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Original Article

Exploring a novel edible mushroom *Ramaria subalpina*: Chemical characterization and Antioxidant activity

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ABSTRACT

Background: During macrofungal inventorisation from an unexplored subalpine forest of Sikkim Himalayas, a new edible mushroom, *Ramaria subalpina* was found and documented in consultation with the forest dwellers. **Objective:** The aim of the present study was to understand the antioxidative potentiality and bioactive constituents of a novel taxon, *Ramaria subalpina,* that is highly prized by the ethnic people of subalpine Sikkim Himalayas, India, for its flavor, texture and gastronomic delicacy since time immemorial. **Methods:** Chemical composition and antioxidant properties of methanolic extract of dried basidiocarps were assessed using HPLC and several *in vitro* assay systems. **Results:** Methanolic extract had phenolics in highest amount, among which pyrrogallol was identified. It also showed potent antioxidant activity. **Conclusion:** The present study suggests that *Ramaria subalpina* has strong medicinal prospects. This novel mushroom can safely be added to the world's edible mushrooms list.

Key words: Antioxidant activity, DPPH, Ferrous ion chelating, HPLC, India.

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INTRODUCTION

Rural communities of India, utilize certain local wild mushrooms for food and medicine, but such indigenous knowledge is either not reported or poorly documented and unsystematically recorded. Claims of their efficacy to be considered as food or medicine need to be scientifically verified prior to their acceptance. It is therefore necessary to know and correctly identify these mushroom species, used by the locals as food or medicine. For this said purpose, the authors are engaged to unveil the hidden macro fungal diversity of the country with an insight to explore their traditional knowledge that is specifically cryptic to the local communities.[1-3]

Choice of food with their myriad usages and cuisine are the signature of cultural, regional and national identity. They are key elements for the dietary patterns in different countries and are consequently important in accurately estimating population dietary intakes. However, throughout Europe, traditional foods are threatened with extinction due to altered lifestyles, and nutritional information of these foods is missing from most current food composition databases.[4,5] In recent days, consumers are looking for new foods with various organoleptic characteristics for their daily lunch basket. Traditional foods such as mushrooms might fulfill this demand, as they are quite rich in carbohydrate, fiber and protein with all essential amino acids and have a low fat content.[6] In a general approximation, the fruit body of a mushroom contain about 56.8% carbohydrate, 25.0% protein, 5.7% fat and 12.5% ash on a dry weight basis.[7] In addition, edible mushrooms are also rich in various polyphenolic compounds that are recognized as an excellent antioxidant due to their ability to scavenge free radicals by single-electron transfer. The enhancement of antioxidant systems for the prevention of cellular oxidative damage via consumption of antioxidant rich foods is of great interest.[8]

In search of wild edible mushrooms (WEM) that are commonly prized by the local forest dwellers across the country, we came across quite a number of mushroom taxa that has been previously identified and reported therein.[3,9,10] *Ramaria subalpina* K. Das & K. Acharya [commonly called as 'thokrechyau' (Nepali; 'thokre' = in bunch and 'chyau' = mushroom)], a newly described taxa,[10] traditionally consumed as an edible mushroom (Figure 1A) by the ethnic people of subalpine Sikkim Himalaya for its flavor, texture and gastronomic delicacy since time immemorial was explored to understand its antioxidative potentiality and bioactive constituents.

