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Free radical scavenging property and antiproliferative activity of *Rhodiola imbricata* Edgew extracts in HT-29 human colon cancer cells

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ABSTRACT

Objective: To investigate the *in vitro* antioxidant and antiproliferative activity of rhizome extracts of *Rhodiola imbricata* (*R. imbricata*) in HT-29 human colon cancer cell line. **Methods:** The successively extracted rhizome of *R. imbricata* using various solvents was analyzed for their total phenolics, tannins and flavonoid contents. *In vitro* antioxidant activity was evaluated by employing different assays, including DPPH, ABTS radical scavenging assays, FRAP, phosphomolybdenum reduction assay, superoxide anion, hydroxyl radical scavenging activities and metal chelating ability. **Results:** Acetone and methanol extracts recorded higher phenolic content and showed comparable antioxidant activity with standard reference. Additionally, they also inhibited the proliferation of HT-29 cells upon treatment at higher concentration (200 μg/mL) (acetone and methanol, 84% and 84%, respectively). On examination acetone extract exhibited antiproliferative activity in a concentration dependent manner whereas, methanol extract showed both dose dependent and time dependent inhibitory activity. **Conclusions:** The results obtained justify the traditional usage of *R. imbricata* from their promising antioxidant activity.

1. Introduction

The use of traditional medicine is widespread and plants provide a large source of natural antioxidants that might serve as leads for the development of novel drugs. Therefore, investigations of natural antioxidants and bioactive compounds for preservation of traditional medicines and use in treating certain human diseases have received much attention[1]. Reactive oxygen species are thought to underline the process of ageing and the pathogenicity of various diseases, such as neurodegenerative disorders and cancer. The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs[2].

While use of synthetic antioxidants (such as butylated hydroxytoluene and butylated hydroxyanisole) to maintain the quality of ready-to-eat food products has become commonplace, consumer concern regarding their safety has motivated the food industry to seek natural alternatives. Phenolic antioxidants can inhibit free radical formation and/or interrupt propagation of autoxidation. Fat-soluble vitamin E and water-soluble vitamin C are both effective in the appropriate matrix. Plant extracts, generally used for their flavoring characteristics, often have strong H-donating activity thus making them extremely effective antioxidants. This antioxidant activity is most often due to phenolic acids, phenolic diterpenes , flavonoids, and volatile oils[3].

Phenolic compounds from natural sources are well known radical scavengers, metal chelators, reducing agents and hydrogen donors. As a result, plants containing high level of polyphenols have attracted greater importance as natural antioxidants worldwide. Besides, the dietary pattern is

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also observed to influence at least 35% of cancers globally. The incorrect dietary pattern accounts to 80% of colon cancer not as a consequence of aging, but is intrinsically linked with eating behavior. A recent report reveals an increase in incidence of cancer in Asia due to an increased adoption of westernized dietary pattern[4]. Scientists are re-examining ancient ideas about the benefit of a healthy diet by isolating beneficial nutrients and providing them in pill form. Many chemicals in foods have been shown to kill cancer cells in laboratory studies and to prevent cancer in animals. Some of the dietary components being studied are selenium, vitamin E, polyphenols (from green tea), lycopene (tomatoes), resveratrol (grapes and red wine) and omega-3 fatty acids (oily fish). These compounds work by many different mechanisms. For example, many nutrients such as epigallocatechin gallate in green tea, resveratrol in red wine, and sulforaphane in broccoli prevent cells from going through the cell cycle, which stops them dividing and giving rise to new cancer cells[5].

Currently research is required to investigate dietary components, as they are the most important modifiers of critical targets in the cancer process. Worldwide, scientists have been working on designing drugs to fight cancer, particularly from plant-derived anticancer compounds[6].

Rhodiola imbricata Edgew. (Syn: Sedum roseum; *S. imbricata; S. rhodiola*) (*R. imbricata*) is a perennial herb of the family Crassulaceae, commonly known as golden or arctic root, grows on rocky slopes, common in drier areas of the western Himalaya at an altitude of 4 000-5 000 m. The genus *Rhodiola* is known to biosynthesize phytochemicals such as flavonoids, coumarins and phenyl glycosides. The aqueous extract of *R. imbricata* root was found to contain gallic acid, p-tyrosol, rosavin and rosin[7]. Rhodiola root have been used extensively since time immemorial for its medicinal properties in traditional folk medicine in China, Tibet, Mongolia and former Soviet Republics to increase physical endurance, work productivity, longevity and to treat fatigue, asthma, hemorrhage, impotence and gastrointestinal ailments[8].

