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Anti-oxidative Effect of Polyphenol-Rich Extract of *Russula albonigra*.

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ABSTRACT

Polyphenol-rich fraction from edible mushroom, *Russula albonigra*, was tested for total phenol, flavonoid, β -carotene, lycopene and ascorbic acid and in vitro antioxidant activity in terms of DPPH radical scavenging and chelating effect of ferrous ion, reducing power and total antioxidant capacity assay. Findings showed that EC₅₀ values were below 1 mg /ml except reducing power test. The extract exhibited 50% reducing power at only 1.2 mg /ml concentration. Estimated putative antioxidant components are in order of phenol > flavonoids > β -carotene > ascorbic acid > lycopene. Results imply that *R. albonigra* can be a potential source of natural antioxidant which may be used as food supplement to treat various oxidative stress related diseases.

Keywords: Antioxidant activity, Antioxidant components, Chelating effect, Reducing power, Scavenging activity.

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INTRODUCTION

Oxidation is essential for the production of energy. However, as a consequence, harmful reactive oxygen species (ROS) are also produced [1] induced by various endogenous and exogenous sources and plays an important role in most major degenerative diseases associated with aging. Although almost all organisms are equipped with antioxidant defence systems, they are often inadequate to completely prevent oxidation stress-induced damage, but evidences indicate that it can be reduced by intake of antioxidant supplements [2]. Many synthetic antioxidants are available in the market, but in recent years, interest has augmented considerably in finding antioxidants from natural sources for use in foods or medicinal materials [3] to replace synthetic antioxidants, which are being restricted due to their carcinogenicity [4]. Recently, much attention has been paid to the therapeutic value of mushroom based on its antioxidative property and interest in the role of antioxidants in human health has prompted research in the fields of food science [5].

Mushrooms accumulate a variety of secondary metabolites including phenolic compounds, polyketides, terpenes, and steroids. Some of these compounds have tremendous importance to humankind in that they display a broad range of useful antibacterial, antiviral, and pharmaceutical activities as well as less toxic effects [6]. They may act directly as antioxidant or prevent underlying oxidative stress related pathological conditions such as cancer [7], heart ailments [8], diabetes [9], inflammation [10], gastric ulcer [11], hepatic damage [12,13], microbial pathogens [14,15] etc. Mushrooms have long been used as medicine by the Chinese, and recently, a lot of attention has been paid to the therapeutic value of mushrooms based on its antioxidative property and their role in human health, prompting extensive research in the fields of food science.

Russula albonigra (Krombh) Fr., commonly known as brittlegill and placed under the family Russulaceae is an edible wild mushroom distributed throughout the lateritic region of West Bengal [16]. However, very little is known about the medicinal application of this mushroom. In the course of our ongoing search we studied the hot water fraction of *R. albonigra* with an objective to evaluate its potency as an antioxidant and the presence of bioactive components.

MATERIALS AND METHODS

Mushroom sampling

Basidiocarps of *R. albonigra* were collected in the month of July from the lateritic region of West Bengal and identified using standard literature [17]. A voucher specimen (AMF - 598) was deposited in the same department of University of Calcutta.

Chemicals

BHT (butylated hydroxyl toluene), L-ascorbic acid, quercetin, gallic acid, EDTA (ethylenediaminetetraacetic acid), potassium ferricyanide, ferrous chloride, Folin-Ciocalteu reagent, NBT (nitrobluetetrazolium), riboflavin, DPPH (1,1-diphenyl 1-2-picrylhydrazyl), TCA (trichloroacetic acid), ammonium molybdate and methionine were purchased from Sigma

chemicals Co. (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Preparation of extract

Polyphenol-rich fraction of *Russula albonigra* was extracted according to the method of Khatua et al (2013) [18] with a slight modification. Dried and powdered basidiocarps of *Russula albonigra* were extracted with ethanol at 25°C for 2 days to eliminate the alcohol soluble materials such as triterpenoids and steroids. 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 eight hrs. The polysaccharides were precipitated by ethanol addition. After centrifugation the pellet was separated and the supernatant was evaporated to remove ethanol. The obtained concentrated polyphenol rich fraction of *Russula albonigra* was stored at 4°C until further analysis.