MATERIALS AND METHODS

Preparation of methanolic extracts

Basidiocarps of the collected specimen were cleaned to remove residual compost and dried at a maximum temperature of 45 ± 3°C. Dried basidiocarps were

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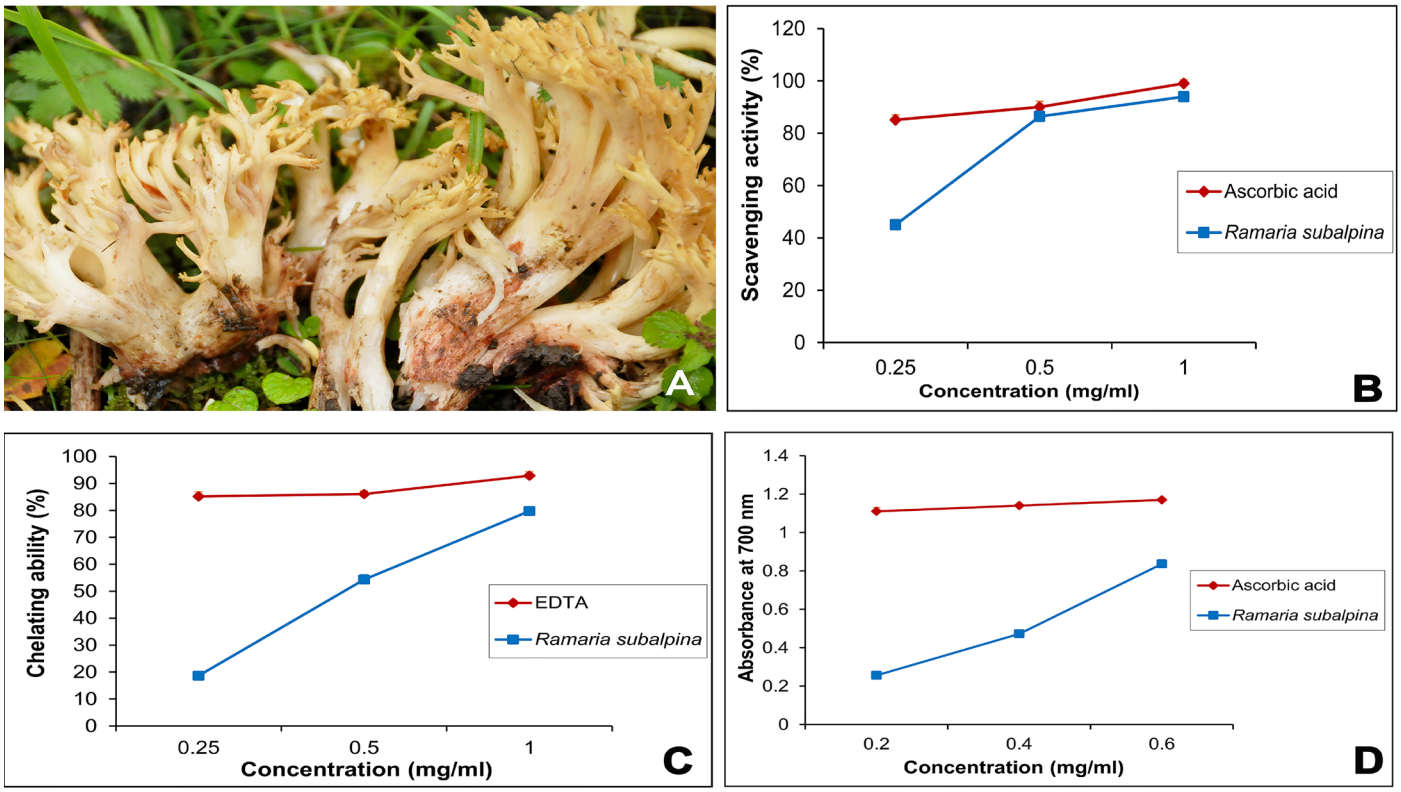

FIGURE 1: *Ramaria subalpina* (A) Fresh basidiocarps in the field (B) DPPH radical scavenging activity (C) Ferous ion chelating ability (D) Reducing power. Results are the mean ± SD of three separate experiments, each in triplicate.

