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Research Article

EVALUATION OF ANTIOXIDATIVE ACTIVITY AND CHEMICAL COMPOSITION OF ETHANOLIC EXTRACT FROM *AMANITA VAGINATA* (BULL.) LAM.: AN *IN VITRO* STUDY

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ABSTRACT

Objectives: The objective of this study was to evaluate free radical scavenging capacity and presence of bio-active components responsible for the activity in ethanolic fraction of *Amanita vaginata* (EfraAva).

Methods: Antioxidant activity of ethanolic extract of *A. vaginata* was determined by using various processes and also investigated for bioactive components such as total phenol, total flavonoid, ascorbic acid, ß-carotene and lycopene.

Results: The results showed that EfraAva has good antioxidant activity and EC_{50} values of the extract was in the order of chelating ability of ferrous ion< reducing power< DPPH radical scavenging.

Conclusion: Thus it can be suggested that fraction of *A. vaginata* may be used as a natural additive in food and pharmaceutical industries.

Keywords: Antioxidant activity, Chelating ability of ferrous ions, DPPH, Edible mushroom, Free radicals, Phenolic compounds, Reducing power

INTRODUCTION

Free radical is an atom or a group of atoms containing one or more unpaired electrons in atomic or molecular orbitals. Being unstable, they attack the nearest stable molecule to capture the needed electron for gaining stability [1]. The family of free radicals generated from oxygen is called ROS which are ions, atoms or molecules that have the ability to oxidize reduced molecules as electron donors. Various forms of ROS are found in animal cells, which include superoxide anions ($O_2^{\cdot-}$) and hydroxyl radicals (OH·), as well as compounds like H_2O_2 and singlet oxygen [2]. During high metabolic states, the production of ROS is increased in an organism, inducing damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrate, which causes serious cell damage leading to a variety of human diseases [3]. Antioxidants are capable of neutralizing free radicals or their actions [4, 5]. In mechanistic terms, an antioxidant can be defined as a hydrogen donor [6].

Mushrooms should be a good alternative foodstuff with an especially balanced healthy nutrition for the human [7], because mushrooms are rich sources of antioxidant compounds like phenolic components (phenolic acids and flavonoids), tocopherols, ascorbic acid, carotenoids and polysaccharide. It also contains low level of protein, fat and important minerals required for normal functioning of the body [1, 8]. Recently, mushrooms have become a source of physiologically beneficial medicines and as well as functional foods with no side effects. Some recent research works on wild edible mushrooms of West Bengal revelled that they have potentiality to fight against cancer, heart ailments, diabetes, inflammation, hepatic damage, gastric ulcer, microbial pathogens etc. [9-15]. Mushroom polysaccharides and extracts have strong antioxidant properties also [16-18].

Amanita vaginata is a Basidiomycetes edible mushroom. It is widely found in the lateritic regions of Sal forests of West Bengal. They are ectomycorrhizal with Sal trees (*Shorea robusta* Gaertn. F.), Local people called them as 'Sal Chattu' [19].

MATERIAL AND METHODS

Chemicals

BHT (butylated hydroxytoluene), L-ascorbic acid, quercetin, galic acid EDTA (ethylenediaminetetraacetic acid), potassium ferricyanide, ferrous chloride, ferric 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).

Preparation of extract

The fruiting bodies of *A. vaginata* were cleaned to remove residual compost and dried to eliminate moisture. Desiccated mushroom samples were ground to obtain fine particles. 30 gm of mushroom powder was soaked in 500 ml ethanol and stirred at 25ºC 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 40ºC under vacuum to acquire ethanolic fraction of *A. vaginata* (EfraAva) and stored in amber coated bottle at 4ºC until further analysis [21].

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

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

Determination of total phenolic compounds

Total phenolics was measured using Folin-Ciocalteu reagent [22]. 1 ml EfraAva 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 the 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 flavonoid content

Flavonoid concentration was determined by the method as described [23]. 1 ml of EfraAva 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 EfraAva 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 activity

The total antioxidant capacity was determined as described by Prieto et al, (1999) [26] 26 with some modification. 0.3 ml of EfraAva 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.

Determination of reducing power

The reducing power of EfraAva was determined according to the method of Oyaizu [27]. Various concentrations of the fraction (0.2, 0.5 and 1 mg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 min and then 2.5 ml of TCA (10%) was added to the mixture. 2.5 ml of the solution was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The reaction mixture was incubated for 15 min and absorbance was measured at 700 nm. A higher absorbance indicates a higher reductive capability. Ascorbic acid was used as standard. EC_{50} value is the effective concentration

at which the absorbance was 0.5 for reducing power.