A medicated herbal health beverage (tea) is successfully developed, patented and technology transferred to national vendors which are developed by using 11 potential medicinal plants of high altitude in India. *R. imbricata* is the major constituent of this herbal tea and rich in antioxidant value. These products are also being introduced in the Indian army as special high altitude rations[9]. Recently, roots of *R. imbricata*, was found to possess radio-protective[10], cytoprotective and antioxidant[11], wound healing[12], immunomodulatory[13], adaptogenic[14], anti-fatigue[15], anti-cancer[16], neuroprotective[17] and hepatoprotective[18] bio-activities. Also, the extract of *R. imbricata* root was found to be free from heavy metal toxicity[19]. The aim of the present study was to investigate the phenolic contents, antioxidant and antiproliferative properties of *R. imbricata* rhizome in order to evaluate its medicinal value and to point to an easily accessible source of natural antioxidants that could be used as a possible food supplement or nutraceuticals.

2. Materials and methods

2.1. Chemicals and regeants

Potassium ferricyanide, ferric chloride, 2,2-diphenyl-1-picryl-hydrazyl, potassium persulfate, azinobis (3-ethylbenzothiozoline-6-sulfonic acid) disodium salt, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, linoleic acid, ferrous chloride, ammonium thiocyanate, hydrogen peroxide, ferrous ammonium sulfate, ethylenediamine tetracetic acid (EDTA) disodium salt, 2,2′-bipyridyl and hydroxylamine hydrochloride, Penicillin G and streptomycin [3,-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-tetrazolium bromide] (MTT) were obtained from Himedia (Mumbai, India), Sigma (St.Louis, USA) and fetal bovine serum, Dulbecco′s modified Eagle medium-Biochrom (Berlin, Germany). All other chemicals and reagents used were of analytical grade.

2.2. Maintenance of cell line

Human colon adenocarcinoma (HT) cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco′s modified Eagle medium ((Biochrom, Berlin, Germany) with 0.45% glucose supplemented with 10% fetal calf serum and 1% penicillin antibiotic solution in tissue culture flask (Sarstedt, USA). Cells were incubated at 37 ℃ in a humidified, 5% CO_2 atmosphere (Hera cell 240, Germany).

2.3. Plant material

The fresh leaves of *R. imbricata* were collected from western Himalaya′s Leh-Ladakh, Kashmir, India during the month of August to September. The plant was identified and authenticated from Defence Institute of High Altitude Research. Freshly collected plant materials were washed under running tap water and distilled water to remove adhering dust and then dried under shade. The dried samples were powdered in a Mechanical grinder and used for solvent extraction.

2.4. Solvent extraction

The air dried powdered plant samples were extracted in Soxhlet extractor for 8-10 h. successively with petroleum ether, chloroform, hot water and aqueous extract. Each time

before extracting with the next solvent, the material was dried in hot air oven at 40℃. The extracts were concentrated by rotary vacuum evaporator (Yamato RE300, Japan) and then dried. The dried extract obtained with each solvent was weighed. The percentage yield was expressed in terms of air dried weight of plant material. The evaporated extracts thus obtained were dissolved in the respective solvents at the concentration of 1 mg/mL and used for assessment of total phenolics and antioxidant activity through various *in vitro* assays.

2.5. Determination of total phenolics, tannins and flavonoids content

The total phenolic content was determined according to the method described by Siddhuraju and Becker[20] and the results were expressed as gallic acid equivalents (GAE). The tannin content of the tested samples was calculated by Siddhuraju and Manian[21] method in terms of GAE. Total flavonoids in the extracts were estimated as rutin equivalent according to the method of Zhishen *et al*[22].

