ANTIOXIDANT ACTIVITIES OF EXTRACTS OBTAINED BY DIFFERENT FRACTIONATION FROM *TRICHOLOMA GIGANTEUM* BASIDIOCARPS

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Summary

The purpose of this study was to elucidate the antioxidant capacities of *Tricholoma giganteum.* Three fractions from the mushroom were evaluated for antioxidant activity against hydroxyl radical, superoxide radical, DPPH radical, ferrous ion chelating ability and reducing power. Among three different fraction (Fa, Fb and Fc), the fraction Fa had a strong antioxidant activity and Fc also presented a relatively strong antioxidant effect. Whereas, the polysaccharide fraction Fb was found to be a weak scavenger of free radicals when compared to Fa and Fc. Cellular damage caused by reactive oxygen species has been implicated in several diseases and hence antioxidants have significant importance in human health. Our result thus indicates that Fa fraction of *T. giganteum* has maximum antioxidant property and may be utilized as a promising source of therapeutics.

Key Words: Antioxidant activity, Chelating ability, Reducing power, *Tricholoma giganteum*.

Introduction

Oxidation is essential to many living organism for production of energy to fuel biological processes. Potentially harmful reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism [1]. Free radicals have been implicated in the pathogenesis of various diseases, including myocardial and cerebral ischemia, atherosclerosis, diabetes, rheumatoid arthritis, inflammation, and cancer- initiation, as well as in the aging process [2, 3]. Almost all organisms are well protected against free radical damage by antioxidant enzymes such as superoxide dismutase (SOD) and catalase or chemical compounds such as ascorbic acid, α-tocopherol, carotenoids, polyphenol compounds and glutathione [4]. Antioxidants are deployed to prevent generation of ROS or to scavenge those formed. Thus, oxidatively induced tissue damage is minimized. When natural defenses are overwhelmed by excessive generation of pro-oxidants, a situation of oxidative stress evolves, and cellular macromolecules might suffer oxidative damage [5]. A number of methods have been developed to measure the efficiency of antioxidants. These methods focus on different mechanisms of the antioxidants defense system such as scavenging or inhibiting free radicals or chelation of metal ions that otherwise may lead to free radical formation. Neutralization of this radical activity by naturally occurring substances mainly by supplementation of food having antioxidant property is becoming one of the most acceptable modes of modern therapy [6]. Amongst them, mushroom or their derivatives or extracts occupy an elite position to perform this function [6–10]. Recently, mushrooms have become attractive as functional foods and as a source of physiologically beneficial medicines, while being devoid of undesirable side effects [11]. Mushrooms are considered as rich food because they contain protein, sugar, glycogen, lipid, vitamins, amino acids and crude fiber [12, 13]. They also contain important minerals required for normal functioning of the body [13]. Edible mushrooms are highly nutritious and could be used as medicines for treatment of cancer, heart ailments, diabetes, inflammation, hepatic damage, high blood pressure, etc [7, 14–19]. Over two third of cancer related death could be prevented through lifestyle modification particularly through dietary means and mushroom consumption may contribute to minimize cancer risk through antioxidant input [20]. *Tricholoma giganteum* of the Family Tricholomataceae, a wild edible mushroom is most conspicuous in the tropical region during rainy season. They are robust in size and popular among the people of these areas because of their gastronomic and nutritional delicacy. In this study, the antioxidant activity of different fractions obtained from *T. giganteum* were evaluated and compared, as assessed by their ability to scavenge free radicals and to protect from oxidative stress.

Materials and methods

Sample collection and preparation

Basidiocarps of *T. giganteum* were collected from the forest and local market of Kolkata and adjoining area.

Powdered *Tricholoma giganteum* (100 g) was extracted with 80% ethanol at room temperature overnight and was repeated 4 times, and then freeze-dried (Fa, 6.232 g). The residual fraction was dissolved in distilled water in a boiling water bath for 4 h. The aqueous phase was evaporated and reduced to half its volume and then mixed with 99% ethanol (1:4, v/v), and the precipitated fraction was freeze-dried (Fb, 5.922 g). The aqueous phase was then evaporated to remove the ethanol and mixed with ethyl acetate (2:1, v/v). The upper ethyl acetate layer was then evaporated and lyophilized (Fc, 240 mg) (Figure 1). The freeze-dried fractions Fa, Fb and Fc were reconstituted in distilled water at a concentration of 10 mg/ml. These stock solutions were kept in the dark at 4 °C for further use [21].

