RESEARCH ARTICLE PHARMACOLOGIA

DOI: 10.5567/pharmacologia.2014.247.255

A Comparative Study on *in vitro* and *in vivo* Antioxidant Properties of *Rubus ellipticus* and *Rubus niveus*

Blassan P. George, Thangaraj Parimelazhagan, Rahul Chandran and Shanmugam Saravanan
Department of Botany, Bharathiar University, Coimbatore, 641 046, Tamil Nadu, India

ABSTRACT

Background: The present investigation has been undertaken to study the *in vitro* and *in vivo* antioxidant potential of *Rubus ellipticus* and *Rubus niveus*. **Materials and Methods:** Leaf, stem and root of the *Rubus* plants was extracted in Soxhlet using petroleum ether, chloroform, acetone and methanol and all the extracts were subjected to *in vitro* antioxidant assays. *R. ellipticus* Leaf Methanol (RELM) and *R. niveus* Root Acetone (RNRA) extracts were administered to test the *in vivo* antioxidant levels after 30 days of treatment. **Results:** RELM and RNRA extracts depicted maximum activity in most of the radical scavenging assays such as DPPH (1,1-diphenyl-2-picryl hydrazyl), ABTS (2,2- azinobis -6-thiosulfonic acid), FRAP (ferric reducing antioxidant power assay), nitric oxide, super oxide, hydroxyl radical etc. Therefore, RELM and RNRA were taken for *in vivo* antioxidant screening. The 250 and 100 mg kg^{-1} of RELM and RNRA showed significant activities for catalase, superoxide dismutase, glutathione peroxidase, glutathione S-transferase, glutathione reductase and reduced glutathione. RNRA extracts showed superior antioxidant activity compared to RELM. **Conclusion:** This study could suggest the use of *R. ellipticus* and *R. niveus* as a valuable natural antioxidant and has immense scope as an effective source to fight against the free radical generated diseases. This study should also encourage the scientific search for more medicinal properties from these species so as to combat the ever increasing demand of medicine for the mankind.

Key words: *In vitro* antioxidant, *in vivo* antioxidant, free radical, *Rubus ellipticus*, *Rubus niveus*

Pharmacologia 5 (7): 247-255, 2014

INTRODUCTION

Antioxidants or "free radical scavengers" are nutrients as well as enzymes that are believed to play a vital role in preventing the development of chronic diseases such as cancer, heart disease, Alzheimer's, diabetics etc., by blocking or slow down the oxidation process by neutralizing free radicals. The antioxidant agents are found in foods such as vegetables and fruits. Well known antioxidants, such as vitamin E (α-tocopherol), vitamin C and polyphenols/flavonoids, have been investigated for their possible use to prevent the diseases (Nunez-Selles, 2005). The link between free radicals and disease processes led to considerable research to develop nontoxic drugs that can scavenge the free radicals. Several plant extracts and products have been shown to possess significant antioxidant potential (Sabu, 2003).

An antioxidant is a compound that inhibits or delays the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidized substrate. The scavenging of Reactive Oxygen Species (ROS) is one of possible mechanism of action. Others include the prevention of ROS formation by metal binding or enzyme inhibition. The antioxidant compounds can be recycled in the cell or are irreversibly damaged, but their oxidation products are less harmful or can be further converted to harmless substances (Halliwell, 1995; Halliwell and Gutteridge, 2007). The antioxidant testing can reveal various mechanisms of action, depending on features of the particular assay. Simple methods include free radical scavenging with use of colored, artificial stable free radicals such as 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS used in the TEAC assay-Trolox equivalent antioxidant capacity) (Re *et al*., 1999) and DPPH (1,1-diphenyl-2-picrylhydrazyl free radical) (Molyneux, 2004), as well as transition metal reduction that can be monitored by colorimetry. The metal based methods include the reduction of ferric ions: FRAP-(ferric reducing ability of plasma) and ferric thiocyanate assays (Halliwell, 1995; Aruoma, 2003) or molybdenum ion-phosphomolybdenum (P-Mo) assay (Prieto *et al*., 1999). These tests are easy and affordable and can be used in high throughput screening.

Corresponding Author: T. Parimelazhagan, Department of Botany, Bharathiar University, Coimbatore, 641 046, Tamil Nadu, India Tel: +04222428305 Fax: +04222425706

© 2014 Science Reuters, UK

The genus *Rubus* is very diverse, includes over 750 species in 12 sub genera and is found on all continents except Antarctica (Finn, 2008). *R. ellipticus* is a weedy raspberry is well established in disturbed wet forests, 1800-5580 ft elevation and thrives in sunny open pastures as well as deep rain forests. *R. niveus* is native from Indian to southeastern Asia, the Philippines and Indonesia (Gerrish *et al.*, 1992). They contain a range of biologically active substance including polyphenolics, flavanols, alkanols, anthocyaninis, lignans and tannins. In the present study we are following the quantification assays such as total phenolics, tannins and flavonoids, *in vitro* antioxidant assays such as DPPH, ABTS, FRAP, P-Mo, nitric oxide, super oxide and hydroxyl radical scavenging, the analysis of *in vivo* antioxidant molecule and enzymes in blood and liver such as Glutathione, Glutathione Peroxidase, Glutathione Reductase, Glutathione-S-Transferase, Superoxide Dismutase and Catalase for the comparative evaluation of antioxidant potential of the selected *Rubus* species. A survey of literature revealed that the antioxidant potentials of these plants have not yet been fully evaluated. Keeping this in view, in the present investigation we have investigated the antioxidant potential of *R. ellipticus* and *R. niveus in vitro* as well as *in vivo* and compared with the known natural and synthetic antioxidants to put forward a scope to develop an effective natural antioxidant supplement to fight against the free radical generated diseases.

