferrous ions by mushroom extract was determined by the modified method (Dinis et al., 1994). The sample (0.5 ml) of different concentrations (5-25 mg/ml) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml). The mixture was shaken vigorously, kept at room temperature for 10 min and absorbance of the resulting solution was measured at 562 nm. The metal chelating activity (MCA) was calculated according to the following equation:

$$\text{Metal chelating activity (\%)} = [(A_{control} - A_{sample})/(A_{control})] \times 100$$

Where, A_{sample} is the absorbance of the solution recorded during addition of extract at a particular level and $A_{control}$ is the absorbance of the $FeCl_2$ and ferrozine solution without addition of extract.

Statistical analysis

The statistical analyses were performed using the statistical package SPSS (Statistical Package for Social Science, SPSS Inc., Chicago, IL). Analyses of variance were performed by ANOVA and significance of each group was verified with one-way analysis of variance followed by Duncan's multiple range test ($P<0.05$). For multifactorial comparison, principal component analyses (PCA) was used to display the correlation between the various parameters and their relationship with the different mushroom extract. Varimax rotation was performed to produce orthogonal transformations to the reduced factors to identify the high and low correlations better. Multifactorial analysis was carried out using the XLStat-Pro 7.5 (Addinsoft, New York, USA) software.

RESULTS AND DISCUSSION

Total polyphenols

The content of phenolic compounds could be used as an important indicator of antioxidant capacity. It has been

reported that the antioxidant activity of plant materials is well correlated with the content of their phenolic compounds (Pan et al., 2008). Total phenolic contents for the analysed mushroom samples evaluated by the Folin–Ciocalteu method are shown in Fig. 1. Results were expressed as mg of gallic acid equivalents/g of mushroom extract. The amount of total phenolics in cap aqueous extract was higher (5.60 ± 0.11 mg GAE/g extract) while other had lower phenolic contents, with stipe methanolic extract showing the lowest amount (2.40 ± 0.11 mg GAE/g extract). In the present study, the extracts from the cap showed higher phenolic content than the stipe, in general (Fig. 1) compared to the reported values in other mushrooms such as cap methanolic extracts for *Lactarius deliciosus* (10.66 ± 0.52 mg GAE/g), *Tricholoma portentosum* (6.57 ± 0.31 mg GAE/g) and *Hypsizygus ulmarius* (26.72 ± 0.50 mg GAE/g) (Ferreira et al., 2007; Babu and Rao, 2013). Mushroom extracts have high levels of phenolic compounds, composed of one or more aromatic rings bearing one or more hydroxyl groups, which can exhibit extensive free radical-scavenging activities as hydrogen donors or electron-donating agents and metal ion-chelating properties. The greater numbers of hydroxyl groups in the phenolics could exhibit higher antioxidant activity (Rangkadilok et al., 2007).

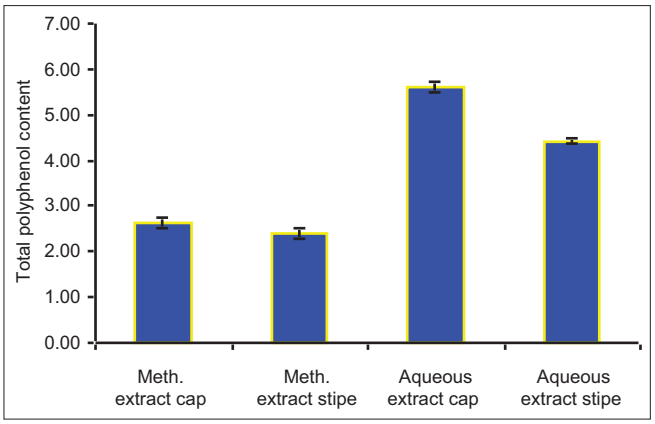

Fig 1. Total phenolics content (mg GAE/g dry weight mushroom) in methanolic and aqueous extract from *L. edodes* cap and stipe. All data are means of three (n=3) independent measurements ± standard error.

