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RESEARCH ARTICLE

Chemical and Biological Studies on a Recently Discovered Edible Mushroom: a Report

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SUMMARY

In our previous publication, we have justified Russula alatoreticula as a novel macrofungus that has long been prized in local people's diet. Thus, the present work was designed to unveil its diverse medicinal prospects and for that a heat stable polyphenol rich fraction was prepared. The extract was found to be rich mainly in phenolics and flavonoids, while carotenoids as well as ascorbic acid were detected in minor amount. Further analysis by HPLC revealed manifestation of at least six phenolic components, of which pyrogallol and cinnamic acid were tentatively recognized. Simultaneously, the fraction presented strong free radical (OH, DPPH, $ABTS^{-}$) scavenging activity and chelating ability of metal ion with EC50 value ranging from 75–3615 µg/ml. In contrast to that, the formulation exhibited moderate antibacterial efficacy as it was able to inhibit only Gram positive bacteria namely Listeria monocytogenes and Staphylococcus aureus. Overall, the present work contributed new record regarding a unique mushroom where its polyphenol rich fraction could be regarded as a potent source of natural therapeutic agents.

Key Words: *Antibacterial property, Antioxidant activity, HPLC, Hydro-ethanol fraction, Russula alatoreticula*

Yeni keşfedilen yenilebilir bir mantar üzerine kimyasal ve biyolojik çalışmalar: Bir Rapor

ÖZET

Daha önceki çalışmalarımızda, uzun zamandır halkın diyetinde değerli olan Russula alatoreticula'nın yeni bir makrofungus olduğu kanıtlanmıştır. Böylece,,çeşitli tıbbi özelliklerini ortaya çıkarmak üzere söz konusu çalışma tasarlanmış, polifenolce zengin ısıya dayanıklı bir fraksiyon hazıelanmıştır.Ekstrede karotenoidler ve askorbik asit az miktarda saptanırken, ana olarak fenolik ve flavonoidlerce zengin olduğu tespit edilmiştir. İleri HPLC analizlerinde en azından 6 farklı fenolik bileşik ortaya çıkarılmış, deneme olarak pirogallol ve sinnamik asit tanımlanmıştır. Aynı anda fraksiyon güçlü serbest radikal (OH, DPPH, $ABTS^{-}$) süpürücü aktivite ve 75-3615 µg/ml aralığında EC50 değeri ile metal iyonu şelasyon özelliği göstermiştir. Bunun tersine, formülasyon orta düzeyde antibakteriyel etkinlik göstermiş, yalnızca Listeria monocytogenes and Staphylococcus aureus isimli gram pozitif bakterileri inhibe etmiştir.sonuç olarak, bu çalışma , polifenoliklerce zengin fraksiyonu güçlü bir doğal terapötik maddelerin kaynağı olan bir özgün bir mantara ilişkin olarak yeni bir yayın olarak katkıda bulunmuştur.

Anahtar Kelimeler: *Antibateriyel özellik, Antioksidan aktivite, HPLC, Hidro-etanol fraksiyon, Russula alatoreticula*

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INTRODUCTION

During recent field inspection, a brightly coloured mushroom was collected from lateritic region of West Bengal, India with the help of tribal mycophagy inhabitants. After detailed study, the taxon was identified as a novel specimen and named as *Russula alatoreticula* (Russulaceae, Basidiomycota). In continuation of that work, a water soluble polysaccaridic fraction was prepared that displayed strong antioxidant, immune-stimulatory and moderate antibacterial activities (Khatua et al., 2017b).

Besides carbohydrate, mushrooms consist another class of therapeutic compounds that are derived from secondary metabolism. These constituents like phenols and flavonoids are synthesized for protection against pathogens like insects, viruses and bacteria; as a result many of them are ideal for embarrassment of microbial growth. At present, these nature derived substances are in high demand due to worldwide escalation of antibiotic resistant pathogens (Tiong et al., 2016). Simultaneously, phenolic components are also ascribed for ability to provide antioxidative protection in biological systems (Khatua et al., 2013a). They are characterized by aromatic rings with one or more hydroxyl groups making them ideal for radical scavenging activity. They possess the capacity of quenching lipid peroxidation, chelating redox active metals and preventing DNA oxidative damage. As a result, great attention has been paid to these bioactive compounds now-a-days due to their ability to promote benefits for human health (Khatua et al., 2013b, c; Khatua et al., 2015a).

