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Research Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING CAPACITY OF PHENOLIC EXTRACT FROM RUSSULA LAUROCERASI

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ABSTRACT

Objectives: Cellular damage caused by reactive oxygen species has been implicated in several diseases; hence antioxidants have significant importance in human health. The objective of this study was to evaluate antioxidant properties of phenolic extract from *Russula laurocerasi* and presence of components responsible for the activity.

Methods: The antioxidant properties were studied using various *in vitro* assays. Qualitative determination of different bioactive constituents such as phenol, flavonoid, β-carotene, lycopene and ascorbic acid were also done.

Results: The extract was strong hydroxyl radical scavenger reflected by its low EC_{50} value i.e. 0.03 mg/ml. EC_{50} values of the extract was in the order of hydroxyl radical scavenging< chelating ability of ferrous ion< DPPH scavenging< superoxide radical scavenging< β-carotene bleaching< reducing power. The extract presented a relatively strong antioxidant effect which was found to be correlated with total phenols (R^2= 0.969) and flavonoids (R^2= 0.888) implying that the polyphenols was partly responsible for the antioxidant activities.

Conclusion: Our result thus indicates that the fraction of *R. laurocerasi* may be utilized as a promising source of therapeutics.

Keywords: Antioxidant activity, edible mushroom, flavonoid, phenol, reactive oxygen species. RUNNING HEAD: Antioxidant capacity of phenolic extract of *Russula laurocerasi*

INTRODUCTION

Reactive oxygen species (ROS) are known to control various normal physiologic functions of organisms by acting as secondary messengers. But excessive stimulation of NAD(P)H by cytokines or mitochondrial electron transport chain, xanthin oxidase and some exogenous sources (such as UV radiation, pathogen invasion, herbicide action, oxygen shortage) can overproduce ROS resulting in numerous diseases [1, 2]. Organisms possess several defense mechanisms to control the level of ROS [3]. When such defense mechanisms become unbalanced, antioxidant supplement can be used to reduce the oxidative damage. Repairing such damages by naturally occurring substances mainly by supplementation of food having antioxidant property is becoming one of the most acceptable modes of modern therapy [4].

For thousands of years, edible mushrooms have been used to maintain health and increase longevity [5]. Mushrooms are rich sources of antioxidant compounds, mainly phenolic compounds (phenolic acids and flavonoids), tocopherols, ascorbic acid, carotenoids and polysaccharide [4]. They may act directly as antioxidant [6] or prevent underlying oxidative stress related pathological conditions such as cancer [7], heart ailments [8], diabetes [9], inflammation [10], hepatic damage [11, 12], microbial pathogens [13, 14] etc.

Some members of Russulaceae family have already been assessed for potent antioxidant activity such as *Russula virescens*, *Russula delica* and antimicrobial activity like *R. delica* [15, 16]. But there are no conclusive reports on chemical composition and antioxidant activities about *Russula laurocerasi*, a well-known mycorrhizal fungus, which is used as food in West Bengal, India. So this study was focused on the antioxidant activity of phenol rich fraction obtained from *R. laurocerasi* by their ability to scavenge free radicals. The contents of antioxidant components were also determined.

MATERIAL AND METHODS

Mushroom sampling

Basidiocarps of *R. laurocerasi* were collected in the month of July from the lateritic region of West Bengal and identified using standard literature [17]. A voucher specimen (AMFH 602) has been deposited in the same department of University of Calcutta.

Standards and reagents

All chemicals used were of analytical grade and freshly prepared before use. L-methionine, nitro-blue tetrazolium (NBT), riboflavin, 2-Deoxy-D-ribose, ferric chloride, hydrogen peroxide (H_2O_2), trichloro acetic acid (TCA), thiobarbituric acid (TBA), ferrous chloride, ferrozine, potassium ferricyanide, β-carotene, Tween 20, linoleic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium bicarbonate, Folin-ciocalteu reagent, aluminium nitrate, potassium acetate, acetone, n-hexane, standards such as L-ascorbic acid, ethylenediamine tetraacetic acid (EDTA), butylated hydroxyanisole (BHA), gallic acid, quercetin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Preparation of extract

Polyphenol rich fraction was extracted according to the method of Cui et al. [18] with slight modification. The fruiting bodies of *R. laurocerasi* were cleaned to remove residual compost and dried to remove moisture content. 10 gram of desiccated material were ground and macerated in ethanol at the ratio of 1:20 (w/v) at 25°C for 2 days to eliminate the alcohol soluble constituents such as coloured material, small organic molecules (steroid, terpenoids etc.) and fat. The extracts were filtered through Whatman No. 1 filter paper and the entire extraction process was repeated on the residue. The filtrate was air dried, extracted by stirring with distilled water at 100°C for 8 hrs. After filtration, 4 volume of ethanol was

added to the supernatant slowly and kept at 4°C overnight. Precipitate was discarded after centrifugation and the supernatant was concentrated under reduced pressure in a rotary evaporator. Now, this concentrated polyphenol rich extract of *R. laurocerasi* (RulaPre) was stored at 4°C until further analysis.