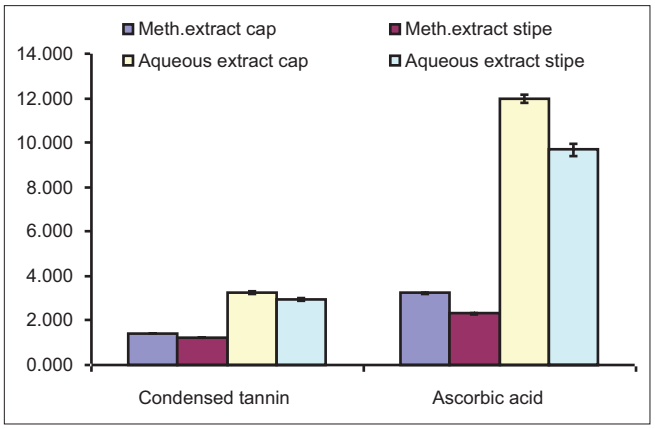

Fig 2. Condensed tannin and ascorbic acid content in methanolic and aqueous extract from *L. edodes* cap and stipe.

Condensed tannin and ascorbic acid content

Flavonoids are the most common and widely distributed groups of plant phenolic compounds which usually are very effective antioxidants. The condensed tannins (catechins) from the mushroom samples were assayed by vanillin-HCl colorimetric assay and expressed in mg catechins/g extract. The result showed that condensed tannins of various extracts varied considerably from 1.23-3.26 mg catechin/g extract (Fig. 2). The amount of total condensed tannins in cap aqueous extract was higher (3.26 ± 0.07 mg catechin/g extract) while stipe methanolic extract showed the lowest amount (1.23 ± 0.01 mg catechin/g extract). Sudha et al. (2012) also reported that total flavonoid content of various extracts of *Pleurotus eous* varied considerably from 6.38-7.79 mg CAE/g extract. Ascorbic acid in the mushroom sample extracts are shown in Fig. 2. Ascorbic acid varied considerably from 2.31–11.96 mg/g extract, which is in agreement with other authors (Mau et al., 2002; Tsai et al., 2009). Ascorbate is a powerful secondary antioxidant, reducing the oxidized form of α-tocopherol, an important antioxidant in non-aqueous phase (Padh, 1990).

Radical scavenging activity on DPPH and ABTS

Free radical scavenging is one of the mechanisms in inhibiting lipid oxidation commonly used to estimate antioxidant activity. The radical scavenging activity (RSA) of mushroom extracts was tested against the ABTS and DPPH. ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain breaking antioxidants (Leong and Shui, 2002). RSA on DPPH and ABTS of different mushroom extracts varied from 4.40-89.58 and 9.32-99.61, respectively (Fig. 3) and increased with increase in concentration. RSA on DPPH was higher for cap aqueous extract (89.58 at 25mg/mL extract concentration) while stipe methanolic extract showed lowest RSA on DPPH (4.40 at 5 mg/mL extract concentration). The scavenging activity was better in aqueous extract when compared to methanolic extract. RSA on ABTS was also better in aqueous extract in comparison to methanolic extract and found to be higher in cap aqueous extract (99.61 at 25 mg/mL extract concentration). The extracts obtained from the mushroom cap proved to have better scavenging effect (Ferreira et al., 2007). Scavenging effects of the acetonic, methanolic and aqueous extracts from the fruiting bodies of *P. salmoneostramineus* and *P. citrinopileatus* increased with increasing concentrations (Alam et al., 2011a and b). With the presence of radical scavenging activity, consumption of this mushroom species might be beneficial to protect human body against oxidative damage, which can be further developed into health related degenerative illnesses.