The percentage yield extracts were calculated based on dry weight as:

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

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

Determination of total phenols

Total phenols in the extract were measured using Folin-Ciocalteu reagent [19]. 1ml of the extract (100 mg/ml) was mixed with 1ml Folin-Ciocalteu reagent and incubated for 3 min at room temperature. After incubation, 1ml of 35 % saturated Na₂CO₃ solution was added in the reaction mixture, volume adjusted to 10 ml with distilled water and incubated in the dark for 90 min, after which the absorbance was monitored at 725 nm with a spectrophotometer. Gallic acid was used as standard. Total phenol content of the sample was expressed as mg of gallic acid equivalents per gram of extract.

Determination of total flavonoid content

Flavonoid concentration was determined by the method as described [20]. The extract (100 mg/ml) was diluted with 4.3 ml of 80% aqueous methanol and 0.1 ml of 10% aluminium nitrate and 0.1 ml of 1 M aqueous potassium acetate were added to it. 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 β-carotene and lycopene content

β-carotene and lycopene was determined by the processes as suggested [21]. In brief, 100 ml of the extract (10 mg/ml) 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. β-carotene and lycopene contents were calculated according to the following equations:

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

$$\beta\text{-carotene (mg/100mg)} = 0.216A_{663} - 0.304A_{505} + 0.452A_{453}$$

Determination of ascorbic acid content

Ascorbic acid content was determined by a method as described [22] with a little modification. Standard ascorbic acid (100 $\mu\text{g/ml}$) 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 extract (w $\mu\text{g/ml}$) was similarly titrated with the dye (V_2 ml). The amount of ascorbic acid was calculated using the formula, Ascorbic acid ($\mu\text{g/mg}$) = $\{(10 \mu\text{g}/V_1\text{ml}) \times V_2 \text{ ml}\} \times w \mu\text{g} \times 1000$.

Total antioxidant capacity assay

The assay is based on the reduction of Mo (VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH [23]. The tubes containing the extract (1 mg/ml) and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95°C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

DPPH radical scavenging activity was tested as follows. Different concentrations of RalPre (0.5 to 2 mg) were added to 0.004% 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.

Reducing power

Reducing power of RalPre was determined following the method of Oyaizu, 1986 [24]. Variable concentrations of RalPre 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% trichloroacetic acid was added to the mixture and was centrifuged at 12000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml 0.1% ferric chloride and absorbance was measured at 700 nm. An increase in absorbance of the reaction mixture was taken to mean an increase in reducing power of the sample.

Chelating effect on ferrous ions

The ability of the extract of RalPre to chelate ferrous ions was estimated by the method of Dinis et al, 1995 [25]. 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-Fe²⁺ 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.

Superoxide radical scavenging assay

Superoxide radical scavenging activity was studied using the riboflavin-light-nitrobluetetrazolium (NBT) system suggested by Martinez et al. (2001) [26] with a little modification. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, sample solution of various concentrations of *Russula albonigra*, 100 μ M EDTA, 75 μ M NBT and 2 μ M riboflavin. One set of reaction mixtures were exposed to light for 10 min to activate the riboflavin-NBT and the absorbance of each mixture was measured at 560 nm against identical mixtures from another set kept in the dark for the same duration. Butylated hydroxyl anisole (BHA) was used as standard.

RESULTS

Recovery per cent and antioxidant components

Table 1 summarises the percent yield, total phenol, flavonoids, ascorbic acid, beta-carotene and lycopene content in the Polyphenol-rich extract of *Russula albonigra* (RalPre). Data shows that total phenols, flavonoids and β -carotene were the major antioxidant components, whereas, ascorbic acid and lycopene were found in vestigial amounts.

Table 1: yield percentage and antioxidant components of polyphenol-rich extract of *Russula albonigra* (RalPre).

	Flavonoids	Total phenols	Ascorbic acid	β - carotene	Lycopene
Yield %	(μ g/mg)				
8.25%	2.125 \pm 0.375	7.94 \pm 0.45	0.074 \pm 0.416	1.67 \pm 0.000006	0.56 \pm 0.0005

Values are the mean \pm standard deviation of three separate experiments, each in triplicate. Total phenols are expressed in gallic acid equivalent (GAE) and flavonoids as quercetin equivalent (QE).

Table 2: EC₅₀ values of the polyphenol-rich extract of *Russula albonigra* for different antioxidant activities.

	Chelating	DPPH	Reducing	SOD
EC ₅₀ (mg/ml)	0.39 \pm .007	0.47 \pm .02	1.12 \pm .03	0.74 \pm 0.012

Total antioxidant capacity

Total antioxidant capacity of RalPre was determined by the formation of green phosphomolybdenum complex. The phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of green phosphate/Mo(V) complex with the maximal absorption at 695 nm. Total antioxidant activity of the extract was estimated, using ascorbic acid as standard. On analysis, it was found that 0.5 mg of RalPre is as functional as approximately 30 \pm 3.7 μ g of ascorbic acid, expressed as 30 μ g ascorbic acid equivalents (AAE).