MATERIALS AND METHODS

Chemicals: 2,2-diphenyl-1-picryl-hydrazyl (DPPH), potassium persulfate, 2,2'-azinobis- (3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), ferrous ammonium sulfate, ethylene diamine tetra acetic acid (EDTA) disodium salt, sodium nitroprusside, hydrogen peroxide, 1-chloro-2,4-dinitrobenzene (CDNB), GSH (γ-glutamyl cysteinyl glycine), 5,5'-Dithio-Bis (2-Nitrobenzoic Acid) (DTNB), Glutathione disulfide (GSSG) and Nitro blue tetrazolium (NBT) were obtained from Himedia, Merck or Sigma. All other reagents used were of analytical grade.

Plant material: The fresh plants of *R. ellipticus* and *R. niveus* were collected during the month of August from Mannavan Shola forest of Marayoor, Idukki dist, Kerala, India. The whole plants were separated in to leaves, stem, root etc., and washed in running tap water to remove dirt and other foreign materials and then shade dried.

Preparation of extracts: The dried, powdered plant samples were successively extracted in Soxhlet with Petroleum ether, Chloroform, Acetone and Methanol. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. The different solvent extracts were concentrated by rotary vacuum evaporator (Yamato BO410, Japan) and then dried. The dry extract obtained with each solvent was weighed to determine the yield of soluble components. The percentage yields were expressed in terms of the air dried weight of sample material.

The extracts thus obtained were used directly to assess the antioxidant potential.

Determination of total phenolics and tannins: The total phenol content was determined according to the method described by Siddhuraju and Becker (2003) and Siddhuraju (2007). The analysis was performed in triplicates and the results were expressed in Gallic Acid Equivalents (GAE). The same extract can be used to estimate the tannin content by treating with polyvinyl polypyrrolidone (PVPP) (Siddhuraju, 2007). From the above results, the tannin content of the sample was calculated as follows:

Tannin (%) = Tannin phenolics (%)- Non tannin phenolics (%)

Estimation of total flavonoids: The flavonoid contents of the extracts were quantified according to Zhishen *et al*. (1999). The pink colour developed due to the presence of flavonoids was read spectrophotometrically at 510 nm. The results were expressed in Rutin Equivalents (RE).

Determination of non-enzymatic antioxidant activities

DPPH radical scavenging activity: The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH according to Blois (1958). The absorbance 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.

Trolox equivalent antioxidant capacity (TEAC) assay: The total antioxidant activity of the samples was measured by ABTS radical cation decolourization assay according to Re *et al*. (1999). 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 plotted as a function of Trolox concentration. The unit of Total Antioxidant Activity (TAA) is defined as the concentration of Trolox having equivalent antioxidant activity expressed as $\mu M\ g^{-1}$ sample extracts.

Ferric reducing antioxidant power (FRAP) assay: The antioxidant capacities of different extracts of samples

© 2014 Science Reuters, UK

were estimated according to Pulido *et al*. (2000). 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.

Phosphomolybdenum assay: The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al*. (1999). The results were expressed in milligrams of ascorbic acid equivalents per gram extract.

Nitric oxide radical scavenging activity: The procedure is based on the method of Sreejayan and Rao (1997), where sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. The inhibition percentage was calculated using the equation:

$$\text{Radical scavenging activity (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Superoxide radical scavenging activity: 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 (Beauchamp and Fridovich, 1971). The scavenging activity on superoxide anion generation was calculated as:

$$\text{Inhibition (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Hydroxyl radical scavenging activity: The scavenging activity of acetone, methanol and water extracts of the plant samples along with the reference standard tannic acid and quercetin on hydroxyl radical was measured according to the method of Klein *et al*. (1991). The analysis was performed in triplicate. The scavenging activity on hydroxyl radical generation was calculated as:

$$\text{Inhibition (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Determination of *in vivo* antioxidant activity of RELM and RNRA extracts: Thirty six Swiss albino male mice were divided into 6 groups of 6 animals and they were treated orally with RELM and RNRA extracts dissolved in Carboxy Methyl Cellulose (CMC) (0.1%) at different doses for 30 days:

Group 1, 2: Normal (Untreated), Control treated with CMC (0.1%)
Group 3, 4: RELM-100, 250 mg kg^{-1} b.wt.
Group 5, 6: RNRA-100, 250 mg kg^{-1} b.wt.

At the end of the experiment, animals were sacrificed and blood was collected by heart puncture and liver was excised and washed in ice-cold Tris-HCl buffer (0.1 M, pH 7.4) and cytosolic samples of liver homogenate were prepared by centrifugation at 10,000 rpm for 30 min at 4°C. Estimation of the total protein was carried out by the method of Lowry, Rosenbrough, Farr and Randall (Lowry *et al*., 1951). The following parameters were assayed in both blood and liver to assess the oxidative stress.

Superoxide dismutase (SOD): The SOD activity was measured by the NBT reduction method of McCord and Fridovich (1969).

Catalase activity (CAT): CAT activity was estimated by the method of Aebi (1974) by measuring the rate of decomposition of hydrogen peroxide at 240 nm.

Glutathione (GSH): GSH (γ-glutamyl cysteinyl glycine) activity was assayed by the method of Moron *et al*. (1979), based on the reaction with DTNB.

Glutathione peroxidase (GPX): The assay of glutathione peroxidase was carried out by the method of Hafeman *et al*. (1974) based on the degradation of Hydrogen peroxide in the presence of GSH.