Therefore, the present study was designed for determination of phenolic composition of the ethnic mushroom, *R. alatoreticula* and for that a polyphenol rich extract was prepared. Further, the extract was evaluated for myco-chemical composition, antioxidant activity and antibacterial potential to predict usefulness of the specimen.

MATERIALS AND METHODS

Chemicals

2-Deoxy-D–ribose, hydrogen peroxide, ferric chloride, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ferrous chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium persulfate, ferrozine, ammonium molybdate, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), 2,6-dichlorophenol indophenol (DCPIP), Folin-Ciocalteu, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), *p*-iodonitrotetrazolium chloride (INT), nutrient broth (NB) and oxalic acid were purchased from Himedia, Mumbai, India. Eleven HPLC standards such as gallic acid, vanillic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, chlorogenic acid, ferulic acid, salicylic acid, quercetin, myricetin, cinnamic acid and pyrogallol were procured from Sigma Aldrich (MO, USA).

Collection of basidiocarps and authentication

Fruiting bodies of *R. alatoreticula* were collected from natural habitat of West Bengal in the month of August, 2013 (collector: Prof K. Acharya). Identity of the gathered basidiome was confirmed by Prof. K. Acharya based on morphological and DNA barcoding analyses (Accession no: CAL 1271) as described in our previous publication (Khatua et al., 2017b).

Preparation of extract

Fruit bodies including both pileus and stipe were desiccated by a field drier at 40°C to make them crispy. Dried basidiocarps were further pulverized using electric blender, sieved through 160 mesh and preserved in an air tight container. 5 gm of powdered basidiocarps were extracted with 50 ml of ethanol overnight at room temperature and subsequently separated by Whatman filter paper. Residue was then re-extracted with 30 ml of ethanol, the filtrate was air dried and subjected to 100 ml distilled water at boiling condition for about 7 hrs. After filtration, 4 volume of ethanol (400 ml) was added to the supernatant slowly and kept at 4°C overnight. Precipitate was separated by centrifugation and the supernatant was concentrated under reduced pressure with the help of rotary evaporator (Rotavapor R-3, Butchi, Switzerland) (Khatua et al., 2013d). The yield percentage of extract was calculated based on dry weight as:

$$\text{Yield } (\%) = (W_1 \times 100) / W_2$$

Where W_1 = weight of extract after solvent evaporation; W_2 = weight of the minced mushroom

Total polyphenol content determination

To determine total phenolic compounds, 1 ml of the studied extract was mixed with 1 ml of Folin-Ciocalteu reagent. After 3 min incubation, 1 ml sodium carbonate solution (35%) was added and adjusted to 10 ml by water. The reaction was kept in dark for 90 min, and absorbance was measured at 725 nm against blank. Gallic acid (10–40 µg) was considered as standard and results were expressed as µg of gallic acid equivalents per mg of extract (Khatua et al., 2017d).

Total flavonoid estimation

1 ml extract was diluted with 4.1 ml of 80% aqueous ethanol, 0.1 ml of 1 M potassium acetate and 0.1 ml of 10% aluminium nitrate to estimate total flavonoid content. After 40 min incubation at room temperature absorbance was detected at 415 nm. Quercetin (5–20 µg) was used to calculate the standard curve and results were expressed as µg of quercetin equivalents per mg of extract (Khatua et al., 2017d).