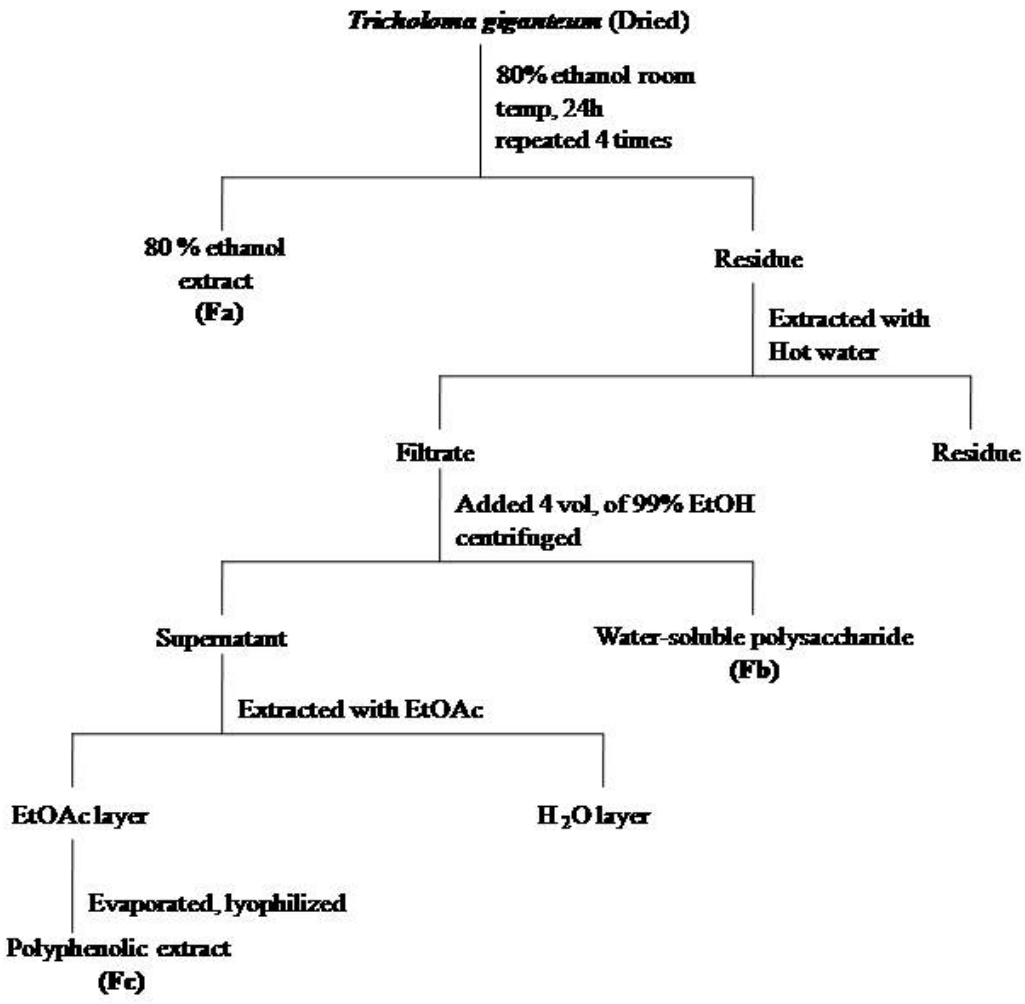

Figure 1 Preparation of *Tricholoma giganteum* fractions [21].

Assay of hydroxyl radical (OH^-) - scavenging activity

Hydroxyl radicals (OH^-) 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 [22]. Reaction mixture (1 ml) contained deoxyribose (2.8 m*M*), KH_2PO_4- KOH (20 m*M*; *p*H 7.4), $FeCl_3$ (100 m*M*), EDTA (104 µ*M*), H_2O_2 (1 m*M*) and ascorbate (100 µ*M*). Reaction mixture was incubated at 37ºC for 1 h and color developed as described above. IC_{50} value of deoxyribose degradation by the fractions Fa, Fb and Fc over the control was measured.