2.6. In vitro antioxidant assays

2.6.1. Free radical scavenging activity on diphenyl-picrylhydrazyl radical (DPPH$^{\bullet}$)

The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH, according 20 μL to the method of Blios[23]. Sample extracts at various concentrations (20 μL, 40 μL, 60 μL, 80 μL and 100 μL) were taken and the volume was adjusted to 100 μL with methanol. About 5 mL of a 0.1 mM methanolic solution of DPPH was added to the aliquots of samples and BHT and rutin were used as a standard compounds. Negative control was prepared by adding 100 μL of methanol in 5 mL of 0.1 mM methanolic solution DPPH. The tubes were allowed to stand for 20 min at 27℃ The absorbance of the sample was measured at 517 nm against the blank (methanol). Radical scavenging activity of the samples was expressed as IC_{50} which is the concentration of the sample required to inhibit 50% of DPPH$^{\bullet}$ concentration.

2.6.2. ABTS radical cation scavenging activity

Antioxidant activity of this plant was measured using an improved ABTS method as described by Re *et al*[24]. ABTS$^{\bullet}$+ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12-16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30℃ to give an absorbance of 0.70±0.02 at 734 nm. The stock solution of the sample extracts were diluted such that after introduction of 10 μL aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 mL of diluted ABTS solution to 10 μL of sample or Trolox (final concentration 0-15 μM) in ethanol, absorbance was measured at 30℃ exactly 30 min after the initial mixing. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated against the blank (ethanol) absorbance at 734 nm and then was plotted as a function of Trolox concentration. The unit of total antioxidant activity is defined as the concentration of Trolox having equivalent antioxidant activity expressed as μM/g sample extracts.

2.6.3. Ferric reducing/antioxidant power (FRAP) assay

The antioxidant capacity of solvent extracts was estimated according to the procedure described by Pulido *et al*[25]. FRAP reagent (900 μL), prepared freshly and incubated at 37℃, was mixed with 90 μL of distilled water and 30 μL of test sample or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37℃ for 30 min in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent was prepared by mixing 2.5 mL of 20 mM TPTZ in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 M acetate buffer (pH-3.6). At the end of incubation, the absorbance readings were taken immediately at 593 nm against the reagent blank, using a spectrophotometer. Methanolic solutions of known Fe (Ⅱ) concentration, ranging from 100 to 2000 μM, ($FeSO_4 \cdot 7H_2O$) were used for the preparation of the calibration curve. The parameter equivalent concentration was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4 \cdot 7H_2O$. Equivalent concentration was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM concentration of Fe (Ⅱ) solution.

2.6.4. Hydroxyl radical scavenging activity

The scavenging activity of the *R. imbricata* extracts on hydroxyl radical was measured according to the method of Klein *et al*[26]. Various concentrations (20, 40, 60 and 80 μg) of extracts were added with 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1 mL of dimethyl sulphoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90℃ for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75 g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed

was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity (HRSA) is calculated by the following formula:
% HRSA = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

2.6.5. Superoxide anion scavenging activity

The effect of scavenging superoxide radical was determined by the nitroblue tetrazolium reduction method[27]. The assay was based on the capacity of various extracts to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH-7.6), 20 μg riboflavin, 12 mM EDTA, 0.1 mg NBT and 100 μL sample solution or standards (BHA and BHT). Reaction was started by illuminating the reaction mixture with sample extract for 90 sec. Immediately after illumination, the absorbance was measured at 590 nm against the reagent blank (reaction mixture without plant sample). Identical tubes with reaction mixture kept in the dark served as negative control. The scavenging activity on superoxide anion generation was calculated as: Scavenging activity (%) = $[(A_0 - A_1) / A_0] \times 100$, where, A_0 is the absorbance of the control, and A_1 is the absorbance of the sample extract/standard.

2.6.6. Phosphomolybdenum assay

The antioxidant activities of samples were evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al*[28]. An aliquot of 100 μL of sample or ascorbic acid in 1 mM dimethyl sulphoxide (standard) or distilled water (blank) was added with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a test tube. The test tubes were covered with foil and incubated in a water bath at 95 ℃ for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the reagent blank. The results reported are mean values expressed as milligrams of ascorbic acid equivalents per gram extract.