DPPH assay

DPPH is a stable free radical that has a characteristic absorption 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 [27]. Such reactivity has been widely used to test the ability of a compound or extract as free radical scavengers. Upon treatment with an increasing concentration of the RalPre, a marked decrease in absorption was observed (Fig. 1), indicating a potent DPPH scavenging ability of the extract. EC₅₀ of DPPH radical scavenging activity was 0.47 ± 0.02 mg/ml.

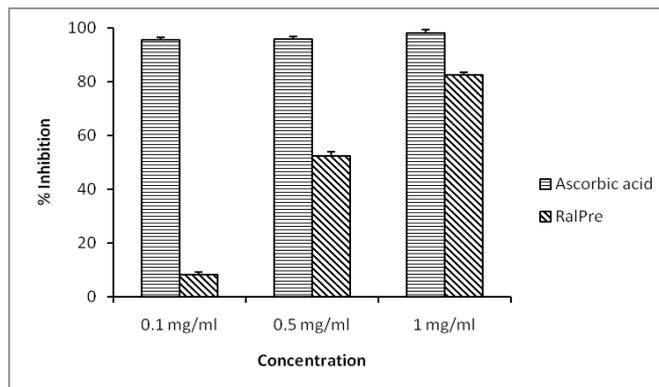


Fig. 1: DPPH radical scavenging activity of RalPre compared with that of the standard, ascorbic acid. Values are the mean ± standard deviation of three separate experiments, each in triplicate.

Chelating effect on ferrous ions

Like many transition metals, ferrous ions in a biological system could catalyse Heber-Weiss and Fenton-type reactions leading to the formation of hydroxyl radicals. Antioxidants chelate these transition metal ions resulting in the suppression of hydroxyl radical generation and inhibition of peroxydation process of biomolecules. The range and the mean of Fe⁺⁺ chelating capacity is directly related with antioxidant potentiality. At 100 – 500 µg/ml the chelating effects of the RalPre was between 24 and 58.7% (fig 2). It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidised forms of metal ions [28].

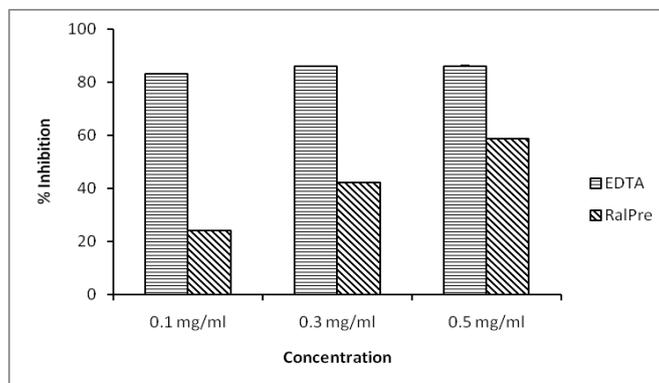


Fig 2: Chelating effects of RalPre on ferrous ions compared with that of EDTA, used as standard. Values are the mean ± standard deviation of three separate experiments, each in triplicate.

Reducing power

Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity [24]. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. In the reducing power assay the presence of antioxidants in the RalPre would effect in the reduction of Fe^{3+} to Fe^{2+} by the donation of an electron. The increasing absorbance at 700 nm by measuring the formation of Perl's Prussian blue indicates an increase in reductive ability. A steady increase in reducing power was observed (Fig. 3). Results showed that EC_{50} for the reducing power of RalPre was of 1.12 ± 0.03 mg/ml.

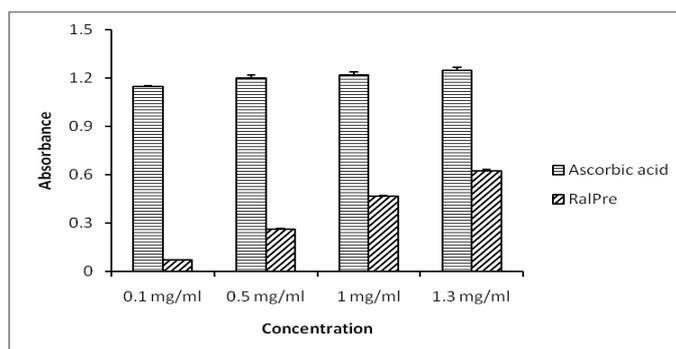


Fig 3: Reducing power of RalPre with respect to that of ascorbic acid used as standard. Values are the mean \pm standard deviation of three separate experiments, each in triplicate.