Assay of superoxide radical (O_2^-) - scavenging activity

The method used by Martinez *et al*. 2001 [23] for determination of the superoxide dismutase was followed with modification in the riboflavin-light-nitrobluetetrazolium (NBT) system [24]. Each 3 ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, 75 µM NBT and 500 µl sample solution of various concentrations of the fractions Fa, Fb and Fc of *T. giganteum*. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination of a fluorescent lamp. Identical tubes with the reaction mixture were kept in the dark and served as blanks.

DPPH radical scavenging assay

The hydrogen atom or electron donation abilities of the fractions of *T. giganteum* and a pure compound were measured from the bleaching of the purple colour methanol solution of 1, 1-diphenyl-2- picryl hydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent [25, 26]. Various concentrations of the fraction Fa and Fc in methanol were added to 2ml of 0.004 % methanol solution of DPPH (w/v) and the mixture was shaken vigorously. After 30 min. incubation period at room temperature in dark, the absorbance was read against a methanol blank at 517 nm. The DPPH scavenging activity was expressed as the percentage inhibition and was calculated in the following way:

$I\ \% = (A_{blank} - A_{sample} / A_{blank}) \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

Chelating ability of ferrous ions

Chelating ability was determined according to the method of Dinis *et al*. (1994) [27]. Different concentration of fractions Fa and Fc (10 mg/ml) in water (1 ml) was mixed with 3.7 ml of methanol and 0.1 ml of 2 m*M* ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 m*M* ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. The percentage of inhibition of ferrozine- Fe^{++} complex formation is given by this formula:

$\%\ \text{inhibition} = \{(A_0 - A_1)/ A_0\} \times 100.$

Where, A_0 was the absorbance of the control and A_1 the absorbance in the presence of mushroom fractions.

Determination of reducing power

The reducing powers of fractions (Fa and Fc) of mushroom were determined according to the method of Oyaizu (1986) [28]. Various concentrations of the fraction Fa and Fc in 1ml of methyl alcohol were mixed with phosphate buffer (2.5 ml, 0.2 *M*, *p*H 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 trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 12000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. A higher absorbance indicates a higher reductive capability.

Analysis of data

In all the cases results is the mean ± SD (standard deviation) of at least three individual experimental data.

Results

Hydroxyl radical scavenging activity

Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at *p*H 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low *p*H [29]. When the test samples were added to the reaction mixture, they removed hydroxyl radical from the sugar and prevented their degradation. All the fractions, i.e., Fa, Fb and Fc showed hydroxyl radical

scavenging activity. With regard to the scavenging ability of hydroxyl radicals, the fractions were effective in order of their IC_{50} value: Fa > Fc > Fb (Figure 2).

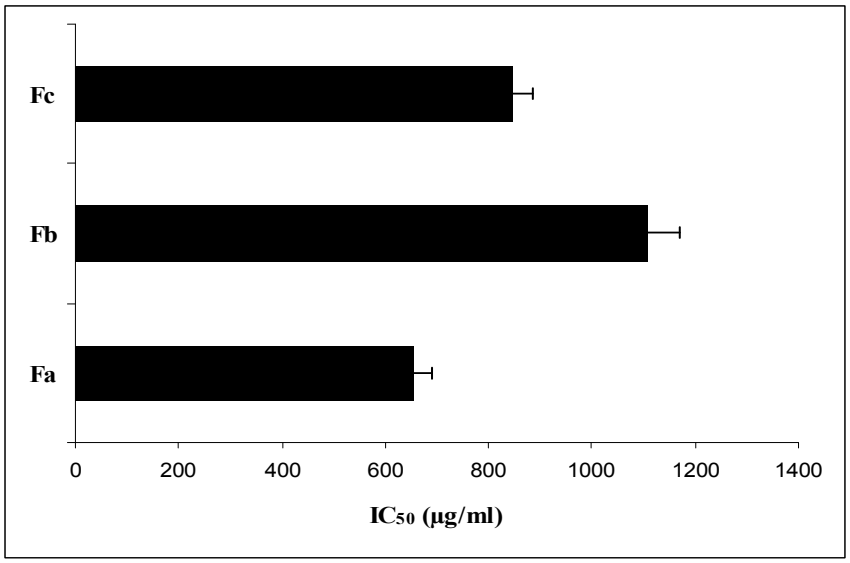

Figure 2 Inhibitory concentration 50% of hydroxyl radical scavenging activity by *Tricholoma giganteum* fractions. Results are the mean ± SD of three separate experiments, each in triplicate.