β-carotene and lycopene estimation

100 mg extract was mixed with 10 ml acetone-hexane solution (4:6) and filtered through Whatman no 4. Absorbance was detected at three different wavelengths simultaneously such as 453, 505, 663 nm (Khatua et al., 2017d). Content of carotenoids were

calculated according to the following equations:

β-carotene (mg/100 ml): 0.216 A_{663}–0.304 A_{505}+0.452 A_{453}

Lycopene (mg/100 ml): –0.0458 A_{663}+0.372 A_{505}–0.0806 A_{453}

Ascorbic acid determination

10 ml ascorbic acid (100 µg/ml) was prepared using 0.6% oxalic acid and titrated against DCPIP. The extent of dye consumed (V_1 ml) represented the quantity of ascorbic acid. Likewise, sample (concentration W µg/ml) was also titrated against dye (V_2 ml) (Khatua et al., 2017d) and amount of ascorbic acid present in the extract was calculated using following formula,

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

Detection of phenolic compounds by HPLC

0.5 mg extract dissolved in 1 ml of HPLC grade methanol was filtered and 20 µl of the filtrate was analysed by HPLC system equipped with an Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm). The mobile phase consisted of eluent A (acetonitrile) and eluent B (0.1% v/v aqueous phosphoric acid). Elution was carried out by using a gradient procedure: 0–5 min, 5% A; 5–10 min, 15% A; 10–15 min, 40% A; 15–20 min, 60% A; 20–22 min, 90% A. The compounds were identified by comparing UV spectra and retention time with authenticate standards namely gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, ferulic acid, myricetin, salicylic acid, quercetin, cinnamic acid and pyrogallol (Chatterjee et al., 2016).

Evaluation of antioxidant potential

Total antioxidant capacity was carried out as described by Prieto et al. (1999) and activity was expressed as number of equivalents of ascorbic acid. Hydroxyl radical ($OH^{\cdot}$) scavenging activity of the polyphenol rich extract (10–100 µg/ml) was evaluated using Fenton's reaction system (Halliwell et al., 1987). In addition, quenching potential of ABTS radicals by the studied extract (50–400 µg/ml) was also determined and absorbance was recorded using Bio-Rad iMarkTM Microplate Reader (USA). Further, the ability of investigated extract (1000–3000 µg/ml) to scavenge DPPH radical was estimated. Finally, the assay of chelating effect on ferrous ion was performed using different concentrations (2000–4000 µg/ml) of the extract (Khatua et al., 2017 a, c).

Estimation of antibacterial activity

Bacillus subtilis ATCC® 6633™ (MTCC 736), *Listeria monocytogenes* ATCC® 19111™ (MTCC 657), *Staphylococcus aureus* ATCC® 700699™, *Escherichia coli* ATCC® 25922™, *Salmonella typhimurium* ATCC® 23564™ (MTCC 98) and *Klebsiella pneumoniae* ATCC® 15380™ (MTCC 109) were utilized for the experiment. The six investigating microbes were cultured freshly and 1×10^5 CFU/ml concentrated dilutions were separately prepared. Reactions were performed in microtiter plate consisting 200 µl of NB, 20 µl of inoculum and different dilutions of studied extract (1–15 mg/ml). Following incubation for 24 h at 37°C, 40 µl of INT (0.2 mg/ml) was added to each reaction mixture and incubated for another 30 min at room temperature. Concentration that inhibited 50% growth of bacteria in comparison with positive control was calculated as minimum inhibitory concentration (MIC). Streptomycin was used as a standard drug. (Khatua et al., 2017b).

Statistical analysis

All data are presented herein as mean ± standard deviation of three independent experiments each in triplicate. Calculations were performed using statistical package for Microsoft® Office Excel (Microsoft®, USA) and differences were evaluated by means of one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