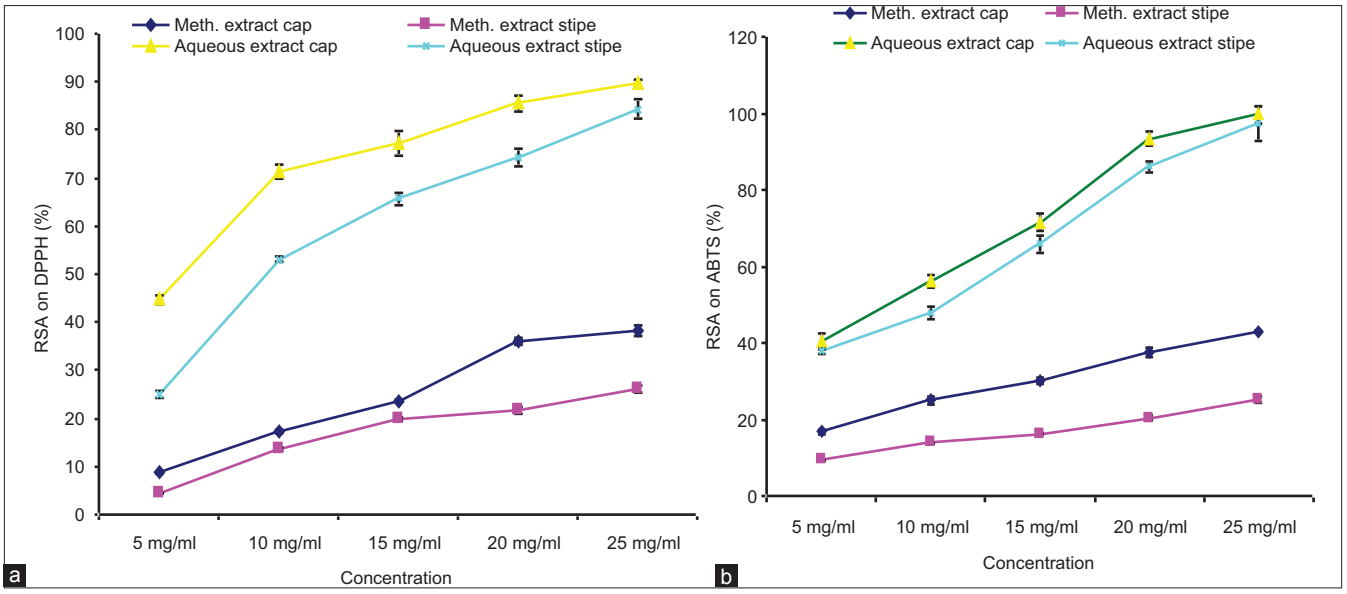

Fig 3. (a & b) Radical scavenging activity on DPPH and ABTS in methanolic and aqueous extract from *L. edodes* cap and stipe. All data are means of three (n=3) independent measurements ± standard error.

Ferric reducing antioxidant power (FRAP)

All the extracts from mushroom species parts showed increased FRAP with the increase in concentration (Fig. 4). At 25 mg/mL concentration, the ferric reducing power of cap aqueous extract was found to be higher (332.77 ± 8.5 µM $FeSO_4$ equivalent) whereas, the lowest FRAP value was obtained in stipe methanolic extract at 5 mg/ml concentration (23.17 ± 0.23 µM $FeSO_4$ equivalent). Excellent FRAP in *Hypsizygus ulmarius* cap compared to other extracts were reported (Babu and Rao, 2013). The results revealed that cap aqueous extract possessed hydrogen-donating capacity indicating the significant reducing power of the extract.

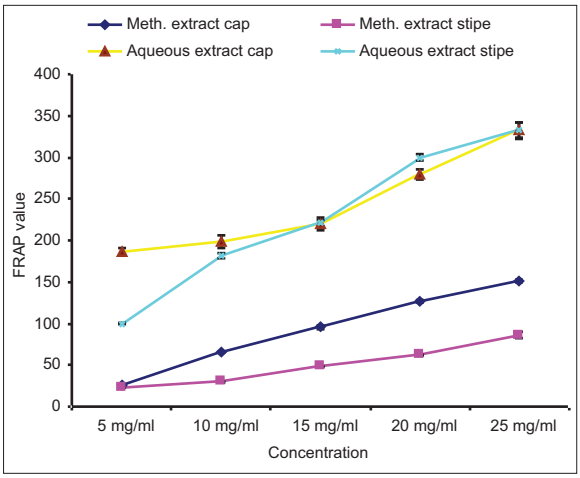

Fig 4. FRAP value in methanolic and aqueous extract from *L. edodes* cap and stipe. All data are means of three (n=3) independent measurements ± standard error.

Total antioxidant activity

Total antioxidant activities of methanolic and aqueous extracts from cap and stipe increased with increase in concentration (Fig. 5). At 25 mg/ml, antioxidant activities were 164.76, 154.42, 154.07, 215.49 µM trolox equivalent for cap methanolic extract, stipe methanolic extract, cap aqueous extract and stipe aqueous extract, respectively. The antioxidant activities of three extracts from fruit bodies, mycelia and fermentation filtrate of *Pleurotus citrinopileatus* increased with concentration (Lee et al., 2007).

Reducing power

Reducing power of the methanolic and aqueous extracts was excellent and increased steadily with the increased concentration (Fig. 6). The reducing powers of methanolic and aqueous extracts of cap were 0.34 ± 0.004 and 0.54 ± 0.005, respectively at 25 mg/mL concentration. The reducing powers of methanolic and aqueous extracts of *Pleurotus eous* were reported to be 0.587 and 0.673 at 2.0 mg/mL, respectively (Sudha et al., 2012), however, cap and stipe methanolic extracts of *Hypsizygus ulmarius* and *Calocybe indica* were 2.46, 2.53, 1.71 and 2.05, respectively (Babu and Rao, 2013).The high reducing power exhibited by the extracts might be indicative of the hydrogen donating ability of the active species present in the extracts.