Assay of superoxide radical scavenging activity

The superoxide radical scavenging activity of the fractions Fa, Fb and Fc of *T. giganteum* were expressed as IC_{50} value (Figure 3). In the present study, the fractions were found to be a notable scavenger of superoxide radicals generated in riboflavin- NBT light system. The Fa fraction showed a lower IC_{50} value (551.56 µg/ml), i.e., higher scavenging activity than Fc (735.08 µg/ml), where as the fraction Fb was comparatively weaker scavenger of superoxide radical with the IC_{50} value of 1213.65 µg/ml.

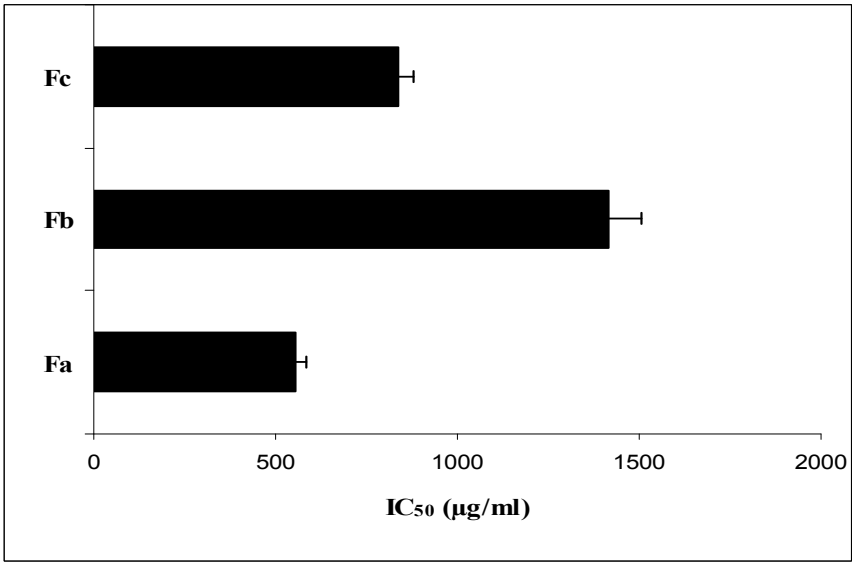

Figure 3 Inhibitory concentration 50% of superoxide radical scavenging activity by *Tricholoma giganteum* fractions. Results are the mean ± SD of three separate experiments, each in triplicate.

The above experiments showed fraction Fb to be a weak scavenger of hydroxyl and superoxide radical and being a polysaccharide fraction, it precipitates in methanol and hence couldn't be taken into account for further experiments. Thus, only fractions Fa and Fc were considered to analyse their DPPH radical scavenging activity, chelating ability of ferrous ion and reducing power.

DPPH radical scavenging activity

The DPPH radical scavenging activities of the fractions were presented in Figure 4. The radical scavenging activity of Fa and Fc fractions of *T. giganteum* were exerted in a dose dependent manner. Both the fractions Fa and Fc showed strong antioxidant effect at concentrations lower than 1 mg/ml.