It is important to select appropriate extraction procedure as the parameters such as solvent type, extraction time etc. play significant role in recovery percentage as well as bioactivity (Hasnat et al., 2013). The naturally occurring antioxidant compounds in mushroom like phenols and flavonoids are polar in nature, thus use of polar solvent would be suitable as extractant. In this context, hydro-ethanol mixture is widely used due to its affinity towards phenolics and safety for human consumption. To prepare such extract in the present study, ethanol was used initially to remove coloured material, steroid, terpenoids and fat. Afterwards, water was applied as extracting solvent and later ethanol was added to discard polysaccharides as well as increase solubility of organic compounds. Finally, an organic fraction from *R. alatoreticula* was prepared that appeared yellowish brown in colour with high recovery percentage (18.5 ± 3.5%). Interestingly, the yield was estimated to be about nine times higher than phenol rich fraction from *Ramaria aurea* (Khatua et al., 2015b) indicating more leaching efficacy of the studied macro fungus. Further analysis indicated that phenol was the major component in the fraction (5.85 ± 0.39 µg gallic acid equivalent/mg of extract) followed by flavonoid (3.09 ± 0.25 µg quercetin equivalent/mg of extract). In contrast, negligible amount of β-carotene and lycopene were quantified such as 0.62 ± 0.18 µg/mg and 0.41 ± 0.13 µg/mg of dry extract respectively. Ascorbic acid was also present in minor amount (0.97 ± 0.22 µg/mg of dry extract).

Further to that, HPLC was performed to insight into molecular constituents. The chromatogram as presented in Figure 1 showed a qualitative profile of the fraction which was composed of minimum six components. Among them pyrogallol was detected as the dominant constituent (44.39 ± 1.23 µg/mg of dry

extract) followed by cinnamic acid (1.36 ± 0.08 µg/mg of extract). Consequently, the finding was in accordance with previous publications where these two phenolics were also reported (Khatua et al., 2017d; Khatua et al., 2014; Khatua et al., 2015a).

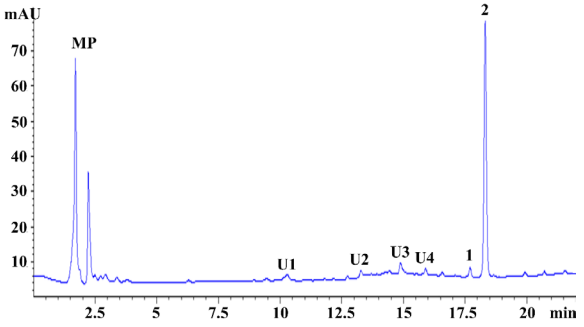

Figure 1. HPLC chromatogram of polyphenol rich extract from *Russula alatoreticula*. (Peaks MP: mobile phase, 1: cinnamic acid, 2: pyrogallol, U1–4: undetermined).

Besides, the extract was subjected to screen for its possible antioxidant activity and the results have been summarized in Table 1. Firstly, $OH^{\cdot}$ scavenging potential was determined as the radical can damage biomolecules immediately after generation (Valko et al., 2007). Hence, it is important to estimate $OH^{\cdot}$ quenching ability of the investigating drug and for that Fe^{2+}-ascorbate- EDTA- H_2O_2 *in vitro* system was followed herein. The studied extract exhibited potent activity which increased gradually in a dose dependent manner. The fraction inhibited 20.71, 28.14, 35.46, 47.23 and 65.9% radicals at the level of 10, 30, 50, 70 and 100 µg/ml (Figure 2A). As a result, the fraction from *R. alatoreticula* was detected to exhibit better antioxidant activity than those of many natural mushrooms like *Auricularia mesenterica*, *A. polytricha* and *A. fuscosuccinea* (Mau et al., 2001).

Table 1: Antioxidant activity of polyphenol rich extract isolated from *Russula alatoreticula*.

Antioxidant parameters		**Extract**	**Standard**
EC_{50} value (µg/ml)	Scavenging ability of hydroxyl radical	75 ± 10[a]	10 ± 0.2[b]
	Scavenging ability of ABTS radicals	126 ± 10[a]	3.18 ± 0.01[b]
	Scavenging ability of DPPH radicals	2450 ± 31[a]	7.69 ± 0.02[b]
	Chelating ability of ferrous ion	3615 ± 30[a]	2.54 ± 0.52[b]
Total antioxidant activity (µg ascorbic acid equivalent/mg of dry extract)		20.35 ± 6.45	–

The effects are presented in terms of EC_{50} values (mean ± standard deviation; n = 3) corresponding to 50% of antioxidant activity except total antioxidant capacity assay. Ascorbic acid was used as standard in hydroxyl radical inhibition, DPPH radical quenching, ABTS radical scavenging and total antioxidant capacity protocols, while EDTA was adopted as a positive control in chelating ability of ferrous ion method. In each row, different letters mean significant differences between the sample and standard ($p < 0.05$).