Glutathione reductase (GR): Glutathione reductase activity was measured by the method of Racker (1955), where the amount of reduced form of NADP consumed during the conversion of GSSG to GSH was estimated.

Glutathione-S-transferase (GST): The method of Habig (1974) was followed to assay the activity of glutathione-S-transferase (GST) based on the rate of increase in conjugate formation between GSH and 1-chloro-2, 4-dinitrobenzene (CDNB).

Statistical analysis: All the values are expressed as Mean±SEM. The values are analyzed using one-way ANOVA and the significance of the difference between means was determined by Duncan's, Tukey Kramer multiple comparisons or Dunnet multiple range test using SPSS software version 17 and GraphPad InStat3 software.

RESULTS AND DISCUSSION

Total phenolics, tannins and flavonoids contents of *R. ellipticus* and *R. niveus* extracts: The non-enzymatic antioxidants like total phenolics, tannins and flavonoids

© 2014 Science Reuters, UK

were estimated and presented in Table 1. The amount of total phenolics, tannins and flavonoid contents in the tested extracts were determined and expressed as Gallic acid equivalents (GAE) and Rutin equivalents. Highest total phenolics and tannin contents were identified in *R. ellipticus* and *R. niveus* root chloroform extract, 80.28 and 66.2 g GAE/100 g extract, respectively. The total flavonoid contents recorded maximum in the *R. ellipticus* and *R. niveus* root petroleum ether extracts (308.89 and 264.76 mg g^{-1} extract) expressed in terms of Rutin Equivalents (RE).

The total phenolic content of methanol and aqueous extracts *Rubus chingii* Hu. Fruit was found to be 4.54 and 4.02 g/100 g DW and the major types of phenolic compounds such as; Phenolic acids (gallic acid) and tannins (ellagic acid) were also reported by Cai *et al*. (2004). The total phenolic and flavonoid contents of *R. sanctus* reported that 4.52 mg g^{-1} plant extract in gallic acid equivalent and 4.66 mg g^{-1} plant extract in Rutin equivalent (Motamed and Naghibi, 2010). Similar to the previous reports our study also showed significant phenolic, flavonoid and tannin contents. The root extracts of the tested *Rubus* species showed higher levels of total phenol, flavonoid and tannin contents.

***In vitro* antioxidant activities of *R. ellipticus* and *R. niveus* extracts:** The stable free radicals such as DPPH and ABTS were effectively scavenged by *R. ellipticus* and *R. niveus* extracts. DPPH radical scavenging activity was expressed as IC_{50} values compared to the standard antioxidants BHT, BHA, Quercetin and Rutin (Fig. 1 and 2) and ABTS by Trolox Equalents (Table 2). Acetone and methanol extracts of both the plants showed comparable activity with standards by scavenging DPPH radical.

Table 1: Total phenolics/tannins/flavonoid contents of *R. ellipticus* and *R. niveus*

		Phenolic (GAE g/100 g extract)		Tannin (GAE g/100 g)		Flavonoid (RE mg g^{-1} extract)	
Plant parts	Extracts	*R. ellipticus*	*R. niveus*	*R. ellipticus*	*R. niveus*	*R. ellipticus*	*R. niveus*
Leaf	Pet. ether	35.44 ± 5.34^b	19.19 ± 0.13^c	27.30 ± 4.69^c	11.07 ± 0.70^c	150.44 ± 0.77^a	128.95 ± 0.66^c
	Chloroform	38.84 ± 7.24^b	19.80 ± 0.13^c	29.80 ± 6.33^c	10.50 ± 0.21	69.11 ± 0.38^b	59.24 ± 0.33^d
	Acetone	58.26 ± 2.24^a	17.28 ± 0.55^b	48.00 ± 1.79^a	15.88 ± 0.76^b	189.11 ± 2.69^a	162.10 ± 2.31^a
	Methanol	56.08 ± 2.72^a	27.93 ± 0.92^a	46.90 ± 1.69^a	15.85 ± 0.65^a	160.89 ± 2.14^a	137.90 ± 1.84^b
Stem	Pet. ether	39.91 ± 1.17^d	11.57 ± 0.33^c	32.30 ± 0.35^b	11.65 ± 0.30^c	220.22 ± 1.54^a	188.76 ± 1.32^b
	Chloroform	62.02 ± 2.4^a	11.01 ± 0.72^c	52.10 ± 1.69^a	10.76 ± 1.14	54.44 ± 5.55^d	46.67 ± 4.76^e
	Acetone	55.79 ± 0.41^b	39.10 ± 0.47^a	40.60 ± 0.71^b	25.74 ± 0.60^a	236.44 ± 6.68^a	202.67 ± 5.72^a
	Methanol	44.11 ± 0.98^c	29.41 ± 0.77^b	27.30 ± 0.36^c	13.61 ± 0.68^b	142.00 ± 9.68^b	121.71 ± 8.30^c
Root	Pet. ether	41.95 ± 3.65^b	10.70 ± 0.25^c	38.70 ± 3.15^b	10.94 ± 0.22	308.89 ± 15.4^a	264.76 ± 3.20^a
	Chloroform	80.28 ± 3.05^a	12.32 ± 0.59^b	66.20 ± 2.65^a	11.12 ± 0.21^b	62.44 ± 5.7^d	53.52 ± 4.93^d
	Acetone	46.23 ± 0.25^b	41.71 ± 1.30^a	25.20 ± 0.55^c	28.40 ± 0.65^a	188.80 ± 3.91^b	161.90 ± 3.35^b
	Methanol	44.01 ± 0.80^c	41.68 ± 1.97^a	19.01 ± 0.06^d	29.22 ± 0.29^a	144.60 ± 2.4^b	124.00 ± 2.06^c

