ANTIOXIDANT ACTIVITY OF *RHODODENDRON ARBOREUM* SSP. *NILAGIRICUM*

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Abstract

Rhododendron arboreum Sm. ssp. *nilagiricum* (Zenker) Tagg (Ericaceae), a tree species endemic to the Palani Hills of Southern Western Ghats of peninsular India. The present investigations evaluate the antioxidant activity of acetone and ethanolic extracts of *R. arboreum* by using established antioxidant methods such as DPPH, Nitricoxide (NO), Hydroxyl scavenging, Metal chelating ability of ferrous ions, Total antioxidant activity, Reducing power assay and Superoxide scavenging assay (SOD. *R. arboreum ssp. nilagiricum* showed a dose-dependent percentage of inhibition on the scavenging assays. At a maximum concentration of 250 and 500 µg/ml of all the assays, both the extracts showed radical scavenging activities. This study determined that acetone and ethanolic extracts of *R. arboreum* ssp. *nilagiricum* showed better antioxidant potential which can be used in development of therapeutic phytomedicine.

Key Words: *Rhododendron arboreum*, Free radical scavenging activity, Phytomedicine.

Introduction

Medicinal plants would be the best source to obtain a wide range of drugs. Therefore such plants should be investigated to better understand their properties, safety and efficacy (Nascimento *et al.*, 2000). Phytochemicals are responsible for medicinal activity of plants. Herbal plants have the presence of antioxidants such as phenolics, flavonoids, tannins, and proanthocyanidins. The antioxidant contents of medicinal plants may contribute to the protection from diseases. Antioxidant agents of biological origin have attracted special interest because of their free radical scavenging abilities (Osawa *et al.*, 1990). Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress (Zengin *et al.*, 2011).The antioxidants may prevent and cure cancer and other diseases. Human body has an inherent antioxidative mechanism and many of the biological

functions such as the anti-mutagenic, anti-carcinogenic and anti-aging responses originate from this property (Gulcin, 2012).

Rhododendron arboreum Sm. ssp. *nilagiricum* (Zenker) Tagg (Ericaceae), a tree species endemic to the southern Western Ghats of peninsular India. The plant has been used as medicine due to their antioxidant activity is being traditionally used by the inhabitants to treat various ailments. The present study aims to spectrophotometric quantification of antioxidant activity of ethanol extract of *Rhododendron arboretum* ssp. *nilagiricum*

Materials and Methods

Sample collection and processing

The plant material of *R. arboreum* was collected from Palani Hills of Southern Western Ghats. The leaves of the plant were properly washed with tap water and then rinsed with distilled water. The leaves were shed dried and crushed to obtain powder.

Extraction:

100 g of the dried powder of *R. arboreum* extracted with 95% ethanol using soxhlet apparatus and then make a concentration of 1mg/ml diluted to prepare the different concentrations for antioxidant assays.

1. DPPH radical scavenging activity assay

The free radical scavenging activity of the fractions was measured *in vitro* by 2,2′- diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier (Williams *et al.*, 1995). The stock solution was prepared by dissolving 24 mg DPPH with 100 ml of acetone and ethanol and stored at 20°C until required. The working solution was obtained by diluting DPPH solution with acetone and ethanol. A 3 ml aliquot of this solution was mixed with 100 µl of the acetone and ethanol extracts of *Rhododendron arboreum* ssp. *nilagiricum* at various concentrations (10, 20, 40, 60, 80 amd 100 µg/ml). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect (\%)} = [(\text{control OD} - \text{sample OD})/(\text{control OD})] \times 100$$

2. Nitric oxide scavenging activity

The nitric oxide radical scavenging activity was done using the method of Alderson *et al.* (2001). 3ml of reaction mixture containing sodium nitroprusside (10mM phosphate buffered saline) and various concentrations (100, 200, 300, 400 and 500 µg/ml) of the acetone and ethanol extracts of *Rhododendron arboreum* ssp. *nilagiricum* were incubated at 37ºC for 4 hours. To the incubation solution, 0.5ml of Griess reagent was added and the absorbance was read at 546nm.The percentage inhibition was calculated using the formula**:**