Table 2. Antibacterial activity of polyphenol rich extract from *Russula alatoreticula*.

Type of bacteria	**Name of bacteria**	**Extract (µg/ml)**	**Streptomycin (µg/ml)**
Gram positive	*Listeria monocytogenes*	8830 ± 5.96[a]	4.68 ± 0.17[b]
	Staphylococcus aureus	7168 ± 89.2[a]	6.29 ± 0.16[b]
	Bacillus subtilis	ND	5.61 ± 0.01
Gram negative	*Escherichia coli*	ND	5.41 ± 0.11
	Salmonella typhimurium	ND	5.09 ± 0.03
	Klebsiella pneumoniae	ND	5.29 ± 0.14

The activity was estimated by determining minimum inhibitory concentration (MIC) value (mean ± SD; n = 3). In each row different letters mean significant differences ($p < 0.05$). ND: No activity was detected within 15 mg/ml of extract treatment

$ABTS^{\cdot-}$ quenching potential was also evaluated for further assessment of antioxidant activity of the extract. The radicals were generated by persulfate oxidation of $ABTS^{2-}$ that can be reduced in presence of antioxidative substance resulting decolourization (Khatua et al., 2017c). Analysis indicated that the extract under investigation possessed strong radical scavenging activity that amplified with increase of concentration (Figure 2B). As the doses ranged from 50, 100, 200, 300 to 400 µg/ml, quenching effects of the extract amplified from 40.12, 48.27, 55.24, 62.47 to 69.46%. However, EC_{50} value of the extract was detected to be higher than *M. lobayensis* indicating lower potential of *R. alatoreticula* (Khatua et al., 2017d).