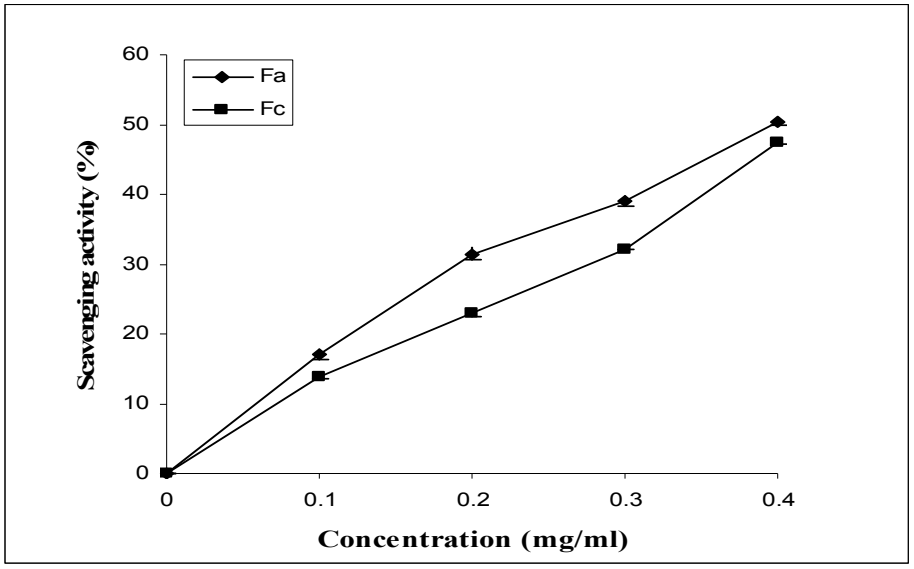

Figure 4 DPPH free radical scavenging activity of fractions Fa and Fc of *Tricholoma giganteum*. Results are the mean ± SD of three separate experiments, each in triplicate.

Chelating ability of ferrous ion

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 color. Reduction therefore allows estimation of the chelating ability of the coexisting chelator [30]. Figure 5 reveals that the fraction Fa and Fc of *T. giganteum* in this study demonstrated a marked capacity for iron binding ability of 66.62% and 49.77% respectively at a concentration of 1 mg/ml. The fraction, Fa was better chelator for ferrous ion compared to Fc. As the ferrous ions are effective pro oxidants in food system [31], so the moderate ferrous ion chelating abilities would be beneficial.

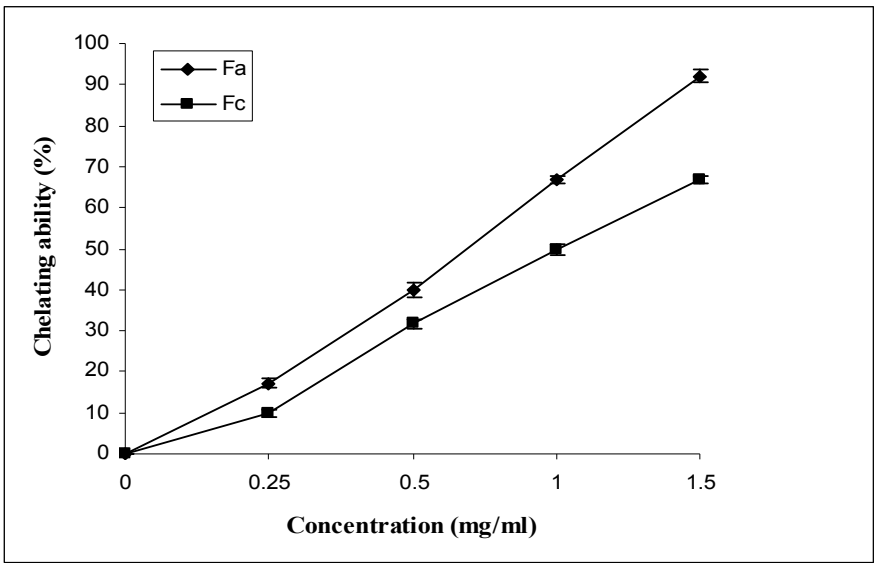

Figure 5 Ferrous ion chelating ability of fractions Fa and Fc from *Tricholoma giganteum*. Results are the mean ± SD of three separate experiments, each in triplicate.

Determination of reducing power

Reducing power of Fa and Fc fractions from *T. giganteum* increased readily alongwith the increased concentrations (Figure 6). At 2 mg/ml, reducing power were in the order: Fa (0.471) > Fc (0.253). The antioxidant activity has been reported to be concomitant with the development of reducing capacity [32]. The variation of the reducing among the fractions might be due to its hydrogen-donating ability as described by Shimada et al. [33].