% inhibition= (OD of control – OD of sample/OD of control) ×100

3. **Hydroxyl radical scavenging activity**

The activity of the acetone and ethanol extracts of *R. arboreum* in the scavenging of hydroxyl free radical was determined by the method described by Elizabeth and Rao (1990) with a slight modification. In brief, plant extracts at different concentration was mixed with a reaction mixture contained, in a final volume of 1 ml: 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM) and ascorbic acid (100 µM). The mixture was then incubated for 1 h at 37°C and 0.5 ml of the reaction mixture was heated at 90°C for 15 min after addition of 1 ml of 2.8 % TCA and 1 ml of 1 % aqueous TBA to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. Hydroxyl radical scavenging ability (%) was calculated by using the formula:

Hydroxyl radical scavenged (%) = (OD of control–OD of sample/OD of control)×100

4. Superoxide anion scavenging assay

The assay for superoxide anion radical scavenging activity was supported by riboflavin-light-NBT system (Beauchamp and Fridovich, 1971). Briefly, 1 ml of acetone and ethanol extracts of *R. arboreum* were taken at different concentrations (25 to 500 µg/ml) and mixed with 0.5 ml of phosphate buffer (50 mM, pH 7.6), 0.3 ml riboflavin (50 mM), 0.25 ml PMS (20 mM), and 0.1 ml NBT (0.5 mM). Reaction was started by illuminating the reaction mixture using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The scavenging ability of the plant extract was determined by the following equation:

Scavenging effect (%) = (1−OD of sample/OD of control)×100

5. Metal chelating ability of ferrous ions

The chelating of ferrous ions by extracts was estimated by the method of Hinneburg *et al.* (2006). The different concentrations of acetone and ethanol extracts of *R. arboreum* was added to a solution of 2mM/L $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5mM/L ferrozine (0.2ml) and the mixture was shaken vigorously and left at 37ºC for 10 min and the absorbance was measured spectrophotometrically at 562nm. The chelating activity of the extracts was evaluated using EDTA as standard and the results were expressed as µg EDTA equivalent /g of extract.

6. Total Antioxidant activity

About 7.4 sulpuric acid (0.6M solution), 0.9942g of sodium sulphate (28mM solution) and 1.2359g of ammonium molybdate (4mM) were mixed together in 250ml distilled water and labelled as Total Antioxidant Capacity (TAC) reagent. Different concentration of acetone and ethanol extracts of *R. arboreum* (100, 200, 300, 400 and 500 µg/mL) was taken in separate test tubes. About 1ml of TAC reagent was added to all tubes. Blank was prepared with distilled water replacing the TAC reagent (control). Absorbance was measured at 695nm in a spectrophotometer. Ascorbic acid was used as a standard (Pourmorad *et al.*, 2006).

Total antioxidant activity= (Control OD-Sample OD/Control OD).

7. Reducing power activity

The reducing power was determined according to the method of Berker *et al*., (2010). The acetone and ethanol extracts *R. arboreum* at different concentrations (100, 200, 300, 400 and 500 µg/ml) were mixed with 2.5ml of 200mM sodium phosphate buffer and 2.5ml of 1% potassium ferricyanide and mixture was incubated at 50ºC for 20 mins. After the addition of 2.5ml of 10% trichloroacetic acid the reaction mixture was centrifuged at 3000 rpm for 10min. About 5ml of the upper layer was mixed with 5ml of deionised water and 1 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. Ascorbic acid was used as a standard.

Determination of $IC_{50}$for antioxidant activity

Inhibition concentration (IC_{50}) value was determined from the plotted graph of scavenging activity versus the concentration of extract (using linear regression analysis), which is defined as the amount of antioxidant necessary to reduce the initial antioxidant concentration by 50%. Lower IC_{50} value indicates the higher radical scavenging effect.

Results and Discussion:

1. DPPH radical scavenging assay

DPPH free radical compound has been widely used to test the free radical scavenging ability of acetone and ethanol extracts of *R. arboreum*. The DPPH radical scavenging activity of extracted material was detected and compared with standard antioxidant ascorbic acid. The extracts tested against DPPH stable radicals which reveals that the radical scavenging activity. The extracts possessed excellent antioxidant capacity by increased with the increasing concentration of the extracts. At a 100µg/ml concentration of acetone and ethanol extracts, the percentage of inhibition was found to be 61.29% and 67.91% respectively (Table 1; Figure 1). However, the scavenging activity of ascorbic acid at the same concentration was 88.17%. The IC_{50} values of acetone and ethanol extracts of *R. arboreum* ssp.*nilagiricum* wasfound at the concentration of 75.83µg/mland 64.28 µg/ml while the standard ascorbic acid was 38.90µg/ml (Table 8).