ground to obtain fine particles. One gm of the powder was extracted by stirring with 50 ml of methanol for overnight and subsequently separated by Whatman filter paper. The residue was then re-extracted with 30 ml of methanol and the combined methanolic extracts were evaporated at 40°C (Rotavapor R3 Buchi, Switzerland) to dryness. The methanolic fraction was stored at -20°C in a dark bottle until analysis, for no more than 1 month. A stock solution of 20 mg/ml of the fraction was prepared. Successive dilutions were made to evaluate chemical characterization and antioxidant activity.[11]

Chemical composition

Determination of total phenolic compound

Total phenolics were measured using Folin-Ciocalteu reagent.[12] 1 ml of methanolic extract was mixed with 1 ml Folin-Ciocalteu reagent and incubated for 3 minutes at room temperature. After incubation, 1 ml 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 flavonoid content

Flavonoid concentration was determined following the method as described by Adebayo *et al.*[13] 1 ml methanolic extract 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 to it. After 40 min of incubation 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.[14] The process, in brief was 100 mg of methanolic extract 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 based on the following equations:

Lycopene (mg/100 ml) = – 0.0458A663 + 0.372A505 – 0.0806A453

ß-carotene (mg/100 ml) = 0.216A663 – 0.304A505 + 0.452A453

Determination of ascorbic acid content

Ascorbic acid was determined by titration as described by Rekha *et al.*[15] With little 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 (V1 ml) is equivalent to the amount of ascorbic acid. The methanolic extract (w µg/ml) was similarly titrated with the dye (V2 ml). The amount of ascorbic acid was calculated using the formula-

Ascorbic acid (µg /mg) = ({(10 µg/V1 ml) × V2 ml} × w µg) × 1000

Determination of phenolic profile by HPLC

For quantitative analysis of phenolic compounds, a 3-level calibration curve was obtained by injection of known concentrations (10–50 µg/ml) of eleven standard compounds: gallic acid ($y = 34.773x–9.2238$; $R^2 = 0.9991$), *p*-hydroxybenzoic acid ($y = 45.79x–7.3583$; $R^2 = 0.9995$) chlorogenic acid ($y = 13.776x–2.9025$; $R^2 = 0.9993$), vanillic acid ($y = 19.225x+0.2588$; $R^2 = 0.9994$), *p*-coumaric acid ($y = 49.773x–10.541$; $R^2 = 0.9994$), ferulic acid ($y = 30.425x–2.8188$; $R^2 = 0.9995$), myricetin ($y = 5.0676x–6.0375$; $R^2 = 0.9937$), salicylic acid ($y = 4.4974x–0.4763$; $R^2 = 0.9994$), quercetin ($y = 5.2478x–5.9763$; $R^2 = 0.9954$), cinnamic acid

(y = 108.07x–111.55; R^2 = 0.9979) and pyrogallol (y = 10.8x+0.3333; R^2 = 0.9999).

For HPLC analysis, 10 mg dried extract was dissolved in 1 mL of HPLC grade methanol and then was filtered through a 0.2 µm membrane, 20 µL of the filtrate was analyzed by HPLC (Agilent, USA). Separation was performed with Agilent Eclipse plus C_{18} column (100 × 4.6 mm, 3.5 µm) using a flow rate of 0.8 mL/min at room temperature. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). Elution was carried out by using a gradient procedure: 0–2 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 their UV spectra recorded with diode array detector and retention time of authenticate standards. The results were expressed in µg per g of extract.

Antioxidant activity of the mushroom fraction

Total antioxidant activity

The total antioxidant capacity was determined as described by Prieto *et al.*[16] With little modification. In brief, 0.3 ml of methanolic extract with varying concentration (0.1–1 mg/ml) was added to 3 ml of the reaction mixture (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.