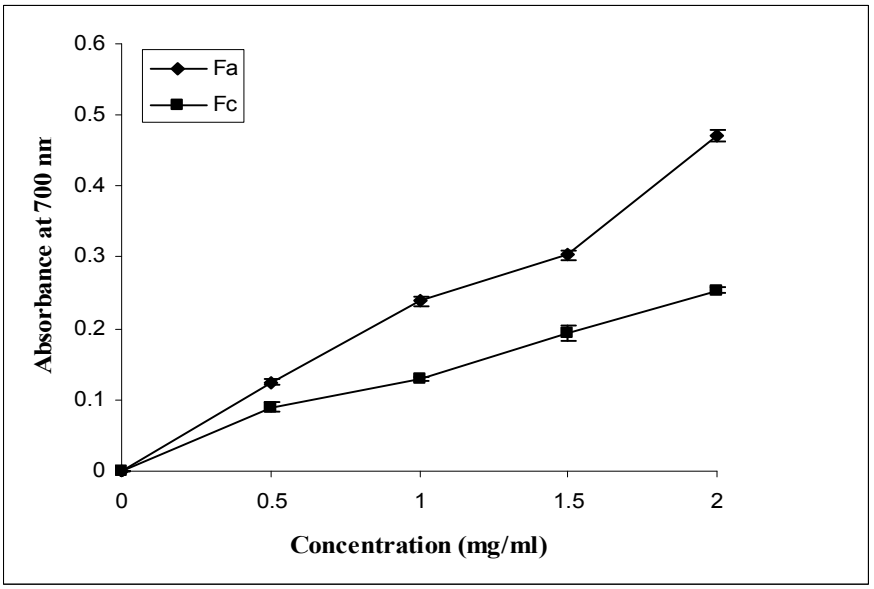

Figure 6 Reducing power of fractions Fa and Fc of *Tricholoma giganteum.* Results are the mean ± SD of three separate experiments, each in triplicate.

Discussion

In the present study, we found that *T. giganteum* fractions exert variable antioxidant activity. Results of the investigation reveal that the Fa fraction showed strongest antioxidant activity.

Earlier Kahlos *et al*. 1989 [34] reported that the Fa extract of a mushroom *Inonotus radiatus*, which contained triterpenoids and steroids including lanosterol, inotodiol, trametenolic acid and ergosterol peroxide, had a strong antioxidant activity. He *et al*. 2001 [35] isolated triterpenoids and steroids from *Inonotus obliquus*. The Fa fraction of *I. obliquus* also had high free radical scavenging activity [21]. The triterpenoids and steroids in the Fa fraction may account for the free radical scavenging effect [36]. The Fc fraction also had relatively strong antioxidant effect. The IC_{50} value of Fa is lowest for hydroxyl radical scavenging activity. The Fc fraction was also effective in low concentration. Water soluble polysaccharide fraction Fb exerts hydroxyl radical scavenging activity with a very high IC_{50} value. Superoxide radical is known to be very harmful to cellular components as a percursor of more reactive species [37]. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxinitrite [38] which is a potent oxidant that causes nitrosative stress in the organ systems. The IC_{50} value for Fa fraction of *T. giganteum* was found to be 551.56 µg/ml. DPPH radical is a stable free radical and possess a characteristic absorbance at 517 nm, which decreases significantly on exposure to radical scavengers by providing hydrogen atom or electron to become a stable diamagnetic molecule [39]. The use of stable DPPH radical has the advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation [40]. The Fa fraction showed higher scavenging ability on DPPH radical than the Fc fraction. Transition metals have been proposed as the catalysts for the initial formation of radicals. Chelating agents may stabilize transition metals in living systems and inhibit generation of radicals, consequently reducing free radical induced damage. To better estimate the antioxidant potential of the mushroom fraction, its chelating activity was evaluated against Fe^{2+} [41]. Fraction, Fa showed the highest ferrous ion chelating activity which chelates ferrous ion by 66.62% at a concentration of 1 mg/ml. Hence, the Fa fraction shows higher interference with the formation of ferrous and ferrozine complex and can be considered as good chelator of ferrous ion. Reducing power of a compound may be serving as a significant indication of its potential antioxidant activity [28]. The presence of reducers (i.e., antioxidants) causes the reduction of Fe^{3+}/Ferrocyanide complex to ferrous form. The yellow color of the test solution was changed to various shades of green and blue, depending on the reducing power of each compound.

From the above investigation it is evident that the fraction Fa of *Tricholoma giganteum* possessed significant antioxidant activity thus suggesting the therapeutic value of this fraction, which could be used as medicine for several killer diseases. These results should encourage further *in vivo* studies which could ultimately lead to an inclusion of this medicinal mushroom in different pharmaceutical formulations. Nevertheless, the results even suggest that the consumption of *T. giganteum* might enhance the antioxidant protection system of human body, against oxidative damage.

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