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Antioxidant Activities and Bioactive Compounds of Polyphenol Rich Extract from Amanita vaginata (Bull.) Lam.

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Abstract: Polyphenol rich fraction from *Amanita vaginata*, a wild edible mushroom, was prepared to investigate its antioxidant activity and presence of various bioactive constituents. The fraction possessed 5 different types of components in a consequence of phenol> ß-carotene> flavonoid> lycopene> ascorbic acid. The extract was extremely good in hydroxyl radical scavenging (EC_{50}= 0.01 mg/ml). EC_{50} values for rest four different antioxidant assays were between 1.2 mg/ml to 1.75 mg/ml. Thus the mushroom species can be used as an easily accessible source of natural antioxidants or in pharmaceutical industry.
Key words: DPPH, free radicals, hydroxyl radicals, phenolic compound, wild edible mushroom.

Introduction:

Free radicals derived from molecular oxygen (O_2) are usually known by reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, hydrogen peroxide etc and represent the most important class of radical species generated in living systems[1]. Free radicals are produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes[2]. However, the uncontrolled production of oxygen derived free radicals can lead to many diseases such as cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes associated with aging. In order to reduce damage to the human body, many synthetic antioxidants are used widely at the present time[3]. Antioxidants in biological systems have multiple functions, including defending against oxidative damage and participating in the major signalling pathways of cells. One major action of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species[4].

Mushrooms have been part of the normal human diet for thousands of years and in recent times, the amounts consumed have risen greatly involving a large number of species. Mushrooms are considered as rich food because they contain protein, sugar, glycogen, lipid, vitamins, amino acids and crude fiber. They also contain important minerals required for normal functioning of the body[5]. Now-a-days, mushrooms have become attractive as functional foods and as a source of physiologically beneficial medicines, while being devoid of undesirable side effects. Recent investigation revelled mushroom has potentiality to combat against cancer, heart ailminats, dibetes, inflammation, hepatic damage, high blood pressure, microbial pathogens etc[6-12] Furthermore polysaccharides and extracts of mushrooms have strong antioxidant properties[13-18].

Amanita vaginata is an edible mushroom widely found in the lateritic regions of Sal forests in West Bengal. Local people called them as 'Sal Chattu', as they are ectomycorrhizal with Sal trees (*Shorea robusta* Gaertn. F.)[19]. Therefore, this paper was concerned with the extraction, composition analysis of polyphenolic rich fraction of *Amanita vaginata* (AvaPre) and explored their antioxidant activities for seeking new biological functional components used in food and pharmaceutical industry.

Materials and Methods:

Chemicals:

BHT (butylated hydroxytoluene), L-ascorbic acid, quercetin, galic acid, EDTA (ethylenediaminetetraacetic acid), potassium ferricyanide, ferrous chloride, Folin-Ciocalteu reagent, NBT (nitroblue tetrazolium), DPPH (1,1-diphenyl 1-2-picrylhydrazyl), TCA (trichlroacetic acid), deoxyribose, aluminum nitrate, potassium acetate, ß-carotene, lycopene, sodium phosphate, ammonium molybdate, methionine and riboflavin were purchased from Sigma chemicals Co. (St. Louis, MO, USA). All other chemicals are of analytical grade.

Sample collection:

The fruit body of *Amanita vaginata* (Bull.) Lam. (Basidomycetes) were collected from the forest of Midnapur, West Bengal, India and identified according to following standerd referance[20]. The voucher specimen was deposited at the Mycological Herbarium of Department of Botany, University of Calcutta, Kolkata, West Bengal, India (AMFH-507).