Table 1. DPPH scavenging assay of acetone and ethanol extracts of *R.arboreum* ssp. *nilagiricum*

Concentration (µg/ml)	**Activity expressed as (%) ±SE**		
	Acetone extract	**Ethanol extract**	**Standard (Ascorbic acid)**
20	25.29±0.56	27.28±0.74	38.07±0.50
40	31.03±0.58	34.7±0.39	47.43±0.24
60	39.85±0.36	51.8±0.46	65.86±0.68
80	54.67±0.38	57.19±0.78	81.04±0.17
100	61.29±0.60	67.91±0.98	88.17±0.57

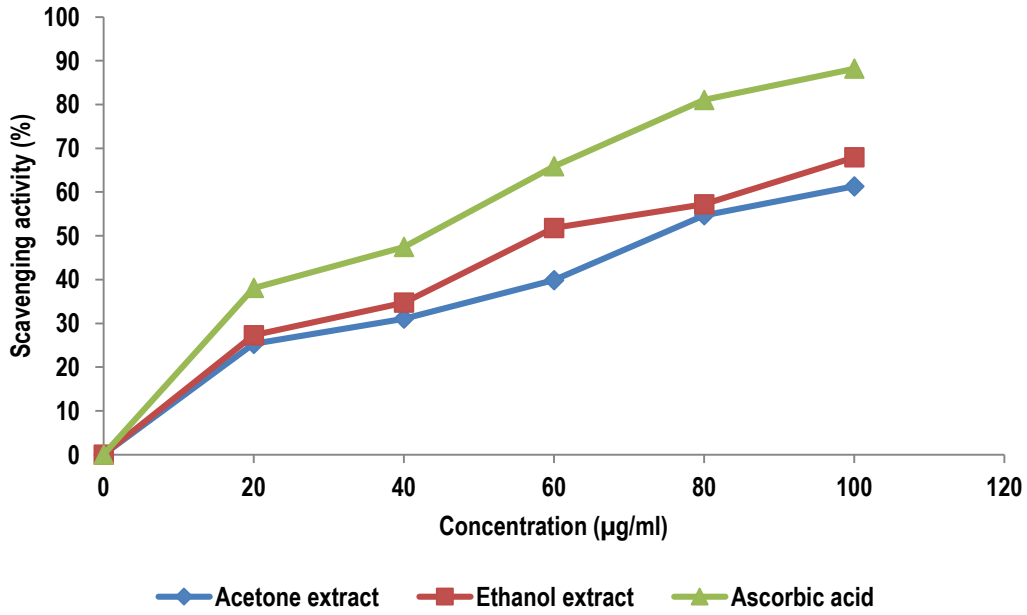

Fig 1. DPPHscavenging assay of acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

Results of the present study revealed thatIC_{50} value of acetone and ethanol extracts of *R. arboreum* ssp. *nilagiricum*showed relatively high DPPH (75 and 64µg/ml). Ethanolic extract of leaves and flowers of *Ageratum conyzoides* showed better antioxidant potential by DPPH radical scavenging method when compared to standard ascorbic acid (Kong *et al*., 2002).

2. Nitric oxide radical scavenging assay

Acetone and ethanol extracts of *R. arboretum* ssp. *nilagiricum* shows the increase in nitric oxide (Table 2; Figure 2). IC_{50} value of acetone and ethanol extract was showed 257.14 µg/ml and 317.34 µg/ml while the standard ascorbic acid was 231.35µg/ml (Table 8). The acetone and ethanol extracts recorded maximum percentage of NO activity of 92.47% and 78.33% at the concentration 500 µg/ml.