Superoxide radical (O_2^-) scavenging assay

The method used by Martinez et al. [19] was followed for determination of radical scavenging ability. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, 75 µM NBT and various concentrations of RulaPre (0.5 – 1.6 mg/ml). After 10 min illumination of a fluorescent lamp, absorbance was measured at 560 nm to monitor production of blue formazan. Identical tubes with the reaction mixture were kept in the dark and served as blanks. BHA was used as a positive control. The degree of scavenging was calculated by the following equation:

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

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample

Hydroxyl radical (OH^-) scavenging assay

The ability of RulaPre to scavenge OH^- radical was determined according to Halliwell et al. [20]. The reaction mixture (1ml) consisted of KH_2PO_4 - KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), variable concentration (0.01 – 0.05 mg/ml) of RulaPre, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbate (100 µM) and H_2O_2 (1 mM). It was incubated at 37°C for 1 h. 2ml TBA-TCA solution (0.375% (w /v) TBA, 15% (w /v) TCA and 0.25 N HCl) was added to stop reaction and incubated at boiling water bath for 15 min. After cooling, absorbance was measured at 535 nm. BHA was used as positive control. The degree of scavenging was calculated by the following equation:

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

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample

DPPH radical scavenging assay

The hydrogen atom or electron donation abilities of RulaPre and a pure compound were measured from the bleaching of the purple coloured methanol solution of DPPH [21]. This spectrophotometric assay uses the stable radical DPPH as a reagent. Various concentrations of RulaPre (0.6 – 1.1 mg/ml) were added to 2ml of 0.004% methanol solution of DPPH (w/v). After 30 min incubation period 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 comparison. The degree of scavenging was calculated by the following equation:

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

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample

Chelating ability of ferrous ions

Chelating ability was determined according to the method of Dinis et al. [22]. Different concentrations of RulaPre (0.3 – 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. 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:

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

Where A0 was the absorbance of the control and A1 was the absorbance in the presence of sample

Determination of reducing power

The reducing power of RulaPre was determined according to the method of Oyaizu [23]. Various concentrations of the fraction (1 – 5 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%) and the 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.

Inhibition of β-carotene bleaching assay

The antioxidant activity of RulaPre was also evaluated by the β-carotene linoleate model system [24]. Reaction mixture consisted of 0.5 mg β-carotene in 1ml HPLC grade chloroform, 25 µl linoleic acid and 200 mg Tween 40. Chloroform was completely evaporated. Then 50 ml distilled water saturated with O_2 was added with vigorous shaking. Aliquots (2ml) of this emulsion were transferred into different tubes containing different concentrations of RulaPre (1 – 3.5 mg/ml) and absorbance was read at 490 nm against water. The tubes were placed at 50°C for 2 h and again absorbance was taken. BHA was used as positive control. The antioxidant activity as percent inhibition rate of β-carotene bleaching relative to control at 2 h was calculated using the equation as follows:

Scavenging effect (%) = {(D0-D1)/ D0} × 100

Where D0 was the β carotene content after 2 h in case of control and D1 was the β carotene content after 2 h in the presence of sample

Total polyphenol contents determination

The content of total phenolic compounds in RulaPre was estimated according to Singleton and Rossi [25]. 1 ml of extract solution was mixed with 1 ml of Folin-ciocalteu reagent. After 3 min incubation, 1 ml of 35% sodium carbonate solution was added to the mixture and it was adjusted to 10 ml by water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm against blank. Gallic acid (10 – 40 µg) was used to calculate the standard curve. Estimation of phenolic compound was carried out in triplicate. The results were expressed as µg of gallic acid equivalents per mg of extract.

Total flavonoid estimation

Total flavonoid content was determined according to Park et al. [26]. 1 ml extract was diluted with 4.1 ml of 80% aqueous ethanol, 0.1 ml of 10% aluminium nitrate and 0.1 ml of 1M potassium acetate. After 40 min incubation at room temperature the absorbance was measured at 415 nm. Quercetin (5 – 20 µg) was used to calculate the standard curve. Estimation of flavonoids was carried out in triplicate. The results were expressed as µg of quercetin equivalents per mg of extract.