2.6.7. Metal chelating activity

The chelating of ferrous ions by various extracts of *R. imbricata* was estimated by the method of Dinis *et al*[29]. Initially, about 100 μL the extract sample was added to 50 μL solution of 2 mM $FeCl_2$. The reaction was initiated by the addition of 200 μL of 5 mM ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm against the blank (deionized water). The metal chelating capacities of the extracts were evaluated using the following equation: Metal chelating capacity (%) = $[(A_0 - A_1) / A_0] \times 100$, where, A_0 is the absorbance of the control, and A1 is the absorbance of the sample extract/standard. The chelating activity of the extracts was evaluated using EDTA as standard and results were expressed as mg EDTA equivalent/g extract.

2.7. Cytotoxicity analysis

Antiproliferative activity of *R. imbricata* methanol extract (dissolved in water) was tested in HT-29 cells, using the MTT assay as described earlier[30]. The cells were seeded in 96-well plates (Sarstedt, USA) with 1×10^6 cells/well and incubated at three time points 24 h, 48 h and 72 h, at 37 ℃. The cells were treated with five different concentrations of the plant extract (50,100, 150,175 and 200 μg/mL). After incubation, 20 μL/ well (50 mg/10 mL PBS) of MTT in phosphate buffered saline was added and incubated for 1 h. 100 μL of Stop mix solutions [10 g SDS (w/v), 0.6 mL aectic acid (v/v) and set volume to 100 mL with DMSO, pH, 4.5] was added each well to stop the reaction and shaked for half an hour. Absorbance was read at 550 nm using a Elisa reader. The percentage growth inhibition and percentage viability of the culture were calculated according to the following equation,

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

2.8. Statistical analysis

The data were subjected to a one way analysis of variance and the significance of the difference between means were determined by Duncan's multiple range tests using the SPSS. Values expressed were means±SD. P-values < 0.05 were regarded to be significant.

3. Results

In the present investigation, different solvent extracts extracted using conventional soxhlet extraction technique in rhizomes of *R. imbricata* was examined for their antioxidants and anticancer activities. This method affords simple and cheap which includes the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix, and there is no filtration requirement after leaching[31]. The percentage of yield, total phenolics, tannins and flavonoid contents of rhizome extracts of *R. imbricata* are presented in Table 1. The highest extract yield (9.73%) was obtained in acetone followed by methanol (8.73%) and water (8.70%). Similarly, acetone extracts recorded the highest total phenolics (41.9 g GAE/100 g extract), tannins (26.1 g GAE/100 g extract) and flavonoid (8.4 g RE/100 g extract) contents

followed by methanol and aqueous extracts. This indicates that the maximum extractable total phenolics, tannins and flavonoids were recorded in acetone extracts followed by methanol extracts. Petroleum ether extract displayed very low phenolics and tannin contents, whereas chloroform extract gave lowest level of flavonoids.

The reduction of DPPH$^{\bullet}$ absorption is indicative of the capacity of the extracts to scavenge free radicals, independently of any enzymatic activity. The results of DPPH radical scavenging activity of the *R. imbricata* rhizome extracts along with the reference standards rutin and BHT are shown in Figure 1. DPPH$^{\bullet}$ bleaching is one of the strategies used to evaluate the antioxidant a property of herbal extracts and this method has shown to be simple. Concentration of the sample necessary to decrease initial concentration of DPPH$^{\bullet}$ by 50% (IC_{50}) under the experimental condition was determined. The lower value of IC_{50} indicates a higher antioxidant activity. Methanol (IC_{50} 62.80 μg/mL) and acetone (IC_{50} 63.80 μg/mL) extracts showed maximum DPPH radical scavenging activity which is comparable to the reference standards BHT (IC_{50} 45.56 μg/mL).

ABTS$^{\bullet}$+ scavenging is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm. Trolox, a water-soluble analog of vitamin E (α-tocopherol), is used as a positive control. The activity is expressed in terms of the Trolox-equivalent antioxidant capacity of the extract (μmol/g sample extracts). The efficacy of ABTS cation radical scavenging activity of various solvent extracts of *R. imbricata* is shown in Table 2. The acetone extract showed as highest scavenger [(9 294.7±132.8) μmol TAA/g extract] of ABTS$^{\bullet+}$.