Extraction procedure:

Polyphenol rich fraction was extracted according to the method of Cui *et al*, (2005)[21] with slight modification. Dried and powdered basidiocarps of *A. vaginata* were extracted with ethanol at 25˚C for 2 days to eliminate the alcohol soluble constituents such as coloured material, small organic molecules (steroid, terpenoids etc.) and fat. After filtration, the residue was then re-extracted with ethanol, as described above. The filtrate was air dried, extracted by stirring with distilled water at 100˚C for 8 hrs. After filtration, 4 volume of ethanol was added to the supernatant slowly and kept at 4˚C overnight. Precipitate was discarded by centrifugation and the supernatant was concentrated under reduced pressure in a rotary evaporator. Now, this concentrated polyphenol rich extract of *A. vaginata* (AvaPre) was stored at 4˚C until further analysis. The percentage yield extracts were calculated based on dry weight as:

Yield (%) = (W1 × 100)/ W2

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

Determination of total phenolic compound:

Total phenolics was measured using Folin-Ciocalteu reagent[22]. 1 ml AvaPre was mixed with 1ml Folin-Ciocalteu reagent and incubated for 3 minutes at room temperature. After incubation, 1ml of 35% saturated Na_2CO_3 solution was added in the reaction mixture and volume was adjusted to 10 ml with distilled water. The reaction mixture was incubated in dark for 90 min, after which the absorbance was read at 725 nm by a spectrophotometer. Gallic acid was used as standard. Total phenol content of the sample was expressed as µg of gallic acid equivalent per mg of extract.

Determination of total flavonid content:

Flavonoid concentration was determined by the method as described[23]. 1 ml of AvaPre containing was diluted with 4.3 ml of 80% aqueous methanol and subsequently 0.1 ml of 10% aluminum nitrate and 0.1 ml of 1M aqueous potassium acetate were added. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as standard.

Determination of total ß-carotene and lycopene content:

Total ß-carotene and lycopene was determined according to Nagata and Yamashita, (1992)[24]. The process, in brief was 100 mg of AvaPre was vigorously shaken with 10 ml of acetone-hexane mixture (4:6) for 1 min and absorbance of the mixture was measured at 453, 505 and 663 nm. ß-carotene and lycopene contents were calculated according to the following equations:

Lycopene (mg/100ml) = $-0.0458A_{663} + 0.372A_{505} - 0.0806A_{453.}$

ß-carotene (mg/100ml) = $0.216A_{663} - 0.304A_{505} + 0.452A_{453}$

Determination of ascorbic acid content:

Ascorbic acid was determined by titration as described by Rekha *et al*, (2012)[25] with some modification. Standard ascorbic acid (0.1 mg/ml) was taken in a conical flask and made up to 10 ml by 0.6% oxalic acid. It was titrated with a dye 2, 6-dichlorophenol indophenol (21 mg sodium bicarbonate and 26 mg of dye in 100 ml water). The amount of dye consumed (V_1 ml) is equivalent to the amount of ascorbic acid. The sample (w µg/ml) was similarly titrated with the dye (V_2 ml). The amount of ascorbic acid was calculated using the formula,

Ascorbic acid (µg /mg) = [{(10 µg /V_1ml) × V_2 ml} × w µg] × 1000.

Antioxidant activity of the mushroom fraction:

Total antioxidant capacity assay:

The total antioxidant capacity was determined as described by Prieto *et al,* (1999)[26] with some modification. 0.3 ml of AvaPre with varying concentration (0.1-1 mg/ml) was added to 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

Superoxide radical scavenging capacity:

The method by Martinez *et al,* (2001)[27] used for determination of the superoxide dismutase was followed with modification in the riboflavin-light-nitrobluetetrazolium (NBT) system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, sample solution of various concentrations (0.5-1.5 mg/ml) of AvaPre, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. Reaction started by illuminating sample with light and the increased absorbance was measured at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in the dark and served as blank. Butylated hydroxyanisole (BHA) was used as a positive control.