β-carotene and lycopene estimation

β-carotene and lycopene were estimated according to the method of Nagata and Yamashita [27]. 100 mg RulaPre was mixed with 10 ml acetone-hexane mixture (4:6) for 1 min and filtered through Whatman no 4. Absorbance was measured at 453, 505, 663 nm. The assays were carried out in triplicate. Content of β-carotene and lycopene 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

Ascorbic acid was determined by titration according to the method described by Rekha et al. [28] with some modification. Standard ascorbic acid (100 µg/ml) was taken in a conical flask and made up to 10 ml by 0.6% oxalic acid. It was titrated against 2, 6-dichlorophenol indophenol dye which was prepared by adding 21 mg sodium bicarbonate and 26 mg dye in 100 ml water. The amount

of dye consumed (V_1 ml) is equivalent to the amount of ascorbic acid. Similarly, sample (concentration w µg/ml) was also titrated against 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.

Statistical analysis

Statistical analysis was done using Excel for Windows Software. A regression analysis, R^2, was established between phenolic and flavonoid contents of the extract using the same software.

RESULTS AND DISCUSSION

Superoxide radical ($O_2^{\cdot-}$) scavenging assay

Superoxide anion is one of the six major reactive oxygen species causing oxidative damage in the human body. Addition of one electron to molecular oxygen forms the superoxide anion radical ($O_2^{\cdot-}$) which again can generate other harmful ROS such as peroxynitrate (ONOO·), peroxyl radicle (LOO·), singlet oxygen, hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide [29].

RulaPre possesses superoxide radical scavenging ability where its activity can be highly compared with BHA (figure 1). At concentrations of 0.5 – 1.6 mg/ml, the scavenging abilities of RulaPre and BHA on superoxide radicals were between 17.9 – 50.9% and 64.4 – 96% respectively. The EC_{50} value of RulaPre (1.56 ± 0.04 mg/ml) was found to be much lower than methanol extract of *Pleurotus squarrosulus* (EC_{50}= 8.63 mg/ml) [30] indicating higher activity of RulaPre.

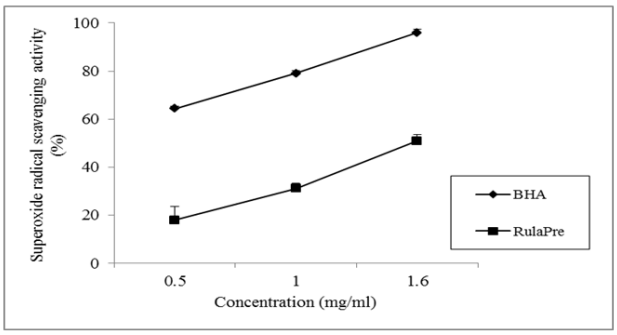

Figure 1: Superoxide radical scavenging activity of phenol rich fraction of *Russula laurocerasi* (RulaPre). Results are the mean ± SD of three separate experiments, each in triplicate.

Hydroxyl radical scavenging assay

Hydroxyl radical is able to damage almost every molecule found in living cell. The DNA strand breaking ability of hydroxyl radical causes mutagenesis and carcinogenesis. Thus it is regarded as extremely reacting free radical in biological systems [1]. In the present study, ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed and detected by their ability to degrade 2-deoxy-D-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH. When the test samples were added to the reaction mixture, they removed hydroxyl radical from the sugar and prevented their degradation [20].

RulaPre exhibited excellent hydroxyl radical scavenging activity in a dose dependent manner (figure 2). EC_{50} value of RulaPre was 0.03 ± 0.004 mg/ml whereas methanol extract of *Russula griseocarnosa* pileus and stipe showed EC_{50} at 7.13 and 11.80 mg/ml respectively [15]. BHA showed tremendous scavenging activity (EC_{50} <0.01 mg/ml).

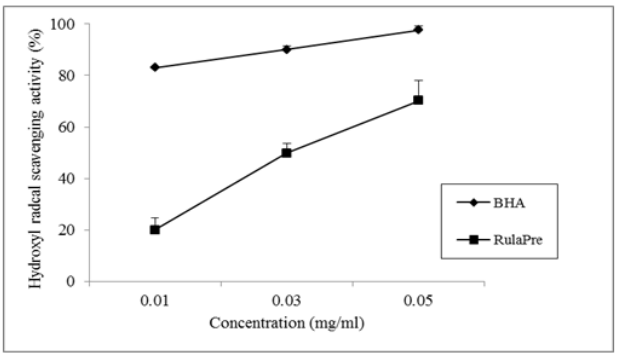

Figure 2: Hydroxyl radical scavenging activity of phenol rich fraction of *Russula laurocerasi* (RulaPre). Results are the mean ± SD of three separate experiments, each in triplicate.