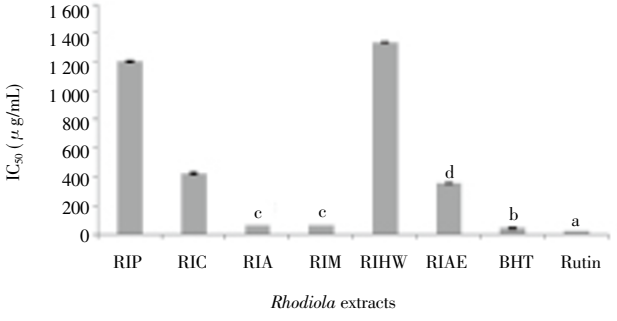

Figure 1. DPPH radical scavenging activity of *R. imbricata* rhizome extracts.
Values followed by different letters are significantly different (a>b>c>d, $P < 0.05$). IC_{50} – Inhibitory concentration, RIP – *Rhodiola* petroleum ether extract, RIC – *Rhodiola* chloroform extract, RIA – *Rhodiola* acetone extract, RIM – *Rhodiola* methanol extract, RIHW – *Rhodiola* hot water extract, RIAE – *Rhodiola* aqueous extract, BHT – butylated hydroxy toluene.

The FRAP values for different solvent extracts of *R. imbricata* are shown in Figure 2. Among the various solvent extracts, acetone extract [1 433.0 mmol Fe (II)/mg extract] showed higher ferric reducing antioxidant activity. Methanol extract possessed moderate levels of ferric reducing antioxidant activity, 1 071.9 mmol Fe (II)/mg extract. The order of FRAP activity of various sample extracts is as follows: RIA>RIM>RIHW>RIAE>RIC>RIP.

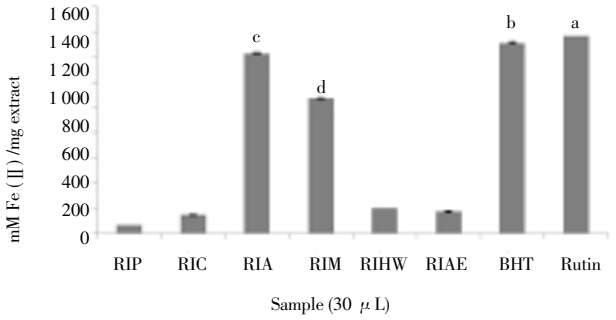

Figure 2. FRAP assay of *R. imbricata* rhizome extract.
Values followed by different letters are significantly different (a>b>c>d, $P < 0.05$). RIP – *Rhodiola* petroleum ether extract, RIC – *Rhodiola* chloroform extract, RIA – *Rhodiola* acetone extract, RIM – *Rhodiola* methanol extract, RIHW – *Rhodiola* hot water extract, RIAE – *Rhodiola* aqueous extract, BHT – butylated hydroxy toluene.

The hydroxyl radical scavenging potential of various solvent extracts of *R. imbricata* is shown in Figure 3. Each extract showing hydroxyl radical scavenging activity increased with increasing concentration of sample extracts. In our study, acetone (37.2%) extract is found to be comparable with reference standards rutin (52.5%) and BHT (43.2%). Aqueous extract showed (33.6%) hydroxyl radical scavenging activity and hot water extract exhibited lowest scavenging activity (7.5%).

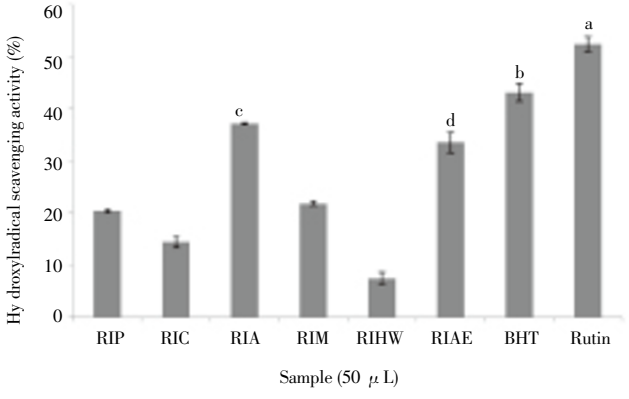

Figure 3. Hydroxyl radical scavenging activity of *R. imbricata* extracts.
Values followed by different letters are significantly different (a>b>c>d, $P < 0.05$). RIP – *Rhodiola* petroleum ether extract, RIC – *Rhodiola* chloroform extract, RIA – *Rhodiola* acetone extract, RIM – *Rhodiola* methanol extract, RIHW – *Rhodiola* hot water extract, RIAE – *Rhodiola* aqueous extract, BHT – butylated hydroxy toluene.