Hydroxyl radical scavenging capacity:

The reaction mixture (1ml) consisted of KH_2PO_4 - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (0.01-0.03 mg/ml) of AvaPre, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbate (100 µM) and H_2O_2 (1 mM). It was incubated at 37°C for 1 h. 2ml TBA-TCA solution (100ml containing 375mg TBA, 15mg TCA, 2ml concentrated HCl added to 98ml of TBA-TCA solution) were added and incubated at 100°C for 15 min. After cooling absorbance was measured at 535 nm against sample where 2ml TBA-TCA was added prior to reaction mixture. BHT was used as positive control[28].

Ferous ion chelating activity:

The ability of the AvaPre to chelate ferrous ions was estimated as described[29]. Briefly, the extract 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), and the mixture was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the

solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_1) / A_0] \times 100$, where A_0 was the absorbance of the control and A_1 of the mixture containing the extract or the absorbance of a standard solution.

Reducing power:

Reducing power of AvaPre was determined following the method of Oyaizu, (1986)[30]. Variable concentrations of AvaPre (0.5-1.5 mg/ml) were added to 2.5 ml 0.2 M phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50˚C for 20 min. 2.5 ml of 10% TCA was added to the mixture. 2.5 ml of the reaction mixture was mixed with 2.5 ml distilled water and 0.5 ml 0.1% ferric chloride and absorbance was measured at 700 nm.

DPPH radical scavinging activity:

Different concentrations of AvaPre (0.5-2 mg/ml) was added to 0.004% methanolic solution of DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark. Absorbance was measured at 517 nm against a blank. EC_{50} value is the effective concentration of extract that scavenged DPPH radicals by 50% and it was obtained by interpolation from linear regression analysis[31].

Results and Discussion:

Bioactive components:

AvaPre was brown in colour and stickey in nature. The extractive value was 1.845%. Naturally occurring antioxidant components namely phenols, flavonoids, ascorbic acid, β-carotene and lycopene were present in AvaPre. Phenols are very important constituents because they have good scavenging ability due to presence of hydroxyl group[32] and it plays an important role in stabilizing lipid peroxidation[33]. AvaPre was found to content significant level of phenolic compound (5.335 ± 0.28 µg gallic acid equivalent/mg of extract). Numerous studies have showed the consumption of foods high in phenolics can reduce the risk of heart disease by slowing the progression of atherosclerosis due to their antioxidative properties[34].

Flavonoids are naturally occurring substances with variable phenolic structures. Flavonoids are very good antioxidant and have free radical scavenging ability. The antiradical property is directed mostly toward hydroxyl and singlet oxygen as well as peroxyl and alkoxyl radicals. Flavonoids content in AvaPre was 0.81 ± 0.06 µg quercetin equivalent/mg. Previous studies on *in vitro* experimental systems also showed that flavonoids possess antiinflammatory, antiallergic, antiviral and anticarcinogenic properties [35, 36].

Lycopene is a highly unsaturated straight chain hydrocarbon with a total of 13 double bonds, 11 of which are conjugated. This unique nature of the lycopene molecule makes it a very potent antioxidant**.** Several studies are being reported in the literature on the effect of lycopene in the prevention of cancers and coronary heart disease. Few

studies have also evaluated its role in hypertension, male infertility and neurodegenerative diseases[37]. β-Carotene is a naturally occurring orange-colored carbon-hydrogen carotenoid, abundant in yellow-orange fruits and vegetables and in dark green, leafy vegetables. It acts as powerful antioxidant and is known to have immunomodulatory effect[38]. AvaPre also contained ß-carotene and lycopene which were 1 ± 0.06 µg/mg, 0.6 ± 0.03 µg/mg of extract respectively.

Ascorbic acid is considered important for its antioxidant propriety. It acts as an electron donor and reducing agent, therefore its antioxidant properties prevent other compounds from being oxidized[39] and it also reportedly reduces the risk of arteriosclerosis, cardiovascular diseases and some forms of cancer[25]. Ascorbic acid was present in AvaPre in an amount of 0.00035 ± 0.00005 µg/mg of extract.