Values are Mean±SD (n=3) , GAE: Gallic acid equivalents, RE: Rutin equivalents, a: $p<0.001$, b: $p<0.01$, c: $p<0.05$, d: Not significant compared with standard

Table 2: ABTS (2, 2'-azinobis (3-ethylbenzothiozoline-6-sulfonic acid)/ferric reducing antioxidant power/phosphomolybdenum activities of *R. ellipticus* and *R. niveus*

		ABTS assay-TEAC (TAC/TE μM/g extract)		FRAP assay (Fe (II) mM/mg extract)		Phosphomolybdenum assay (TAC/AAE mg/g extract)	
Plant part	Extracts	*R. ellipticus*	*R. niveus*	*R. ellipticus*	*R. niveus*	*R. ellipticus*	*R. niveus*
Leaf	Pet. ether	1842.7 ± 40.4^b	2558.24 ± 19.83^c	1168.88 ± 48.9^b	2072.22 ± 73.43^c	31.16 ± 13.8^b	23.81 ± 11.88^b
	Chloroform	1903.4 ± 46.3^b	3280.48 ± 22.55^c	824.44 ± 25.01^b	2218.89 ± 58.91^c	36.41 ± 13.0^b	24.47 ± 10.40^b
	Acetone	52514.0 ± 93.2^a	6277.46 ± 38.30^b	7368.88 ± 75.12^a	4643.33 ± 95.13^b	75.60 ± 10.9^a	48.34 ± 16.95^{ab}
	Methanol	53155.9 ± 75.1^a	7890.70 ± 18.65^a	7524.44 ± 73.13^a	6217.78 ± 54.68^a	78.19 ± 23.3^a	57.61 ± 14.73^a
Stem	Pet. ether	1403.9 ± 23.8^e	2713.48 ± 60.87^d	773.33 ± 54.05	2213.33 ± 91.25^c	28.44 ± 19.36^b	23.34 ± 10.68
	Chloroform	2618.9 ± 46.8^d	3442.48 ± 88.98^{cd}	986.66 ± 43.71^d	2296.67 ± 43.33^c	42.12 ± 16.55^b	38.78 ± 15.87^{bc}
	Acetone	22409.8 ± 29.8^a	11974.43 ± 60.63^a	7548.88 ± 46.26^a	6782.22 ± 37.88^a	71.69 ± 10.92^a	83.18 ± 13.73^a
	Methanol	20303.8 ± 97.4^b	9200.20 ± 65.31^b	6135.55 ± 31.22^b	5798.89 ± 37.66^b	53.60 ± 14.62^{ab}	70.21 ± 16.72^{ab}
Root	Pet. ether	1424.2 ± 73.4^d	2308.49 ± 41.03^b	658.88 ± 29.87^d	2040.00 ± 20.28^d	25.05 ± 12.20^c	33.40 ± 19.86^c
	Chloroform	10313.0 ± 49.7^c	2794.48 ± 36.31^b	1470.0 ± 17.09^c	2214.44 ± 6.94^d	33.70 ± 24.26^{bc}	40.55 ± 18.09^{bc}
	Acetone	48565.9 ± 59.5^a	12548.18 ± 72.22^a	7513.33 ± 75.05^a	8024.44 ± 81.81^a	71.99 ± 15.5^a	89.68 ± 18.55^a
	Methanol	21937.3 ± 65.5^b	12253.20 ± 57.24^a	6334.44 ± 47.38^b	6837.78 ± 29.93^b	61.24 ± 23.06^{ab}	78.35 ± 30.64^{ab}

Values are Mean±SD (n = 3), TEAC: Trolox equivalents antioxidant capacity, TE: Troloxequivalent, TAC: Total antioxidant capacity, FRAP: Ferric reducing antioxidant power assay, AAE: Ascorbic acid equivalents, EDTA: Ethylene diamine tetra acetic acid, a: $p<0.001$, b: $p<0.01$, c: $p<0.05$, d: Not significant compared with standard

© 2014 Science Reuters, UK

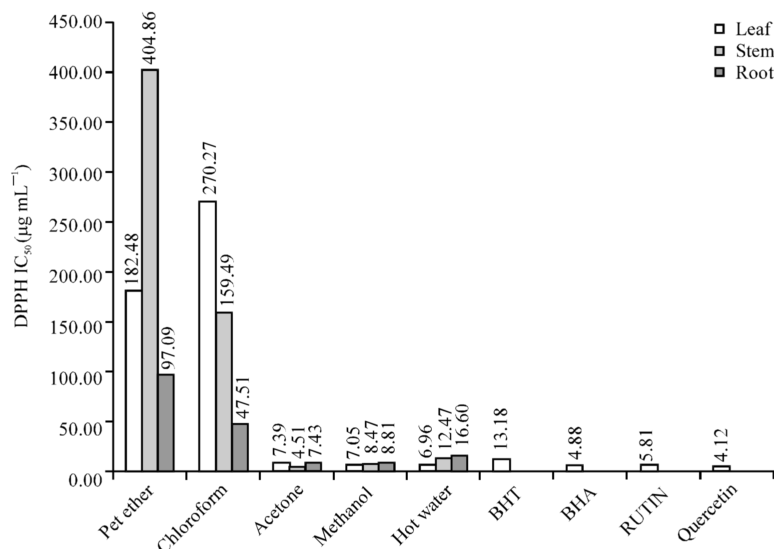

Fig. 1: DPPH radical scavenging activity of *R. ellipticus* extracts, synthetic and natural compounds