The scavenging abilities of *R. imbricata* extracts on superoxide radicals inhibition are shown in Figure 4.

Table 1

Solvent extract recovery, total phenolics, tannins and flavonoid contents of *R. imbricata* extracts.

Samples	Yield of crude extracts (%)	Total phenolics(GAE g/100 g extract)	Tannins(GAE g/100 g extract)	Flavonoid(RE g/100 g extract)
RIP	1.31	2.4±0.2	0.8±0.3	2.5±0.4[d]
RIC	1.03	8.2±0.7[d]	6.8±0.1[d]	2.3±0.5
RIA	9.73	41.9±4.7[a]	26.1±0.7[a]	8.4±0.3[a]
RIM	8.73	39.6±1.7[b]	22.5±2.2[b]	7.7±0.4[b]
RIHW	5.25	4.0±0.1	2.7±0.2	2.2±0.1
RIAE	8.70	11.1±0.4[c]	9.4±0.1[c]	2.7±0.1[c]

Values are means of three replicate determinations (n=3)±standard deviation, values followed by different letters are significantly different (a>b>c>d, $P < 0.05$).

RE: Rutin equvalents, RIP: *R. imbricata* Petroleum ether extract; RIC: *R. imbricata* Chloroform extract, RIA: *R. imbricata* acetone extract; RIM: *R. imbricata* methanol extracts, RIHW: *R. imbricata* hot water extract; RIAE: *R. imbricata* aqueous extract.

Table 2

$ABTS^{\bullet+}$ cation radical scavenging activity, phosphomolybdenum assay and metal chelating activity of *R. imbricata* .

Sample	TAA (μ mol/g extract)	AEAC(g AA/ 100 g extract)	Metal chelating(mg EDTA/g extract)
RIP	1 906.2±56.9	11.2±0.9	9.4±0.1
RIC	3 150.9±24.3	11.4±1.1	11.2±1.2
RIA	9 294.7±132.8[c]	37.1±0.4[c]	34.0±0.3[a]
RIM	6 621.7±146.0[d]	35.1±1.3[d]	21.3±0.6[b]
RIHW	3 315.6±59.7	13.4±2.2	16.0±0.9[c]
RIAE	3 458.7±53.1	12.8±1.3	14.0±1.1[d]
BHT	10 398.0±18.5[b]	56.7±0.8[b]	–
Rutin	10 398.0±18.5[a]	63.2±1.0[a]	0

Values are means of three replicate determinations (n=3)±standard deviation, values followed by different letters are significantly different (a>b>c>d, $P < 0.05$).

RE: Rutin equvalents, RIP: *R. imbricata* Petroleum ether extract; RIC: *R. imbricata* Chloroform extract, RIA: *R. imbricata* acetone extract; RIM: *R. imbricata* methanol extracts, RIHW: *R. imbricata* hot water extract; RIAE: *R. imbricata* aqueous extract, BHT: Butylated hydroxy toluene. TAA: Total antioxidant activity (μ mol trolox equivalents per g extract), AEAC: Ascorbic acid equivalent antioxidant capacity (g ascorbic acid equivalents per 100 g extract), EDTA: Ethylene diamine tetra acetic acid (mg EDTA equivalents per g extract).

Acetone extract showed highest superoxide radicals scavenging activities (75.1%) at a level of 100 μg in the reaction mixture, which is comparable to rutin and BHT. Methanol extracts showed next higher (72.7%) scavenging activity, whereas chloroform extracts revealed low scavenging activity.

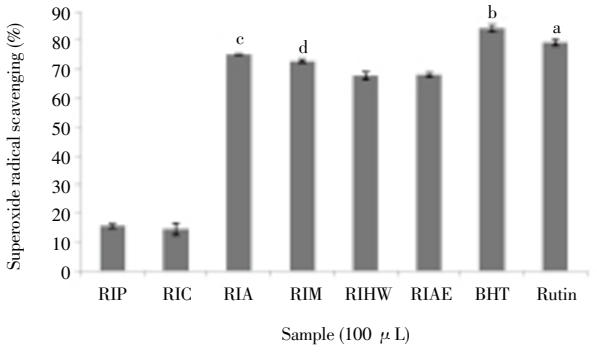

Figure 4. Superoxide radical scavenging activity of *R. imbricata* extracts.