Ferous ion cheleting ability

Chelating ability was determined according to the method of Dinis et al [28]. Different concentrations of EfraAva (0.2, 0.5 and 0.7 mg/ml) were mixed with 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by addition of 0.2 ml of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. EDTA was used as positive control. EC50 value is the effective concentration at which ferrous ions were chelated by 50%. The percentage of inhibition of ferrozine-Fe^{2+} complex formation is given by this formula:

Scavenging effect (%) = {(A0-A1)/ A0} × 100

DPPH radical scavenging activity

Radical scavenging activity was determined using a DPPH assay as described by Shimada et al [29]. Various concentrations of EfraAva (0.5, 1 and 1.5 mg/ml) were added to 2ml of 0.004% methanol solution of DPPH (w/v). After 30 min incubation at room temperature in dark, the absorbance was read against a methanol blank at 517 nm. EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for standard. The degree of scavenging was calculated by the following equation:

Scavenging effect (%) = {(A0-A1)/ A0} × 100

A0 and A1 were the absorbance of the control and absorbance in presence of sample respectively.

RESULTS AND DISCUSSION

Bioactive components

Phenolic compounds are well known secondary metabolites commonly found in high amounts in plants and mushrooms and it has vital biological functions including antioxidant activity [30]. Polyphenols protect against cardiovascular diseases and act as brain-protective factors, anti-ageing and anti-inflammatory substance. It also helps in initiation and progression of cancer [31]. It was found that EfraAva contained different type of antioxidant compounds namely phenols, flavonoids, ascorbic acid, β-carotene and lycopene. Significant level of phenolic compound (11.296 ± 0.74 µg gallic acid equivalent/mg of extract) was present in EfraAva whereas same fraction of *Russula delica* [32] and *Pleurotus flabellatus* [33] contained 12.54 ± 2.84 and 6.875 ± 0.45 µg gallic acid equivalent/mg of extract of phenolic compounds.

Another widely available secondary metabolite is flavonoid that encompasses more than 10,000 structures. They have indeed the capacity to absorb most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit generation of ROS, [34] for this reason it has been used in traditional eastern medicine for thousands of years and constitutes an unavoidable component of diet [35]. Flavonoids content in EfraAva was 2.29 ± 0.44 µg quercetin equivalent/mg. Ethanolic fraction of *R. delica* [32] and *P. flabellatus* [33] contained 0.387 ±0.01 and 1.0625 ± 0.375 µg/mg of extract of flavonoid.

β-carotene is a naturally occurring orange-colored carbon-hydrogen carotenoid and it has potential antioxidant properties due to its chemical structure. β-carotene protects against cancer and cardiovascular diseases and helps formation of retinal [36]. Lycopene is one of the most potent antioxidant and is a natural pigment synthesized by plants and microorganisms but not by animals. It helps to prevent carcinogenesis and atherogenesis by protecting critical biomolecules including lipids, low-density lipoproteins, proteins and DNA [37]. EfraAva also contained ß-carotene and lycopene which were 1.7205 ± 0.265 µg/mg, 0.637 ± 0.098 µg/mg of extract respectively whereas same fraction of *P. flabellatus* [33] contained 0.00717 ± 0.000006 and 0.00459 ± 0.0005 µg/mg.

Ascorbic acid is important water soluble antioxidant vitamin in cells which is able to scavenge reactive oxygen species (ROS) effectively. It prevents scurvy and maintains healthy skin, gums, blood vessels,

helps in collagen formation, absorbs inorganic iron and also reduces risk of arteriosclerosis, cardiovascular diseases and some forms of cancer [25]. EfraAva contained 0.729 ± 0.00031 µg/mg ascorbic acid equivalent

ANTIOXIDANT ACTIVITY

Total antioxidant activity

Antioxidants, capable of neutralizing free radicals or their actions, act at different stages like prevention, interception and repair.

Preventive antioxidants attempt to stop the production of ROS [38]. Result showed 1 mg of EfraAva fraction is equivalent to 213 ± 9.5 µg of ascorbic acid. Total antioxidant activity suggests that the electron donating capacity of EfraAva and thus it may act as radical chain terminator, ultimately transforming reactive free radicals into more stable non reactive products.

Ferrous ion chelating ability

Ferrous ion chelating abilities would be beneficial for the health because it is an effective pro-oxidants in food system. 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 [39]. Figure 1 reveals that the EfraAva demonstrated a marked capacity for iron binding ability of 50% at a concentration of 0.73 mg/ml. In earlier work, Chatterjee et al, 2011 reported a similar type of EC_{50} value of ethanolic extract of *Tricholoma giganteum*[40].