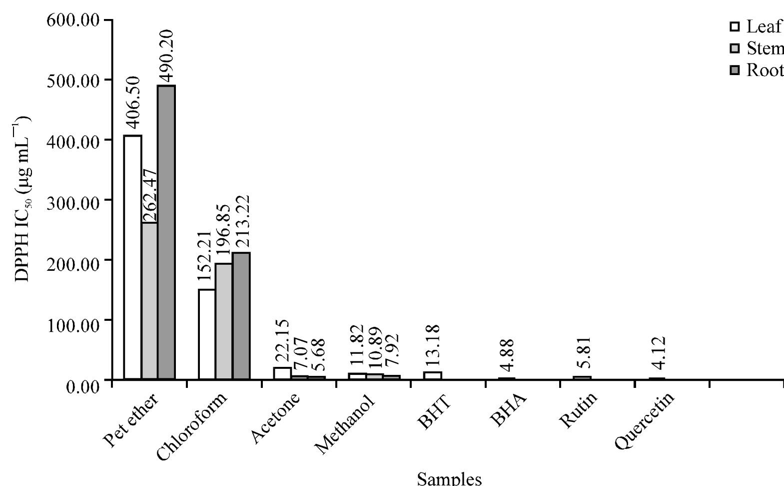

Fig. 2: DPPH radical scavenging activity of *R. niveus* extracts, synthetic and natural compounds

However, stem acetone extract of *R. ellipticus* and root acetone extract of *R. niveus* showed significant activity. Similar activities were observed in *R. sanctus* plant extracts which were found to scavenge the DPPH free radical by 83.27% when compared with Vitamin C and BHT (97.15 and 96.47%) (Motamed and Naghibi, 2010). In case of ABTS assay the methanol extract of leaf and acetone extract of root showed significant activity for *R.ellipticus*, where as for *R. niveus*, the acetone and methanol extract of root showed significant activity. Cai *et al*. (2004) reported the total antioxidant capacity TEAC values of the methanolic and aqueous extracts of *R. chingii* Hu fruits which were found to be 946.1 and 817.0 (μmol Trolox/100 g DW). The DPPH and ABTS radical scavenging activities of *R. ulmifolius* (TEAC 3.8±0.3 mM Trolox and DPPH 5.10±0.5 μg mL^{-1}) were reported by Dall'Acqua *et al*. (2008). Raspberry (*R. idaeus*) leaves, collected in different locations of Lithuania were extracted with ethanol and were tested for their antioxidant activity by using ABTS and DPPH scavenging methods. All extracts were active, with radical scavenging capacity at the used concentrations from 20.5-82.5% in DPPH reaction system and from 8.0-42.7% in ABTS reaction (Venskutonis *et al*., 2007).

Antioxidant potential of *R. ellipticus* and *R. niveus* was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) and are given in Table 2. Among the different extracts methanol and acetone

© 2014 Science Reuters, UK

Table 3: Nitric oxide/super oxide/hydroxyl radical scavenging activities of *R. ellipticus* and *R. niveus*

		Nitric oxide radical scavenging percentage inhibition (100 μg mL^{-1})		Super oxide radical scavenging percentage inhibition (100 μg mL^{-1})		Hydroxyl radical scavenging percentage inhibition (100 μg mL^{-1})	
Plant parts	Extracts	*R. ellipticus*	*R. niveus*	*R. ellipticus*	*R. niveus*	*R. ellipticus*	*R. niveus*
Leaf	Pet. ether	26.54±2.02^{d}	42.76±4.22^{b}	59.23±3.32^{b}	49.91±3.98^{b}	38.11±1.12^{d}	58.23±1.02^{c}
	Chloroform	34.09±4.43^{c}	26.84±5.23^{c}	45.34±2.31^{c}	41.23±2.37^{c}	28.30±1.21^{e}	60.06±2.00^{c}
	Acetone	70.08±2.45^{ab}	24.82±5.43^{c}	66.08±2.43^{b}	60.13±4.94^{b}	46.52±2.02^{c}	50.27±0.92^{d}
	Methanol	71.37±9.02^{a}	55.88±2.54^{b}	68.71±4.35^{b}	60.72±4.32^{b}	62.37±2.1^{b}	41.81±0.99^{e}
Stem	Pet. ether	34.92±2.0^{d}	23.87±5.23^{c}	63.44±3.02^{b}	62.33±3.89^{b}	31.01±1.92^{e}	65.03±1.02^{c}
	Chloroform	38.30±6.01^{d}	31.41±6.87^{c}	50.04±3.00^{c}	49.67±4.25^{b}	22.80±1.03^{f}	64.32±2.01^{c}
	Acetone	68.76±7.34^{c}	60.10±8.42^{b}	65.80±2.32^{b}	62.22±4.83^{b}	38.31±1.03^{d}	51.82±0.97^{d}
	Methanol	70.25±9.43^{ab}	59.44±9.13^{b}	65.97±2.09^{b}	62.15±3.93^{b}	58.01±1.05^{c}	39.92±1.24^{e}
Root	Pet. ether	34.92±8.98^{c}	26.37±2.98^{c}	60.83±2.21^{b}	63.14±3.02^{b}	28.91±1.23^{c}	61.38±1.09^{c}
	Chloroform	43.11±8.95^{c}	40.97±3.23^{b}	51.80±1.09^{c}	52.90±2.95^{b}	29.01±2.02^{c}	60.27±1.29^{c}
	Acetone	67.87±5.99^{ab}	44.48±4.21^{b}	62.89±1.21^{b}	59.51±4.32^{b}	31.75±2.00^{c}	55.80±1.12^{c}
	Methanol	69.36±5.43^{a}	54.10±4.43^{b}	61.05±1.22^{b}	64.72±4.02^{b}	46.41±2.32^{b}	59.73±0.93^{c}
	BHA	-	-	94.35±2.93^{a}	94.35±2.93^{a}	-	-
	BHT	90.50±3.43^{a}	90.50±3.43^{a}	94.10±2.12^{a}	94.10±2.12^{a}	-	-
	Rutin	92.00±3.22^{a}	92.00±3.22^{a}	-	-		
	Tannic acid	-	-	-	-	71.90±2.31^{ab}	71.90±2.31^{ab}
	Quercetin	-	-	-	-	78.31±1.95^{a}	78.31±1.95^{a}