Table 2. Nitric oxide scavenging assay of acetone and ethanol extractof *R.arboreum* ssp. *nilagiricum*

Concentration	**Activity expressed as (%)±SE**		
(µg/ml)	**Acetone extract**	**Ethanol extract**	**Standard (Ascorbic acid)**
100	14.29±0.05	13.29±0.04	21.49±0.32
200	44.62±0.23	21.25±0.14	48.44±0.25
300	55.15±0.57	50.00±0.86	59.88±0.34
400	85.71±0.06	71.43±0.60	90.00±0.03
500	92.47±0.60	78.33±0.28	95.31±0.18

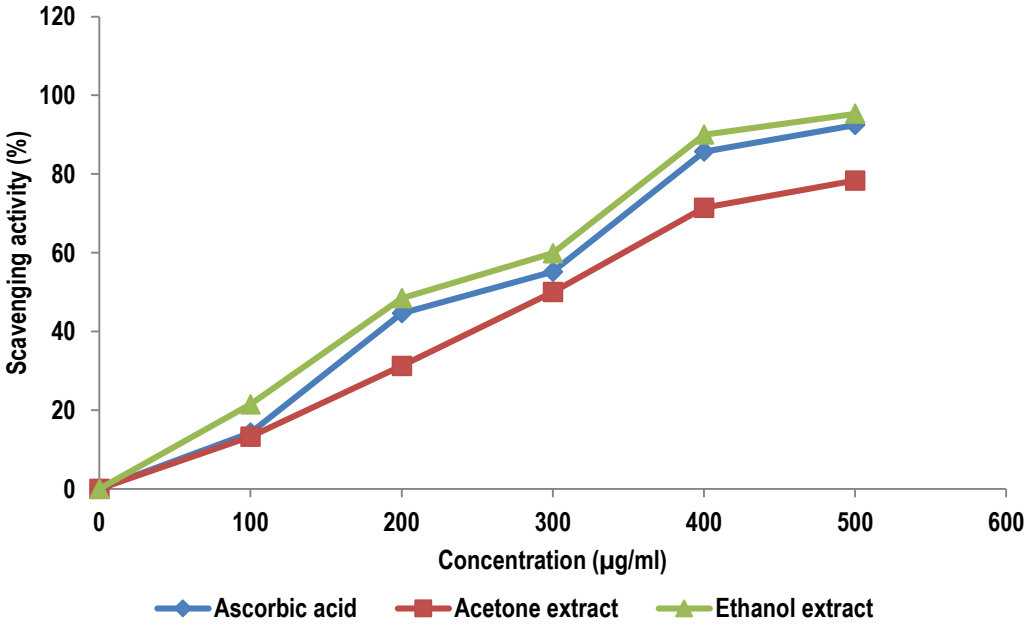

Fig 2. Nitric oxide scavenging assay of acetone and ethanol extracts of *R. arboreum* ssp. *nilagiricum*

In this study, the extracts in sodium nitroprusside (SNP) solution decreased levels of nitrite, a stable oxidation product of NO liberated from SNP in a dose dependent manner. The highest concentration (500 µg/ml) of acetone and ethanol extracts having 92.47% and 78.33%. Previous reports were studied the nitric oxide scavenging activity in various medicinal plants such as Sanja *et al.*, (2009) in *Portulaca oleracea*; Lee *et al*. (2011) in *Rhododendron yedoense*; Jagetia *et al,*. (2004) in some herbal formulations

3. Hydroxyl radical scavenging assay

The maximum hydroxyl radical scavenging effect of the acetone extract was 68.50% and ethanol extract was 53.65% in a concentration of 500 µg/ml and ascorbic acid was used as a standard since it is reported to be significantly effective in inhibition of hydroxyl radicals, which shows 73.49 % scavenging effect at the same concentration (Table 3; Figure 3). The IC_{50} value of acetone and ethanol extracts was found at the concentration of 466.86µg/ml and 457.12µg/ml respectively and standard was showed 226.60 µg/ml (Table 8).

Table 3. Hydroxyl radical scavenging assay of acetone and ethanol extracts of *R. arboreum* ssp. *nilagiricum*

Concentration (µg/ml)	**Activity expressed as (%)±SE**		
	Acetone extract	**Ethanol extract**	**Standard (Ascorbic acid)**

100	19.12±0.06	13.45±0.11	36.98±0.56
200	29.23±0.14	24.45±0.02	45.83±0.35
300	35.16±0.28	30.65±0.20	60.28±0.28
400	47.80±0.11	46.93±0.06	68.49±0.28
500	68.50±0.23	53.65±0.02	73.82±0.06