Values followed by different letters are significantly different (a>b>c>d, $P < 0.05$). RIP – *Rhodiola* petroleum ether extract, RIC – *Rhodiola* chloroform extract, RIA – *Rhodiola* acetone extract, RIM – *Rhodiola* methanol extract, RIHW – *Rhodiola* hot water extract, RIAE – *Rhodiola* aqueous extract, BHT – butylated hydroxy toluene.

The phosphomolybdenum method is based on the reduction of Mo (Ⅵ) to Mo (Ⅴ) by the antioxidant compounds and the formation of green phosphate/Mo (Ⅴ) complex at acidic pH with the maximal absorption at 695 nm. Among the various extracts evaluated, the acetone extract had the strongest phosphomolybdenum reduction [(37.1±0.4) g AAE/100 g extract] and followed by methanol showed maximum [(35.1 ±1.3) g AAE/100 g extract] activity. The other entire sample extracts registered moderate phosphomolybdenum reduction (Table 2).

The chelating effect on the ferrous ions by the various solvent extracts of *R. imbricata* is shown in Table 2. Metal chelating activity increased with increasing concentration of the extracts. All the sample extracts exhibited the ability to chelate metal ions. Among the different sample extracts, the acetone extract showed higher activity [(34.0±0.3) mg EDTA/ g extract] and methanol and hot water extract registered moderate activity.

R. imbricata acetone, methanol, hot water and aqueous extracts were selected to study the antiproliferative activity on HT-29 cells based on the antioxidant activity results (Figure 5 and 6). It was found that the acetone (84%) and

methanol (84%) extracts inhibited the proliferation of HT-29 cells when it was treated at high concentration (200 μg/mL).

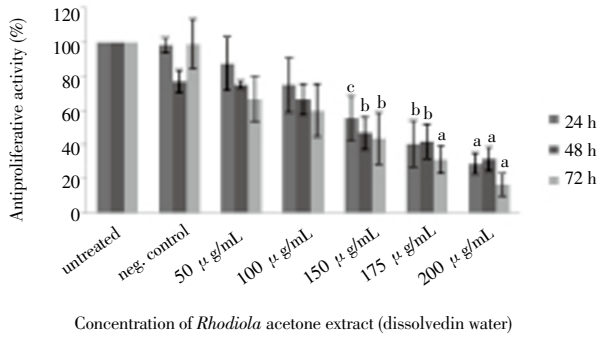

Figure 5. Antiproliferative activity of *R. imbricata* acetone extract in HT-29 cell line.
Values followed by different letters are significantly different (a>b>c>d, $P < 0.05$).

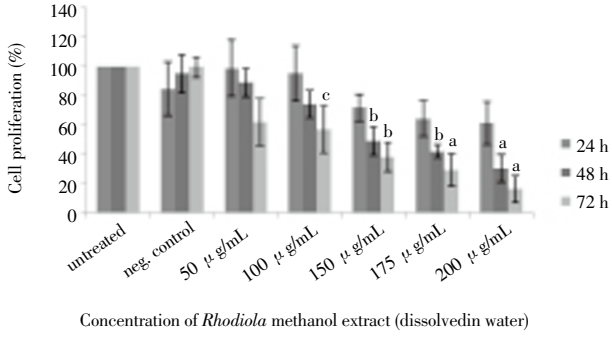

Figure 6. Antiproliferative activity of *R. imbricata* methanol extract in HT-29 cell line.
Values followed by different letters are significantly different (a>b>c>d, $P < 0.05$).

4. Discussion

Polyphenols form a large and diverse class of compounds, many of which occur naturally in a wide range of food and plants. The flavonoids are the largest and best-studied group among polyphenols. They are increasingly recognized as playing potentially important roles in health including but not limited to their roles as antioxidants. A range of plant polyphenols is either being actively developed or already currently sold as dietary supplements and/or herbal-derived medicines. A strong relationship between total phenolic content and antioxidant activity in fruits, vegetables and grain products has been reported[32-36].