Metal chelating activity

The metal chelating activity of different mushroom species extracts on ferrous ions increased with increase in concentration (Fig. 7). The chelating activity of cap aqueous extract (88.86 ± 1.2% at 25 mg/mL extract concentration) was higher followed by stipe aqueous extract (78.93 ± 1.9% at 25 mg/mL extract concentration) while lowest was shown by cap methanolic extract (7.6 ± 0.09% at 5 mg/mL extract

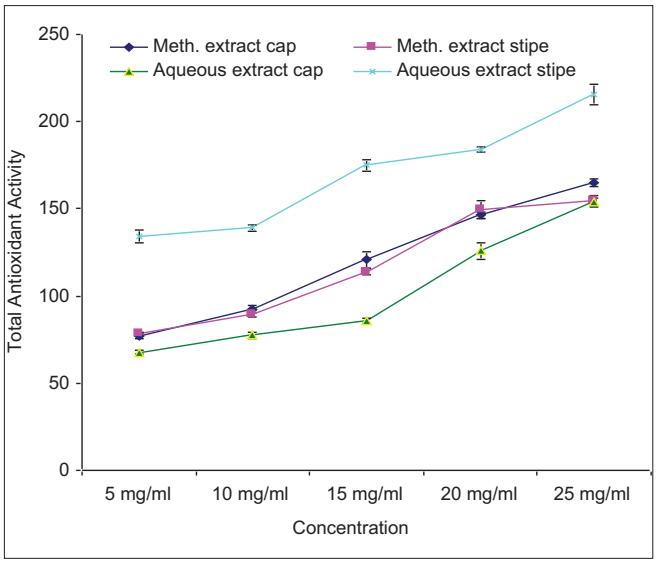

Fig 5. Total antioxidant activity in methanolic and aqueous extract from *L. edodes* cap and stipe. All data are means of three (n=3) independent measurements ± standard error.

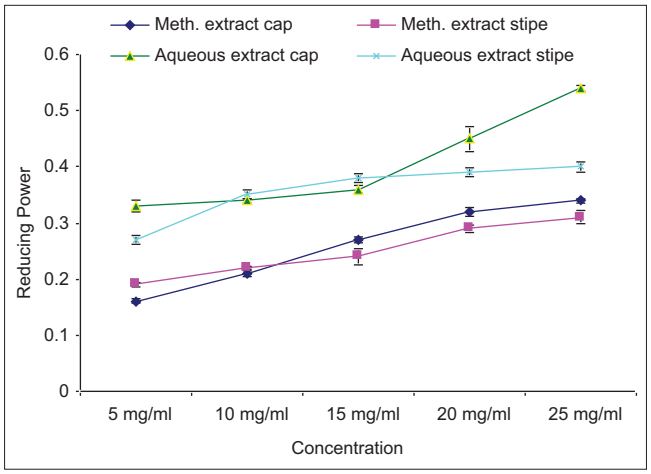

Fig 6. Reducing power in methanolic and aqueous extract from *L. edodes* cap and stipe. All data are means of three (n=3) independent measurements ± standard error.

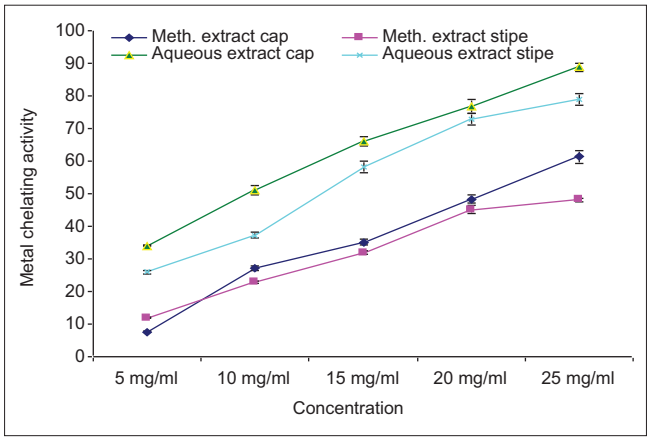

Fig 7. Metal chelating activity in methanolic and aqueous extract from *L. edodes* cap and stipe. All data are means of three (n=3) independent measurements ± standard error.

concentration). Many workers also reported 75.6-92.6% chelating ability of *H. marmoreus* at 1~5 mg/mL (Lee et al., 2007). Chelating agent may serve as secondary anti-oxidants as they reduce redox potential and stabilize the oxidized forms of metal ions. Since ferrous ions are the most effective pro-oxidants in food system, the high ferrous ion chelating abilities of the extracts would be beneficial. This study could provide valuable information to support this two mushroom species as an excellent source of antioxidants in human diet.