DPPH radical scavenging activity

The characteristic absorbance at 517 nm of DPPH, a stable free radical, decreases significantly on exposure to radical scavengers by providing hydrogen atom or electron to become a stable diamagnetic molecule [31]. The use of DPPH radical has the advances of being unaffected by side reaction, such as enzyme inhibition and metal chelation [32]. Such reactivity has been widely used to test the ability of the compound or extract.

With regard to scavenging ability of DPPH radicals, RulaPre performed well as evidenced by its low EC_{50} value (1.09 ± 0.03 mg/ml) (figure 3). Methanol extract of *Agaricus bisporus* showed much lower activity (EC_{50}= 0.139 mg/ml) [33]. Ascorbic acid was established to be an excellent scavenger (EC_{50} 4.3 ± 0.3 µg/ml).

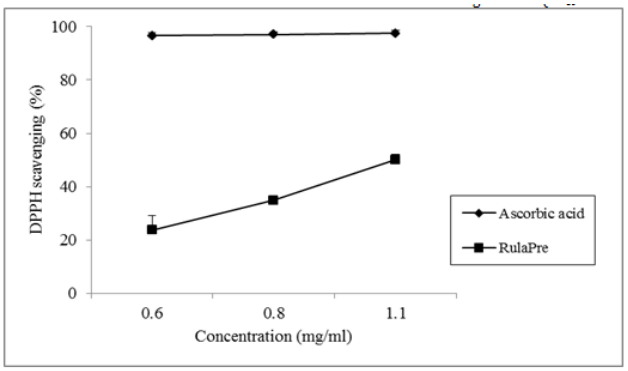

Figure 3: DPPH radical scavenging activity of phenol rich fraction of *Russula laurocerasi* (RulaPre). Results are the mean ± SD of three separate experiments, each in triplicate.

Chelating ability of ferrous ion

Like many transition metals, ferrous ions are effective pro-oxidants in food system, so the moderate ferrous ion chelating abilities would be beneficial. 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 4 reveals chelating ability of RulaPre at 0.3 mg/ml and 0.7 mg/ml were 29.7% and 55.4% respectively (EC_{50} at 0.58 ± 0.04 mg/ml). At the same concentrations the chelating capacities of the standard metal chelator, EDTA, were 94.9% and 98% respectively.

Methanol extract of pileus and stipe of *Russula griseocarnosa* showed EC_{50} at 2.33 and 5.99 mg/ml concentration respectively [15].

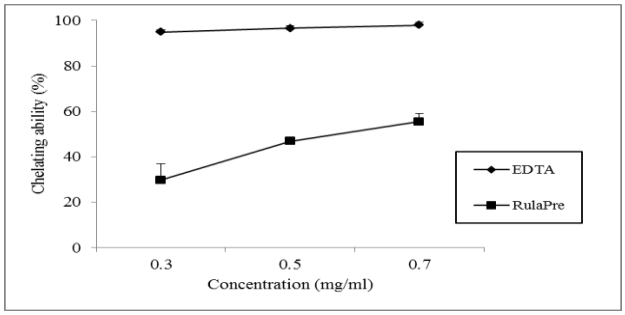

Figure 4: Ferrous ion chelating ability of phenol rich extract of *Russula laurocerasi* (RulaPre). Results are the mean ± SD of three separate experiments, each in triplicate.

Determination of reducing power

Previous report had indicated that the reducing power of any bioactive compound is directly related with the electron donation capacity. Antioxidant as a reducer can be described as redox reaction in which one reaction species is reduced at the expanse of the oxidation of the other. The increasing absorbance at 700 nm by measuring the formation of Perl`s Prussion Blue indicates increase in reducing capacity [34].

Figure 5 shows the reducing power of RulaPre as a function of its concentration. Result displayed that reducing power was 0.5 at 4.483 mg/ml. The EC_{50} value is much lower than methanol extract of *Pleurotus squarrosulus* i.e. 13 mg/ml [30] but higher than methanol extract of *Agaricus arvensis*, *Leucopaxillus giganteus* and *Sarcodon imbricatus* (EC_{50} at 2.86, 1.71 and 2.79 mg/ml concentration respectively) [34].