Total antioxidant capacity:

Result showed 1 mg of sample fraction is equivalent to 225 ± 3.5 µg of ascorbic acid. Total antioxidant activity suggests that the electron donating capacity of AvaPre and thus it may act as radical chain terminator, ultimately transforming reactive free radicals into more stable non-reactive products.

Superoxide radical scavenging capacity:

Superoxide radical is known to be very harmful to cellular components as a percursor of more reactive species. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxy nitrite which is a potent oxidant that causes nitrosative stress in the organ systems[5]. In the present study, AvaPre was found to be a notable scavenger of superoxide radicals generated in riboflavin- nitroblue tetrazolium (NBT) light system (Fig.1). The EC_{50} value of the fraction was determined as 1.2 mg/ml.

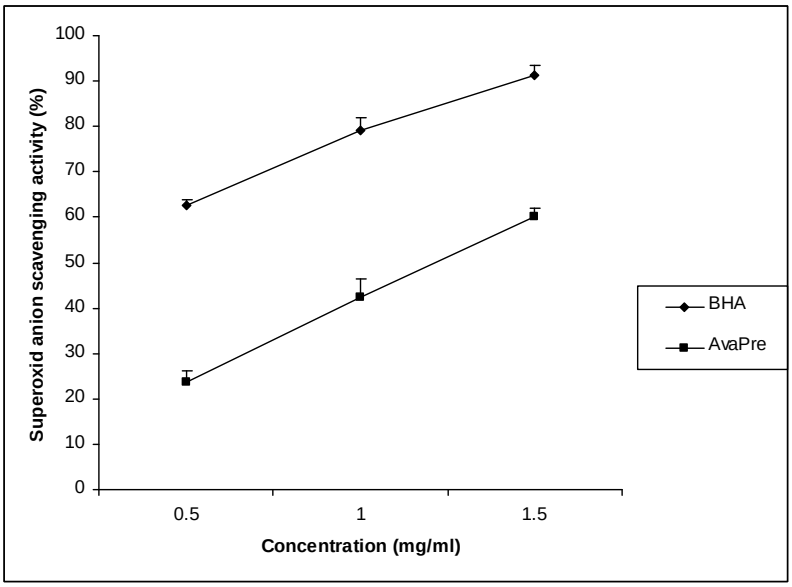

Fig 1: Superoxide radical scavenging activity of AvaPre.
Results are the mean ± SD of three separate experiments, each in triplicate.

Hydroxyl radical scavenging capacity:

Hydroxyl radical ($OH^{\cdot}$) has a very short life time but is considered to be the most toxic among all ROS. It can damage DNA by attacking purines, pyrimidines and deoxyribose. Hydroxyl radicals are formed by an electron transfer from transition metals to H_2O_2 and interact with biomolecules immediately after formation[40]. In our experiment, hydroxyl radicals are generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535 nm[41]. When test sample was added to reaction mixture, they removed hydroxyl radicals from sugar and prevented their degradation. The extract showed potent hydroxyl radical scavenging activity which raised gradually with the increase of concentration (Fig. 2) and the EC_{50} value of the AvaPre was found to be 0.01 mg/ml.