DPPH radical scavenging activity

Radical scavenging activity was determined using a DPPH assay as described by Shimada *et al.*[17] Various concentrations of the methanolic extract (0.25, 0.5 and 1 mg/ml) were added to 2 ml 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:

$$\text{Scavenging effect (\%)} = \{(A0–A1) / A0\} \times 100$$

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

Ferrous ion chelating ability

Chelating ability was determined according to the method of Dinis *et al.*[18] Different concentrations of methanolic extract (0.25, 0.5 and 1 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. EC_{50} 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:

$$\text{Scavenging effect (\%)} = \{(A0–A1)/A0\} \times 100$$

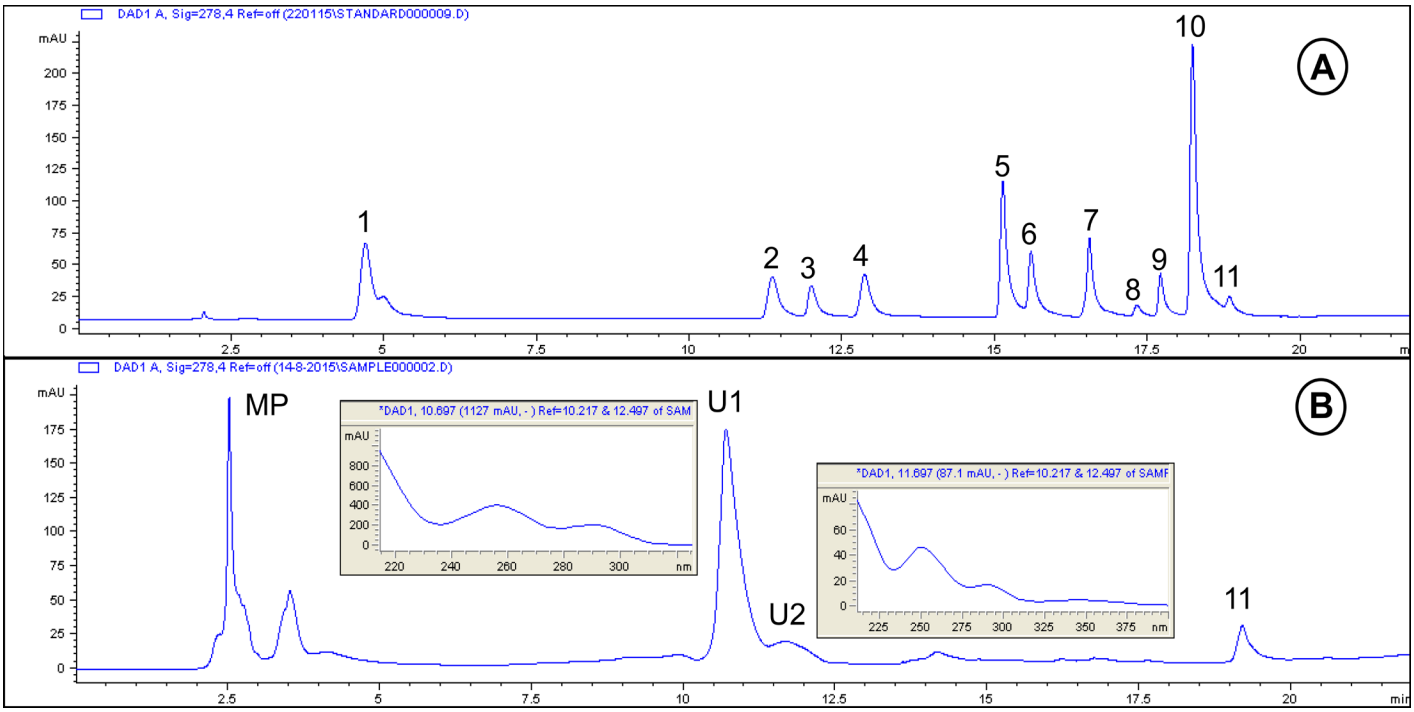

FIGURE 2. HPLC chromatogram of standards and methanolic extract of *Ramaria subalpina* (A) Standards (B) Phenolic profile of the extract (peaks: MP: mobile phase; 1: gallic acid (RT 4.695 min); U1: unidentified peak (RT 10.697 min); 2: p-hydroxybenzoic acid (RT 11.357 min); U2: unidentified peak (RT 11.692 min); 3: chlorogenic acid (RT 11.997 min); 4: vanillic acid (RT 12.867 min); 5: p-coumaric acid (RT 15.128 min); 6: ferulic acid (RT 15.588 min); 7: myricetin (RT 16.547 min); 8: salicylic acid (RT 17.327 min); 9: quercetin (RT 17.711 min); 10: cinnamic acid (RT 18.237 min); 11: pyrogallol (RT 18.837 min).