Multivariate analysis

Principal Component Analysis (PCA) is a useful statistical technique which has found application in reduction of the original variables (total phenolics, condensed tannins, ascorbic acid content, total antioxidant activity, reducing power, FRAP, RSA on ABTS and DPPH, and metal chelating activity) in a smaller number of underlying variables (Principal Component) in order to reveal the interrelationships between the different variables and to find the optimum number of extracted principal components. The first principal component (PC1) accounted for 82.16% variability in the data set while second principal component (PC2) accounted for 16.57% variability. The principal component analysis (PCA) and their correlation are shown in Fig. 8. All parameters occupied the right side of the biplot and among the parameters the total antioxidant activity was observed on the right upper side of the biplot with high positive loading for both PC1 and PC2, while total polyphenols, RSA on ABTS and DPPH, ascorbic acid, reducing power, FRAP, metal chelating activity, condensed tannins were grouped together with slightly lesser positive loadings on the right side of the biplot. This suggests total antioxidant activity is having positive correlation with aqueous extract from stipe of *L. edodes*. PCA revealed that methanolic and aqueous extract from mushroom cap and stipe were grouped into four clusters (Fig.8). The first group consisting of aqueous extract from cap of *L. edodes* showed strong positive correlation with both PC1 and PC2 while second group contained aqueous extract from cap was positively correlated with PC1 and negatively correlated with PC2. Third group was negatively correlated with both PC1 and PC2 having methanolic extract from stipe, however, fourth group contained methanolic extract from cap indicating high positive loading with PC2 and negative to PC1.

The results also showed that aqueous extract from cap contained higher total polyphenols, RSA on ABTS and DPPH, ascorbic acid, reducing power, FRAP, metal chelating activity, condensed tannins.

In the present investigation, we found that *Lentinula edodes* present antioxidant potential especially higher for cap. It appears that the cap is the material that most contributes

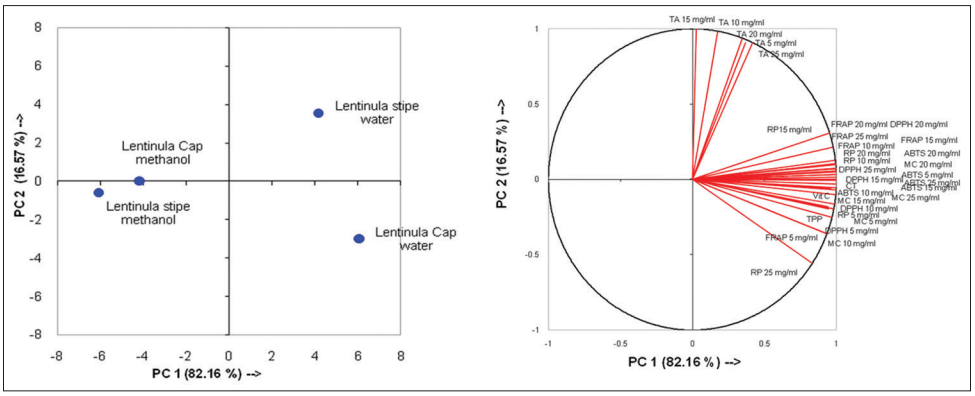

Fig 8. Multifactorial comparison of methanolic and aqueous extracts from cap and stipe of *L. edodes* and various parameters using principal component analysis (PCA).

to the antioxidant activity. The variety of compounds and the antioxidant potential revealed by the analyzed mushroom species contribute to the knowledge of this mushroom species of great consumption in India and possible beneficial effect for human health.

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Author contributions

K. K. M. made a major contribution in culture maintenance and cultivation of *Lentinula edodes*, sample preparation, manuscript writing, editing and finally communicating the article. R. S. P. carried out all the antioxidant activities determination, writing of biochemical part. J. C. B. facilitate to carry out the experiment, was involved in overall planning and supervision.

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