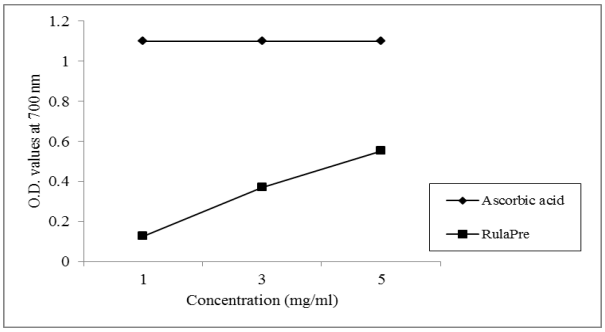

Figure 5: Reducing power of phenol rich extract of *Russula laurocerasi* (RulaPre). Results are the mean ± SD of three separate experiments, each in triplicate.

Inhibition of β-carotene bleaching assay

Inhibition of lipid peroxidation by donation of a hydrogen atom is the basis of β-carotene–/linoleic acid bleaching test. Oxidation of linoleic acid releases linoleic acid peroxide as free radical that oxidizes β-carotene resulting in discoloration and thereby decreasing absorbance value [24].

A linear correlation was found between the ability of RulaPre and the antioxidant capacity (figure 6). EC_{50} of RulaPre was found to be 3.5 ± 0.55 mg/ml which is lower than methanol extract of *Agaricus arvensis*, *Agaricus bisporus*, *Agaricus romagnesii*, *Agaricus silvaticus* and *Agaricus silvicola* (EC_{50} value at 48.30, 21.39, 4.36, 3.72, 14.75 and >5 mg/ml respectively) [35].

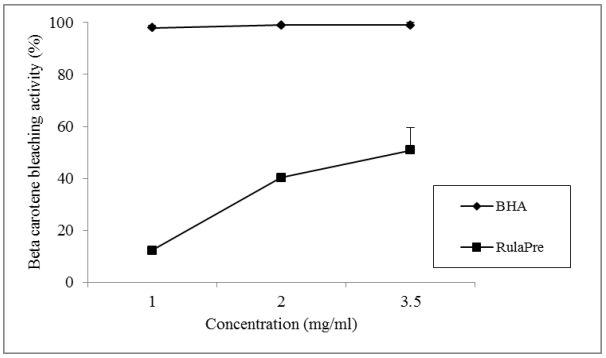

Figure 6: Inhibition of β-carotene bleaching activity of phenol rich fraction of *Russula laurocerasi* (RulaPre). Results are the mean ± SD of three separate experiments, each in triplicate.

Table 1: Correlation coefficients for relationships between antioxidant activities and bioactive compounds such as phenol and flavonoid

Assay	Phenol	Flavonoid
Superoxide radical scavenging	0.968	0.961
Hydroxyl radical scavenging	0.978	0.928
DPPH scavenging	0.963	0.746
Chelating effect of ferrous ion	0.979	0.866
Reducing power	0.99	0.96
Inhibition of β-carotene bleaching	0.934	0.867
Mean	0.969	0.888

Antioxidant components

In the present study, table 2 shows the total phenol, flavonoid, ascorbic acid, β-carotene and lycopene content in the extract. Data exhibits that phenol and flavonoid as the major antioxidant components whereas others are found in vestigial amounts.

Table 2: Total phenol, flavonoid, ascorbic acid, β-carotene and lycopene contents of polyphenol rich extract of *Russula laurocerasi* (RulaPre). Values are mean ± SD of three separate experiments each in triplicate. Total phenols are expressed in gallic acid equivalent (GAE), and flavonoids as quercetin equivalent (QAE).

Phenol (µg /mg)	Flavonoid (µg-/mg)	β-carotene (µg-/mg)	Lycopene (µg-/mg)	Ascorbic acid (µg /mg)
7.05 ± 0.26	2.4 ± 0.32	0.01 ± 0.00003	0.007 ± 0.00001	1.03 ± 0.13

CONCLUSION

The results of the study indicated that phenol rich extract of *R. laurocerasi* (RulaPre) has antioxidant property. The antioxidant effect of the extract was resistant to high temperature, even after boiling. The extract showed excellent hydroxyl radical scavenging activity (EC_{50}= 0.03 mg/ml). While EC_{50} values of the extract for other assays ranged from 0.58 mg/ml for chelating ability of ferrous ion to 5 mg/ml for reducing power. RulaPre contained mixture of bioactive components which is in the order of phenol< flavonoid< ascorbic acid< β-carotene< lycopene of which phenol and flavonoid showed strong association with antioxidant properties of the extract. Thus it can be suggested that RulaPre can be used as a natural additive in food and pharmaceutical industries.

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