Table 1: Total phenolic compound, flavonoid, ß-carotene, lycopene and ascorbic acid concentrations of the methanolic extract of Ramaria subalpina.

Total phenolic compound (µg/mg)	Total flavonoid content (µg/mg)	Total ß-carotene content (µg/mg)	Total lycopene content (µg/mg)	Ascorbic acid contain (µg/mg)
17.5±2.5	2.5±0.5	0.0239±0.002	0.0122±0.0016	0.083±0.007

Values are mean ± SD of three separate experiments each in triplicate. Total phenols are expressed in GAE, and flavonoids are QAE, GAE: Gallic acid equivalent, QAE: Quercetin equivalent.

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

Determination of reducing power

The reducing power of the methanolic extract was determined according to the method of Oyaizu[19]. Different concentrations of the fraction (0.2, 0.4 and 0.6 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 50°C 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 indicates the effective concentration at which the absorbance was 0.5 for reducing power.

RESULTS AND DISCUSSIONS

Chemical composition

Table 1 shows total phenolic compound, flavonoid, ß-carotene, lycopene and ascorbic acid concentrations of the methanolic extract of *Ramaria subalpina*. Total phenolic compound are the major bioactive component found in extract; followed by flavonoid, ascorbic acid, ß-carotene, and very small concentration of lycopene. The results revealed that *R. subalpina* had exhibited quite higher amount of total phenolic compound and total flavonoid content as compared to previously reported methanolic extract of edible *Ramaria flava* (Schaeff.) Quél. which showed 10.51 ± 0.47 µg/mg of total phenolic compound and 0.51 ± 0.01 µg/mg of total flavonoid.[20]

HPLC helps to predict phenolic composition of the extract. Figure 2A depicts a typical HPLC chromatogram of eleven phenolic compounds and Figure 2B represents HPLC chromatogram of the extract at 10 mg/ml concentration. The results showed a qualitative profile of extract where 1 peak had been identified. The component in the fraction was found to be pyrrogalol that being present as much as 6.287 ± 2.473 µg/mg of extract. In addition, two unrecognized phenolic substances (λ_{max} in inset) had been detected in the fraction at 10.697 min and 11.692 min possessing area of 3966.797 and 587.216 respectively.

Antioxidant activity of the mushroom fraction

All living organisms are equipped with stress-response systems that regulate the processes of somatic maintenance and repair.[21] Administration of dietary supplements help to combat oxidative stress and related ailments. Result showed that, 1 mg of *Ramaria subalpina* was equivalent to 230 µg of ascorbic acid. Total antioxidant activity of *R. subalpina* suggests the electron donating capacity, which could act as a radical chain terminator by transforming reactive free radicals into more stable non-reactive products.

DPPH radical scavenging activity can be used to evaluate the antioxidant activity of an extract in a short span of time at room temperature by converting it to 1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine. The amount of yellowing indicates the scavenging potentials of an antioxidant compound.[22,23] The utilization of stable DPPH radical has the benefit for being unaffected by side reactions, such as enzyme inhibition and metal chelation.[24] Thus a lower absorbance at 517 nm indicates a higher radical scavenging activity of the extract. Result indicated that *R. subalpina* exhibited significant radical scavenging activity with EC_{50} value of 0.3±0.05 mg/ml (Figure. 1B). As compared to the previously reported *R. flava* (94.78 ± 0.06% at 12 mg/ml) and *R. formosa*,[20,25] the methanolic extract of *R. subalpina* showed grater electron donating capacity.