Values are Mean±SD(n = 3) a: $p<0.001$, b: $p<0.01$, c: $p<0.05$, d: Not significant compared with standard

Table 4: Effect of *R. ellipticus* leaf methanol and *R. niveus* root extracts on antioxidant systems in blood

Group	Glutathione (nmol mg^{-1} protein)	Glutathione reductase (NADPH nmol min^{-1} mg^{-1} protein)	Catalase (U mg^{-1} protein)	Superoxide dismutase (U mg^{-1} protein)
Normal (Untreated)	34.26±1.32	2.65±1.36	107.32±2.21	529.19±2.04
Control (0.1% carboxy methyl cellulose)	46.44±2.01	2.89±1.09	93.21±2.45	547.32±2.81
RELM 100 mg kg^{-1} b.wt.	51.64±1.42	3.37±1.12	99.78±1.91	539.09±2.73
RELM 250 mg kg^{-1} b.wt.	59.12±2.21*	5.32±1.00	138.81±2.86**	790.02±2.29**
RNRA 100 mg kg^{-1} b.wt.	52.31±1.23	4.76±0.34	109.12±1.22**	612.43±3.08**
RNRA 250 mg kg^{-1} b.wt.	61.24±1.87*	6.32±0.87*	155.42±2.01**	730.21±2.09**

Values are as Mean±SEM (n = 6), Significantly different at *$p<0.05$, **$p<0.01$, ***$p<0.001$, when compared to control, RNRA: *R. niveus* root acetone, RELM: *R. ellipticus* leaf methanol

extracts showed comparable ferric reducing ability. The root acetone extract of *R. niveus* showed higher activity (8024.44 mM Fe (II)/mg extract), followed by stem acetone and leaf methanol extracts (7548.88, 7524.44 mM Fe (II)/mg extract). The ferric reducing antioxidant power assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. The total antioxidant capacity observed for the extracts of *R. ellipticus* and *R. niveus* can be correlated with its free radical scavenging activity equivalent to that of natural antioxidant ascorbic acid. The total antioxidant capacities of different extracts are shown in Table 2. *R. niveus* root acetone extract showed superior activity (89.68 mg AAE/g extract) followed by *R. niveus* stem acetone, *R. ellipticus* leaf methanol and acetone extracts (78. 19 and 75. 60 mg AAE/g extract). The acetone extracts of all the parts showed better radical scavenging capacity.

FRAP values in blueberries were highly correlated with phenol content (r = 0.981), implying that the antioxidant activity of blueberries is largely due to presence of phenolic compounds (Koca and Karadeniz, 2009). Similar results have been reported by other researchers (Wang and Lin, 2000), who found a linear correlation between total antioxidant activity and phenol content in blackberries (r = 0.961). In addition, Deighton *et al*. (2000) reported that there were apparent linear relationships between antioxidant activity (assessed by FRAP) and total phenols (r = 0.965), whereas anthocyanin content had a minor influence on antioxidant activity (r = 0.588) of *Rubus* juices. The leaf methanol extracts of *R. ellipticus* showed better results for nitric oxide (71.37%), superoxide (68.71%) and hydroxyl radical (62.37%) scavenging activities. Whereas, in case of *R. niveus*, stem acetone showed best results for nitric oxide (60.10%), root pet ether (63.14%) for super oxide and stem pet ether (65.03%) for hydroxyl radical scavenging activities (Table 3).

Effect of RELM and RNRA extracts on antioxidant enzymes and Glutathione: RELM and RNRA extracts showed commendable *in vitro* antioxidant activities. The effect of RELM and RNRA extract on *in vivo* antioxidant enzymes and Glutathione in the blood and serum of mice after a period of thirty days is shown in Table 4. The Catalase and SOD were found to be increased significantly in animals treated with 250 mg kg^{-1} b.wt. of RELM, 250 and 100 mg kg^{-1} b.wt. of RNRA extract ($p<0.01$). GR in the serum was found to be significantly

© 2014 Science Reuters, UK

Table 5: Effect of *R. ellipticus* leaf methanol and *R. niveus* root extracts on antioxidant systems in liver