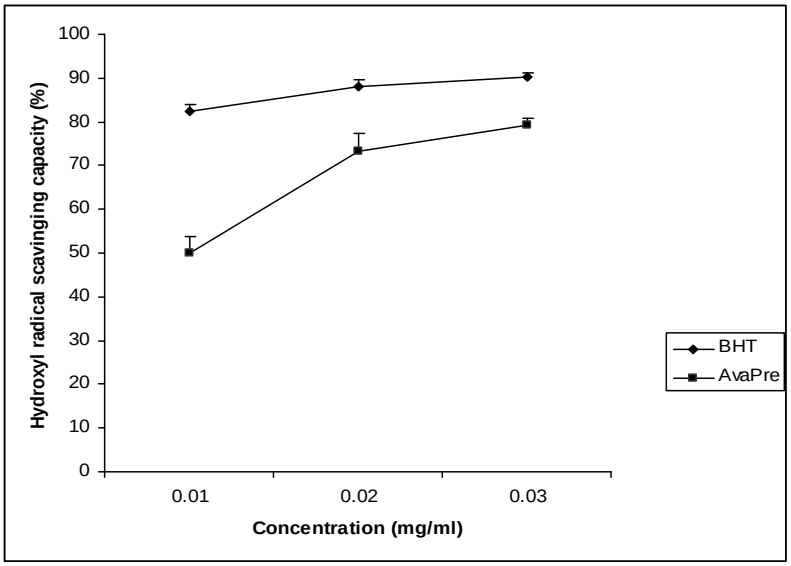

Fig 2: Hydroxyl radical scavenging activity of AvaPre.

Results are the mean ± SD of three separate experiments, each in triplicate.

Ferous ion cheleting ability:

Dietary nutrients containing metal chelators may act as preventive antioxidant because some transition metals e.g. Fe^{2+}, Cu^{+}, Pb^{2+}, Co^{2+} and so on could trigger process of free radical reaction[42]. Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agent, the complex formation is disrupted, thus resulting in the reduction of red colour. Reduction therefore allows estimation of the chelating ability of the coexisting chelator[13]. Fig. 3 reveals that the AvaPre demonstrated a marked capacity for iron binding ability of 50% at a concentration of 1.75 mg/ml.

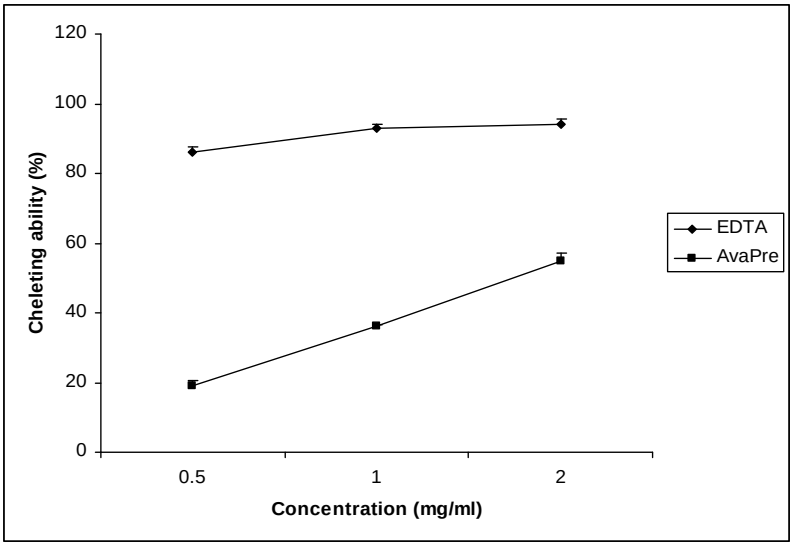

Fig 3: Ferrous ion cheleting activity of AvaPre.

Results are the mean ± SD of three separate experiments, each in triplicate.

Reducing power:

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants[43].

Presence of reducers causes the conversion of the $Fe^{3+/}$ferricyanide complex used in this method to the ferrous form. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentation of Fe^{3+} ion[44]. Fig. 4 reveals that at concentration of 1.55 mg/ml AvaPre showed reducing power of 0.5.