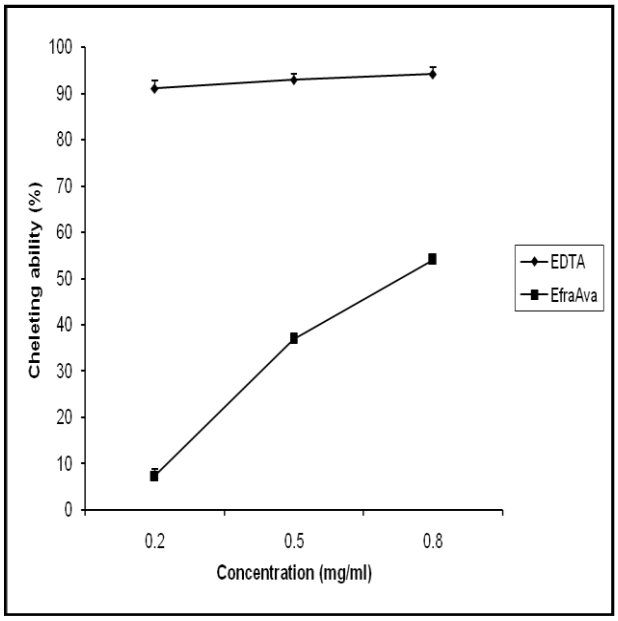

Fig.1: Ferrous ion chelating ability of ethanolic extract of *Amanita vaginata* (EfraAva). Results are the mean ± SD of three separate experiments, each in triplicate.

Determination of reducing power

Antioxidant compounds are able to break free radical chains by donating hydrogen atoms and it also prevents peroxide formation [41]. In this assay, presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex to ferrous form. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of Fe^{3+} ion. Greater absorbance at 700 nm indicated greater reducing power [42]. Figure 2 reveals that at concentration of 0.91 mg/ml EfraAva showed absorbance of 0.5 indicating 50% inhibition. Same fraction of *R. delica* [32] and *P. flabellatus* [33] showed higher reducing power (0.56 and 0.84 mg/ml respectively), whereas EfraAva showed better reducing capability than *T. giganteum* (2.2 mg/ml) [40].

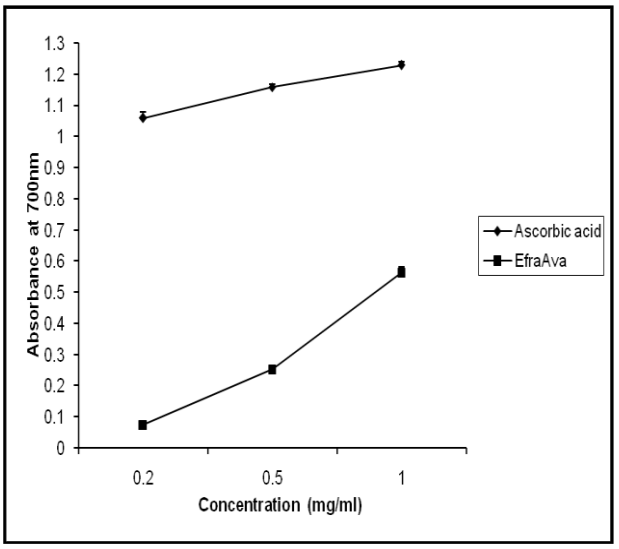

Fig. 2: Reducing power of ethanolic extract of *Amanita vaginata* (EfraAva). Results are the mean ± SD of three separate experiments, each in triplicate.

DPPH radical scavenging activity

DPPH radical is a stable free radical at room temperature and possess a characteristic absorbance at 517 nm. The use of stable DPPH radical has the advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation, which decreases significantly on exposure to radical scavengers by providing hydrogen atom or electron to become a stable diamagnetic molecule [43, 44]. Figure 3 Showed that EC_{50} of DPPH radical scavenging activity was 1.48 mg/ml. In related studies on ethanolic extract of *R. delica* [32] *T. giganteum* [40], *Volvariella volvacea* [17], *Ramaria aurea* [16] and *P. flabellatus*[33] EC_{50} values at 1.2, 0.75, 0.265, 0.857 and 1.8 mg/ml respectively.

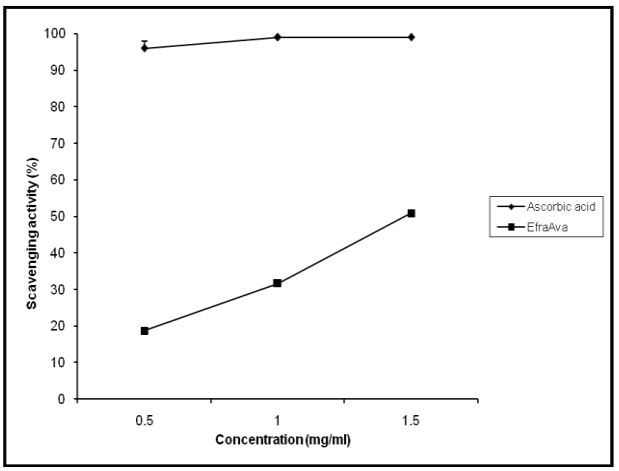

Fig.3: DPPH radical scavenging activity of ethanolic fraction of *Amanita vaginata* (EfraAva). Results are the mean ± SD of three separate experiments, each in triplicate.

CONCLUSION

From the above investigations it is evident that the ethanolic fraction of *Amanita vaginata* (EfraAva) possessed significant antioxidant activity and contained different bio-active components like phenolic compounds, flavonoids, ascorbic acid, β-carotene and lycopene. Thus it can be suggested that this fraction may have therapeutic value which could be used as medicine against several killer diseases.

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