Group	Glutathione (nmoL mg^{-1} protein)	Glutathione peroxidase (U mg^{-1} protein)	Glutathione-S-transferase (nmoL mg^{-1} protein)	Glutathione reductase (NADPH nmol min^{-1} mg^{-1} protein)	Catalase (U mg^{-1} protein)	Superoxide dismutase (U mg^{-1} protein)
Normal	10.76±1.08	9.73±1.50	44.16±2.93	62.58±1.67	3.64±1.66	0.87±1.23
Control (0.1% carboxy methyl cellulose)	9.33±1.22	10.28±1.82	48.93±4.28	75.23±3.98	3.55±1.03	0.91±2.62
RELM 100 mg kg^{-1} b.wt.	11.89±0.53*	12.89±1.55*	51.52±2.48	87.76±3.23	4.01±0.70	1.01±0.42
RELM 250 mg kg^{-1} b.wt.	12.78±0.98**	14.09±1.09**	60.46±2.82	103.01±4.23**	5.23±0.55*	1.29±0.93
RNRA 100 mg kg^{-1} b.wt.	12.31±1.21**	13.34±1.31*	105.23±2.43**	85.56±3.98	3.98±1.94	1.09±1.78
RNRA 250 mg kg^{-1} b.wt.	13.16±1.66***	16.01±1.83***	142.16±5.39**	98.24±4.56**	5.68±0.07*	1.48±1.54*

Values are as Mean±SEM (n = 6), Significantly different at *$p<0.05$, **$p<0.01$, ***$p<0.001$, when compared to control, RELM: *R. ellipticus* leaf methanol, RNRA: *R. niveus* root acetone

increased only in 250 mg kg^{-1} b.wt. of RNRA ($p<0.05$). Similarly GSH level was found to be significantly elevated ($p<0.05$) in the blood of animals treated with 250 mg kg^{-1} b.wt. of RELM and RNRA extracts.

The effect of RELM and RNRA extracts on the antioxidant enzymes in mice liver after treatment for 30 days is given in Table 5. Catalase was found to increase significantly in 250 mg kg^{-1} b.wt. of RELM and RNRA extracts ($p<0.05$). SOD was found to be increased significantly only in 250 mg kg^{-1} b.wt. RNRA extracts treated group. GPX depicted an increase in 250 mg kg^{-1} b.wt. RNRA ($p<0.001$), 250 mg kg^{-1} b.wt. of RELM ($p<0.01$), 100 mg kg^{-1} b.wt. of RELM and RNRA groups ($p<0.05$). GR activity was also increased in 250 mg kg^{-1} b.wt. of RELM and RNRA extract ($p<0.01$) treated groups. Significantly increase in GST level was found in both 100 and 250 mg kg^{-1} b.wt. of RNRA treated groups ($p<0.01$). GSH level was found to be increased significantly in both 100 and 250 mg kg^{-1} b.wt. of RNRA groups ($p<0.01$ and 0.001), 100 and 250 mg kg^{-1} b.wt. of RELM groups ($p<0.05$ and 0.01).

The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. These act on different oxidants as well as in different cellular compartments. One important line of defence is a system of antioxidant enzymes including SOD, catalase, GPX, GST and GR. SOD is a metalloprotein, converts two superoxide radicals into hydrogen peroxide and O_2. To eliminate H_2O_2, before the Fenton reaction which can create highly reactive hydroxyl radicals, organisms use catalase-a homotetrameric ferri heme containing enzyme and/or GPX-a selenium dependent enzyme. The Km value for GPX is lower than that for catalase and hence GPX is considered most important in physiological conditions. GSH abundant in most cells is an important substrate for GPX and Glutathione S-Transferase (GST) and also act by quenching free radicals. GST is GPX like enzyme and its function is to eliminate various hydroperoxides. Glutathione Reductase (GR) is a member of the disulphide oxidoreductase family, catalyses the NADPH-dependent reduction of glutathione disulphide (GSSG) to GSH. In plants the role of glutathione was reported as free radical scavenger and membrane stabilizer, keeping this in view the high levels of GSH and GPX in the RELM and RNRA extracts plays an important role in the lipid peroxidation (Firdous *et al*., 2010). The survey of literature showed that related species of *Rubus* possess promising antioxidant and pharmacological activities; the anti-inflammatory, analgesic and antipyretic properties of *R. niveus* root acetone extract and *R. ellipticus* leaf methanol extract have been reported (George *et al*., 2013a, b). Therefore *R. ellipticus and R. niveus* may also have the potentiality to treat other free radical generated disorders since it contains significant antioxidant activities.

CONCLUSION

The present study confirms the promising antioxidant activities of *R. ellipticus* and *R. niveus.* This is the first report available in the comparative *in vitro* and *in vivo* antioxidant studies on these two *Rubus* species. Four different extracts from the leaf, stem and root of these plants were screened for its *in vitro* antioxidant potentials and two which gave maximum activity (RELM and RNRA) from both the plants were selected for testing the *in vivo* antioxidant potentials. Among the 24 different extracts tested for seven *in vitro* antioxidant assays and three quantification tests, RELM and RNRA extracts showed superior activities in five and six assays such as RELM showed superior activities in ABTS, Phosphomolybdenum, NO, SO, OH etc., and whereas RNRA stood first in quantification of phenolics and tannins, ABTS, FRAP, DPPH, Phosphomolybdenum etc. The *in vivo* results indicated that RELM and RNRA 100 and 250 mg kg^{-1} extract administration significantly increased the levels of SOD, CAT, GSH, GPX, GST and GR in both blood and liver of the 30 days extract treated mice. This antioxidant effect could be due to the phenolic/flavonoid constituents. The increased exposure to free radicals or the impaired efficiency of the protective enzymes and molecules may lead to many free radicals generated diseases including cancer. Further studies are warranted in order to characterize the exact compound responsible for the antioxidant activity. In summary, *R. ellipticus* and *R. niveus* can act as a natural antioxidant to fight against free radical related diseases.

© 2014 Science Reuters, UK

REFERENCES

Aebi, H., 1974. Catalase Estimation. In: Methods of Enzymatic Analysis, Bergmeyer, H.U. (Ed.). Verlag Chemic, Weinheim, NewYork, pp: 673-684.