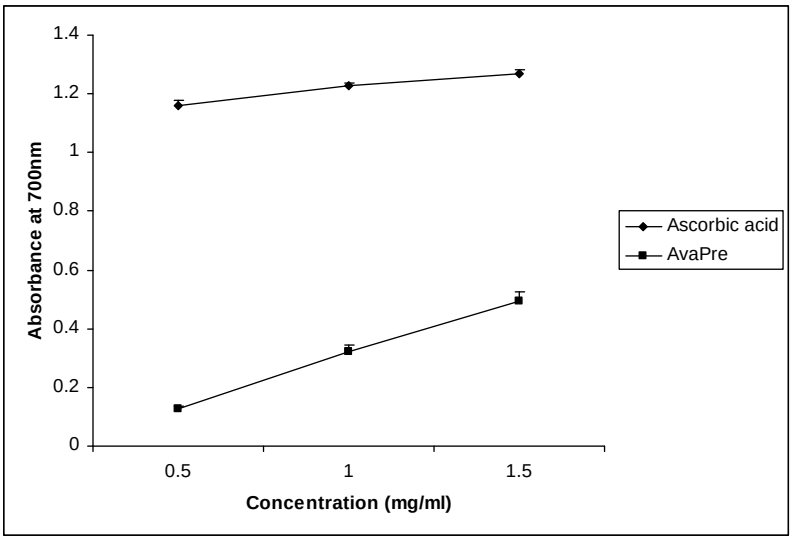

Fig 4: Reducing activity of AvaPre.

Results are the mean ± SD of three separate experiments, each in triplicate.

DPPH radical scavenging activity:

The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time[45]. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[46]. Upon reduction, solution of DPPH fades from purple to yellow. Thus a lower absorbance at 517 nm indicates a higher radical scavenging activity of extract[47]. The effect of the concentration of antioxidant compounds on the DPPH radical was observed as a function of reducing power[48]. As shown in fig 5, AvaPre exhibited significant radical scavenging activity with EC_{50} value at 1.45 mg/ml.

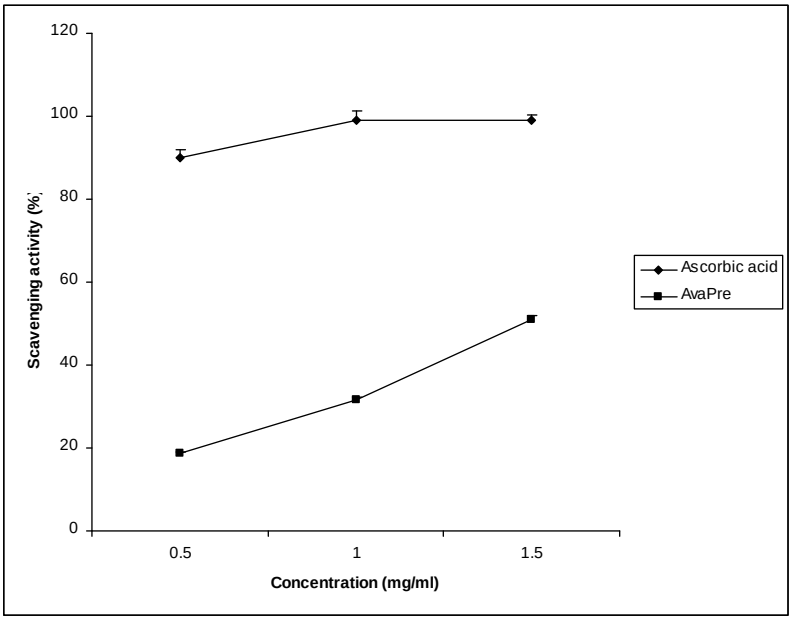

Fig 5: DPPH radical scavenging activity of AvaPre.

Results are the mean ± SD of three separate experiments, each in triplicate.

Conclusion:

The results of this study indicated that investigated polyphenolic rich fraction of *Amanita vaginata* (AvaPre) possesses antioxidant activity and different bioactive components. EC_{50} values of different antioxidant assays were in the following order: hydroxyl radical scavenging < superoxide radical scavenging < DPPH radical scavenging < reducing power < chelating ability of ferrous ion. Phenolic compounds seem to be the main component responsible for the antioxidant activity of the mushroom specie extract. So, the mushroom species can be used as an easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry.

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