SUPEROXIDE RADICAL SCAVENGING

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive species. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxynitrite which is a potent oxidant that causes nitrosative stress in the organ systems [29]. In the present study, the polyphenol-rich fraction of *Amanita vaginata* was found to be a notable scavenger of superoxide radicals generated in riboflavin-nitrobluetetrazolium (NBT) light system (Fig.4). The EC_{50} value of the fraction was 0.74 mg/ml.

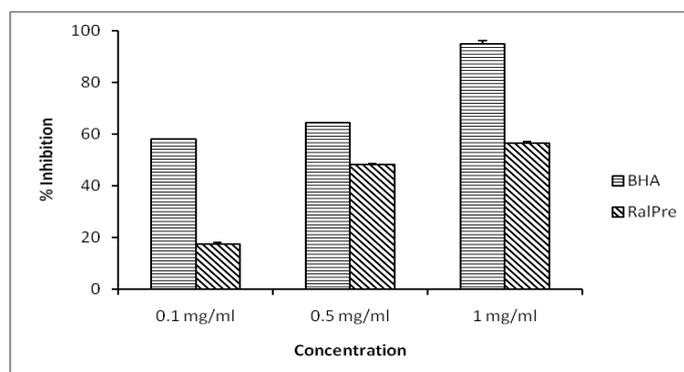


Fig 4: Superoxide radical scavenging activity of RalPre with respect to that of BHA, used as standard. Values are the mean \pm standard deviation of three separate experiments, each in triplicate.

DISCUSSION

Total antioxidant capacities of RalPre was analysed by the phosphomolybdenum method. A high absorbance value of the sample indicates high antioxidant activity. The total antioxidant activity of RalPre was found to be equivalent to the activity of $30 \pm 3.7 \mu\text{g}$ of ascorbic acid. The total antioxidant capacity of RalPre may be attributed to their chemical composition and phenolic content. A recent study [30] showed that some bioactive compounds from citrus fruits had strong total antioxidant activity, which was probably due to the presence of flavonoids, carotenoids and ascorbic acid.

DPPH is a stable free radical and possesses 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. The use of stable DPPH radical has the advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation. Compared with our earlier investigations, the EC_{50} value of polyphenol-rich fraction of *Russula albonigra* (RalPre) was lower than the same fraction of *Russula laurocerasi* [18], *Russula delica* [31], and *Amanita vaginata* [32]. On the other hand, *Tricholoma giganteum* had almost similar activity [29], while *Inonotus obliquus* was reported to have a lower EC_{50} value than RalPre [33]. Therefore, the DPPH radical scavenging activity of the polyphenol-rich extract from different basidiocarps were in descending order *Inonotus obliquus* [33] > *Tricholoma giganteum* [29] > *Russula albonigra* > *Russula delica* [31] > *Russula laurocerasi* [18] > *Amanita vaginata* [32]. Thus it can be said that the polyphenol-rich extract of *Russula albonigra* has significant DPPH radical scavenging ability.

Iron can stimulate lipid peroxidation by the Fenton reaction and accelerate peroxidation by decomposing lipid peroxide into peroxy and alkoxy radical that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The main mechanism of the ferrous ion chelating activity is the ability to deactivate and/or chelate Fe^{2+} which can promote Fenton reaction and hydroperoxide decomposition. Iron toxicity is associated with an increased risk of free radical damage and cancer. Chelation therapy may possibly reduce iron related free radical damage and increase the overall survival in cardiovascular diseases [34]. The ferrous ion chelating ability of RalPre was effective and the EC_{50} value was found to be 0.39 mg/ml. Our previous investigations have shown that the EC_{50} value of the polyphenol-rich extract for *Russula laurocerasi* [18] and *Russula delica* [31] were 0.58 and 0.68 mg/ml respectively, which are comparatively higher, while the EC_{50} value for *Amanita vaginata* [32] and *Tricholoma giganteum* [29] were much higher than that of RalPre. Hence, the studied mushroom extract shows higher interference with the formation of ferrous and ferrozine complex and can be considered as a good chelator of ferrous ions.