Aruoma, O.I., 2003. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant food. Mutation Res. Fundam. Mol. Mech. Mutagen., 523-524: 9-20.

Beauchamp, C. and I. Fridovich, 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal. Biochem., 44: 276-287.

Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature, 181: 1199-1200.

Cai, Y., Q. Luo, M. Sun and H. Corke, 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci., 74: 2157-2184.

Dall'Acqua, S., R. Cervellati, M.C. Loi and G. Innocenti, 2008. Evaluation of *in vitro* antioxidant properties of some traditional sardinian medicinal plants: Investigation of the high antioxidant capacity of *Rubus ulmifolius*. Food Chem., 106: 745-749.

Deighton, N., R. Brennan, C. Finn and H.V. Davies, 2000. Antioxidant properties of domesticated and wild *Rubus* species. J. Sci. Food Agric., 80: 1307-1313.

Finn, C.E., 2008. *Rubus* spp., Blackberry. In: The Encyclopedia of Fruits and Nuts, Janick, J. and R.E. Paull (Eds.). CABI, Cambridge, MA., USA., pp: 348-351.

Firdous, A.P., K.C. Preethi and R. Kuttan, 2010. Antioxidant potential of meso-zeaxanthin a semi synthetic carotenoid. Food Chem., 119: 1096-1101.

George, B.P., T. Parimelazhagan and S. Saravanan, 2013a. Anti-inflammatory, analgesic and antipyretic activities of *Rubus ellipticus* Smith. Leaf Methanol Extract. Int. J. Pharm. Pharmaceutical Sci., 5: 220-224.

George, B.P., T. Parimelazhagan, S. Saravanan and R. Chandran, 2013b. Anti-inflammatory, analgesic and antipyretic properties of *Rubus niveus* Thunb. Root Acetone Extract. Pharmacol., 4: 228-235.

Gerrish, G., R.L. Stemmermann and D.E. Gardner, 1992. The distribution of *Rubus species* in the State of Hawaii. Cooperative Park Studies Unit Technical Report No. 85. University of Hawaii, Manoa, Honolulu, HI., pp: 33. http://scholarspace.manoa.hawaii.edu/handle/10125/7177

Habig, W.H., M.J. Pabst and W.B. Jakoby, 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem., 249: 7130-7139.

Hafeman, D.G., R. A. Sunde and W. G. Hoekstra, 1974. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. J. Nutr., 104: 580-587.

Halliwell, B., 1995. Antioxidant characterization methodology and mechanism. Biochem. Pharmcol., 49: 1341-1348.

Halliwell, B. and J.M.C. Gutteridge, 2007. Free Radicals in Biology and Medicine. 4th Edn., Oxford University Press, Oxford.

Koca, I. and B. Karadeniz, 2009. Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. Scient. Hortic., 121: 447-450.

Klein, S.M., G. Cohen and A.I. Cederbaum, 1991. Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating systems. Biochemistry, 20: 6006-6012.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

McCord, J.M. and I. Fridovich, 1969. Superoxide dismutase: An enzymic function for erythrocuprein (*hemocuprein*). J. Biol. Chem., 244: 6049-6055.

Molyneux, P., 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol., 26: 211-219.

Moron, M.S., J.W. Depierre and B. Mannervik, 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochimica Biophys. Acta-General Subjects, 582: 67-78.

Motamed, S.M. and F. Naghibi, 2010. Antioxidant activity of some edible plants of the Turkmen Sahra region in Northern Iran. Food Chem., 119: 1637-1642.

Nunez-Selles, A.J., 2005. Antioxidant therapy: Myth or reality? J. Brazil Chem. Soc., 16: 699-710.

Prieto, P., M. Pineda and M. Aguilar, 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal. Biochem., 269: 337-341.

Pulido, R., L. Bravo and F. Saura-Calixto, 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J. Agric. Food Chem., 48: 3396-3402.

Racker, E., 1955. Glutathione Reductase (Liver and Yeast). In: Methods in Enzymology, Colowick, S.P. and N.O. Kaplan (Eds.). Vol. 2, Academic Pres, New York, pp: 722-725.

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol. Med., 26: 1231-1237.

© 2014 Science Reuters, UK

Sabu, M.C., 2003. Antioxidant activity of Indian herbal drugs in rats with aloxan-induced diabetes. Pharm. Biol., 41: 500-505.

Siddhuraju, P. and K. Becker, 2003. Studies on antioxidant activities of mucuna seed (*Mucuna pruriens* var utilis) extract and various non-protein amino/imino acids through *in vitro* models. J. Sci. Food Agric., 83: 1517-1524.

Siddhuraju, P., 2007. Antioxidant activity of polyphenolic compounds extracted from defatted raw and dry heated *Tamarindus indica* seed coat. LWT Food Sci. Technol., 40: 982-990.

Sreejayan, N. and M.N. Rao, 1997. Nitric oxide scavenging by curcuminoids. J. Pharm. Pharmacol., 49: 105-107.

Venskutonis, P.R., A. Dvaranauskaite and J. Labokas, 2007. Radical scavenging activity and composition of raspberry (*Rubus idaeus*) leaves from different locations in Lithuania. Fitoterapia, 78: 162-165.

Wang, S.Y. and H.S. Lin, 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry and strawberry varies with cultivar and developmental stage. J. Agric. Food Chem., 48: 140-146.

Zhishen, J., T. Mengcheng and W. Jianming, 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64: 555-559.

© 2014 Science Reuters, UK