Ferrous ions are considered to be effective pro-oxidants in food system that increases the risk of free radical damage and cancer. So, moderate ferrous ion chelating abilities would be beneficial for the human life.[26,27] Sowndhararajan *et al.*[28] Reported that chelating agents are helpful as secondary antioxidants, as they decreases the redox potential and thereby stabilises the oxidised forms of metal ions. Figure. 1C reveals that the *R. subalpina* exhibited a marked capacity for iron binding ability of 50% at a concentration of 0.46 ± 0.03 mg/ml.

Reducing power of any bioactive compound is directly related to the electron donating capacity and can reduce the oxidized intermediates of lipid peroxidation processes in such a way so that they can act as primary and secondary antioxidants.[29,30] Presence of reducers causes the conversion of Fe^{3+}/ferricyanide complex to the ferrous form. Figure 1D revealed that *R. subalpina* showed reducing power of 0.5 at concentration of 0.44 ± 0.02 mg/ml.

CONCLUSION

Exploration of the medicinal properties of dried basidiocarps of *Ramaria subalpina* were subjected for preparation of heat stable phenol rich extract using methanol as solvent system. The fraction showed its potentiality in chelating ferrous ion, DPPH radical scavenging, reducing power and total antioxidant. Total phenols were also found to be the key bioactive component of the extract; followed by flavonoid, ascorbic acid, ß-carotene, and lycopene. Molecular phenolic profiling of the extract through HPLC-UV indicated probable existence of pyrrogalol in a higher amount. All these results led to consider the potentiality of the studied mushroom to have a good source of bioactive molecules such as phenolic components for dietary supplements and functional food.

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CONFLICT OF INTEREST

There is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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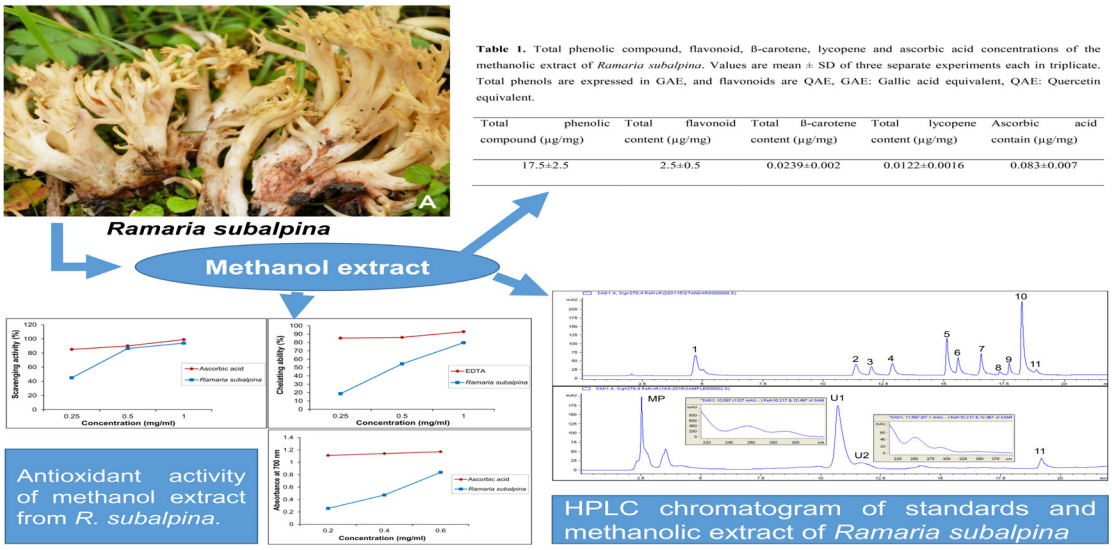
Graphical Abstract



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