Reducing power of a compound serves as a significant indication of its potential antioxidant activity. The presence of reducers (i.e. antioxidants) causes the reduction of Fe^{3+} /ferrocyanide complex to ferrous form. The yellow colour of the test solution changes to various shades of green and blue, which depends on the reducing power of each compound. RalPre was found to be a potent reducing agent, with an EC_{50} value of 1.12 mg/ml. Compared with the reducing powers of previously studied edible mushrooms from our previous reports, the polyphenol-rich extract of *Russula albonigra* was an excellent reducer

of ferric ions. The reducing power of polyphenol-rich extracts of different edible mushrooms in descending order are *Russula albonigra*>*Amanita vaginata* [32]>*Tricholoma giganteum* [29]>*Russula laurocerasi* [18]. In a similar study, it was reported that the hot water extract of *Ganoderma tsugae* [35] had a similar reducing power compared to that of *Russula albonigra* (EC₅₀ 1.12 mg/ml). Apparently the polyphenol-rich extract of *R. albonigra* is an excellent reducing agent.

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive species. One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxynitrite which is a potent oxidant that causes nitrosative stress in the organ systems [29]. In the present study, the EC₅₀ value of RalPre for superoxide radical-scavenging activity was found to be 0.74 mg/ml. Compared with an earlier investigation, the EC₅₀ value of RalPre was lower than the polyphenol-rich extract of *Russula laurocerasi* [18] (1.56 mg/ml). It was also significantly lower than the EC₅₀ value of *Pleurotus squarrosulus* [36] (8.63 mg/ml) and *Amanita vaginata* [32] (1.2 mg/ml). In comparison, *Tricholoma giganteum* [29] showed similar (0.735 mg/ml) superoxide anion scavenging activity, while *Russula delica* [31] showed a little better activity (0.675 mg/ml) than that of RalPre. Thus it can be inferred that RalPre is an effective scavenger of superoxide radicals.

Phenolic compounds are known to be powerful chain-breaking antioxidants and they possess scavenging ability due to their hydroxyl groups. The phenolic compounds contribute directly to the antioxidative action. In the present study, the total phenolic content of RalPre (2.125 µg/mg) was found to be lower than that of the hot water extract of *P. eous* [37], which was reported to be 8.77 µg/mg, *Russula laurocerasi* [18] (7.05 µg/mg), *P. Squarrosulus* [36], which was 18.1 µg/mg and *P. citrinopileatus*, that being 8.62 µg/mg [38].

Ascorbic acid is reported to interact directly with radicals such as O₂⁻ and OH[•] in plasma, thus preventing damage to red cell membranes, it also assists α-tocopherol in inhibition of lipid peroxidase by recycling the tocopherol radical [39]. In the present study, the ascorbic acid content of *R. albonigra* extract was relatively higher (0.74 µg/mg) when compared to the values reported from alcoholic extracts of *P. ostreatus* [39] (25 ng/1 mg) and *P. citrinopileatus* [38] (31 ng/mg), but was comparatively lower than the polyphenol-rich extract of *Russula laurocerasi* [18] (1.03 µg/mg). Many other naturally occurring antioxidant components, including β-carotene, lycopene and flavonoids are known to possess strong antioxidative characteristics [40]. In this study β-carotene was found in significant amounts, i.e. 1.67 µg/mg while lycopene content was significantly lower (0.56 µg/mg), which are higher than that of the methanolic extract of *P. Squarrosulus*, which were 570 ng and 225 ng per mg respectively [36], and polyphenol-rich extract of *R. Laurocerasi* [18] (0.01 and 0.007 µg/mg respectively). The estimated flavonoid content of RalPre is 7.94 µg/mg, which is higher than that of *R. laurocerasi*, reported to be 2.4 µg/mg [18]. Total phenols, flavonoids and β-carotene were the major naturally occurring antioxidant components estimated in this study. The higher amounts of these components in this extract might explain its more effectiveness in antioxidant activities.

CONCLUSION

As a result of the study, the polyphenol-rich extract of *Russula albonigra* (RalPre) was found to be an effective antioxidant in different in vitro assays. RalPre was an excellent

chelator of ferrous iron, scavenger of DPPH free radicals and superoxide anions. The EC₅₀ values for these tests were all well below 1 mg/ml. However, RalPre was also a potent reducer of ferric iron, with an EC₅₀ value just over 1 mg/ml (1.12 mg/ml). Thus, it can be suggested as a natural additive in food and pharmaceutical industries.

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