In the present study, the acetone and methanol extracts also recorded considerable amounts of phenolic compounds and flavonoids. The strong scavenging capacity of methanol and acetone extract on DPPH might possibly due to the phenolic compounds which could act as a hydrogen donor antioxidant. The antioxidant potential of polyphenols has been correlated to the capacity of donating hydrogen radicals. The number and the configuration of H-donating hydroxyl groups are both important structural features influencing the antioxidant capacity of phenolic compounds[37].

$ABTS^{\bullet+}$ decolourization assay measures the total antioxidant capacity in both lipophilic and hydrophilic substances. The effect of the antioxidant concentration and the duration of the inhibition of the radical cation's absorption are taken into account when the antioxidant activity is determined. The advantages of this radical are its water-solubility and high absorption coefficient at long wavelengths, allowing the determination of its rate of consumption with minimal interferences[38]. In the present investigation, the acetone extract showed as highest scavenger of ABTS [(9 294.7±132.8) μmol TAA/g extract] which has the ability to scavenge the ABTS radical by the capacity of antioxidant species to donate electrons or hydrogen atoms to inactivate this radical cation. The petroleum ether extract showed lower level of activity while the methanol extracts revealed moderate activity.

The FRAP method is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine Fe $(TPTZ)^{3+}$, to the intensely blue coloured Fe^{2+} complex Fe $(TPTZ)^{2+}$ by antioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard. The reducing power property indicates that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process, so that they can act as primary and secondary antioxidants[39].

Hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage *in vivo*. The hydroxyl radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition, this species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids. Hydroxyl radicals also play a major role in lipid oxidation. Therefore, the antioxidant activity of acetone extract is considered of importance due to its scavenging activity on the hydroxyl radical.

The superoxide radical assay was based on the capacity of the samples to enhance the aerobic photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin. When the riboflavin is photochemically activated, it reacts with the NBT to give NBTH that leads to formazan according to the reaction. In presence of oxygen, concentrations of radical species are controlled by the quasi equilibrium. Thus, superoxide anions appear indirectly when the test is

performed under aerobic conditions. In the presence of an antioxidant that can donate an electron to NBT, the purple color typical of the formazan decays, a change that can be followed spectrophotometrically at 560 nm. All of the extracts had a scavenging activity on the superoxide radicals in a dose dependent manner.

In phosphomolybdenum method, variations in antioxidant capacity of acetone and other extracts may be attributed to differences in their phenolic contents. According to Jayaprakasha *et al*[40], the antioxidant activity of different extracts may depend on the presence of polyphenols which may act as reductones.

Transition metals have been proposed to be the catalysts for the initial formation of radical. Chelating agents may stabilise transition metals in the living systems and inhibit radical generations, consequently reducing free radical damage. To better estimate the potential antioxidative properties of the extracts, chelating activity of each extract was evaluated against Fe^{2+} and expressed as EDTA equivalents on a dry weight basis. Metal chelating capacity is significant, since it reduced the concentration of the catalysing transition metal in lipid peroxidation. Chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion[41].

The acetone and methanol extracts exhibited potent antiproliferative activity on HT-29 cells. Acetone extract inhibits the cell proliferation in dose dependent manner whereas methanol extract had both time and concentration dependent activity. When compared with untreated cells that were maintained as control, treated cells showed a dose and time dependent inhibitory activity. The proliferation of cells was not inhibited by treatment with the hot water and aqueous extract. It can be concluded that the antiproliferative activity of the acetone and methanol extracts may be due to the presence of polyphenolics in *Rhodiola* rhizome.

In the present investigation, the overall results from *in vitro* experiments, including determination of total phenolics, tannins and flavonoids contents, DPPH, ABTS radical scavenging activity, FRAP assay, hydroxyl radical, superoxide anion scavenging activity, phosphomolybdenum assay, metal chelating activity, antiproliferative activity on HT-29 cells demonstrated that acetone and methanol extracts of *R. imbricata* rhizome have a significant effect on antioxidant and antiproliferative activities. Further investigation is currently underway to figure out the mode of action and to identify specific phytochemicals responsible for their antioxidant and anti-proliferative activities.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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