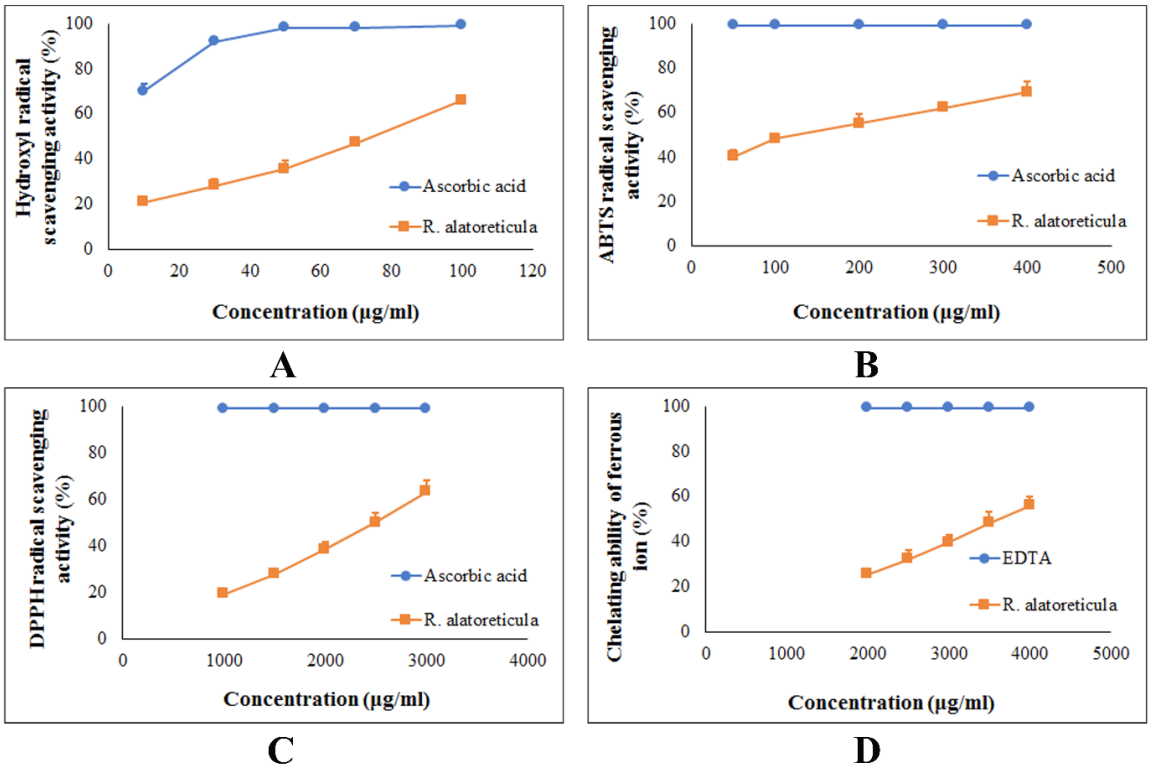

Figure 2. Antioxidant activity of polyphenol rich extract from *Russula alatoreticula*. (A) Hydroxyl radical scavenging activity (B) ABTS radical scavenging activity (C) DPPH radical scavenging activity (D) Chelating ability of ferrous ion.

In addition, DPPH· scavenging assay was also performed being the most easy method for estimation of antioxidant activity. As presented in Figure 2C, the extract was able to reduce DPPH· to yellow coloured diphenyl-picrylhydrazine in a concentration wise pattern. At the level of 1000, 2000 and 3000 µg/ml, the fraction quenched radical at the rate of 19.39, 38.67 and 63.2% respectively. Data suggested that extract from *R. alatoreticula* exhibited radical scavenging activity quite similar to *Macrocybe crassa* (Acharya et al., 2015b) but lower than *Russula senecis* (Khatua et al., 2015a), *Grifola frondosa* (Acharya et al., 2015a), *Laetiporus sulphureus* (Acharya et al., 2016) and *Pleurotus djamor* (Acharya et al., 2017b).

Another popular method for determination of antioxidant activity is Fe^{2+} chelating assay. The technique depicts ability of antioxidants to capture the metal ion that helps in generation of OH· by Fenton's reaction (Khatua et al., 2017c). At 2000, 3000 and 4000 µg/ml concentrations the fraction showed chelating ability of 25.3, 39.67 and 55.73% respectively (Figure 2D). Studies with relevant literature proved that extract from *R. alatoreticula* exhibited lower activity than *Ramaria subalpine* (Acharya et al., 2017a).

Finally, phosphomolybdenum method was performed for evaluation of total antioxidant capacity. The assay is based on formation of green phosphate/Mo (V) complex due to reduction of Mo (VI) to Mo (V) by the antioxidant compound (Khatua et al., 2017d). The formulation showed 20.35 ± 6.45 µg ascorbic acid equivalent/mg of dry extract antioxidant capacity. As a result, *R. alatoreticula* presented higher activity in this regard than *G. frondosa* (Acharya et al., 2015a).

The antibacterial activities of polyphenol rich extract from *R. alatoreticula* was evaluated against six bacterial strains, some of them cause diseases in respiratory and gastrointestinal tracts. The extract presented moderate inhibitory potential particularly against Gram positive bacteria except *B. subtilis*. The extract was found to be more active against *S. aureus* and *L. monocytogenes* where MIC value ranged from 7168 to 8830 µg/ml. However, all three Gram negative species under investigation were found to be resistant even when challenged by 15 mg/ml of sample. This could be explained by the fact that the cell wall structure of Gram negative strains serve as efficient barrier to various compounds (Matijašević et al., 2016).

CONCLUSION

Overall, the present study provides novel information regarding chemical composition, antioxidant effect and antibacterial potential of a polyphenolic extract from an ethnically popular yet scientifically less explored myco-food, *R. alatoreticula*. The extract was found to be rich in phenolic compounds (pyrogallol> cinnamic acid) where flavonoid represented a major portion. Consequently, it was proved as valuable source of free radical scavenging, reducing and metal ion chelating compounds. However, the extract failed to execute effective antibacterial activity particularly against Gram negative species. Taken together, *R. alatoreticula* could be regarded as safe medicinal mushroom and thus warrants further research for development of new pharmaceuticals.

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