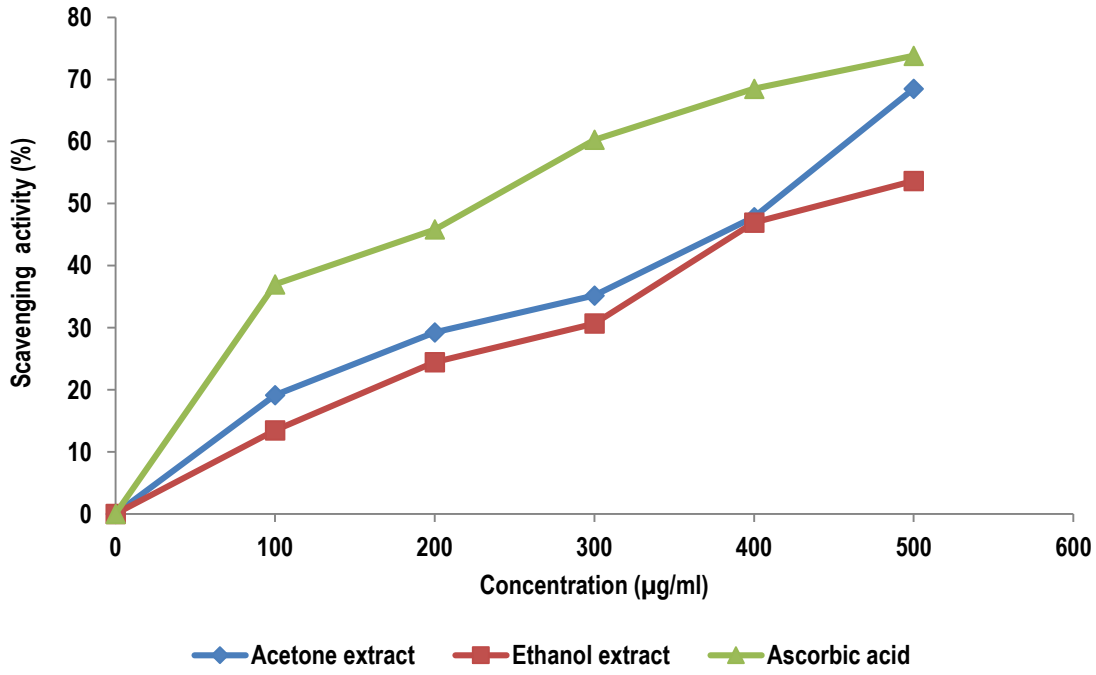

Fig 3. Hydroxide radical scavenging assay of acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

At 500µg/ml concentration of *Leucas linifolia* extracts showed 78% inhibition, with an IC_{50} value of 150 µg/ml in hydroxyl radical assay (Ramakrishna *et al.*, 2012). In the present study, the acetone and ethanol extracts of *Rhododendron arboreum* ssp. *nilagircum* showed 68 % and 53 % inhibition with an IC_{50} value of 466.86 µg/ml and 457.12 µg/ml respectively.

4. Superoxide radical scavenging assay

The superoxide radical (SO) scavenging activity obtained for the extract of *R.arboreum* ssp. *nilagiricum* dose dependent free radical scavenging activity and the percentage inhibition was shown in Table 4; Figure 4. In the present study, the acetone and ethanol extracts was found to be a notable scavenger of superoxide radicals generated in the riboflavin-NBT light system. The scavenging activity of acetone and ethanol extracts was compared with the ascorbic acid as standard. The highest percentage of inhibition of

acetone and ethanol extracts was 60% and 64%respectively. The maximum inhibition of standard was 77% in the concentration of 250 µg/ml. The IC_{50}value of acetone and ethanol extracts was found to be at the concentrations of 202.08µg/ml and 185.17µg/ml and standard ascorbic acid concentration was 139.84 µg/ml (Table 8).

Table 4. Superoxide radical scavenging assay of acetone and ethanol extractof *R.arboreum* ssp. *nilagiricum*

Concentration (µg/ml)	**Activity expressed as (%)±SE**		
	Acetone extract	**Ethanol extract**	**Standard (Ascorbic acid)**
50	20±0.86	27.1±0.43	27±0.63
100	33±0.11	38.3±0.69	41±0.35
150	38±0.63	41.2±0.69	49±0.37
200	49±0.17	49.5±0.75	69±0.17
250	60±0.34	64.0±0.11	77±0.05

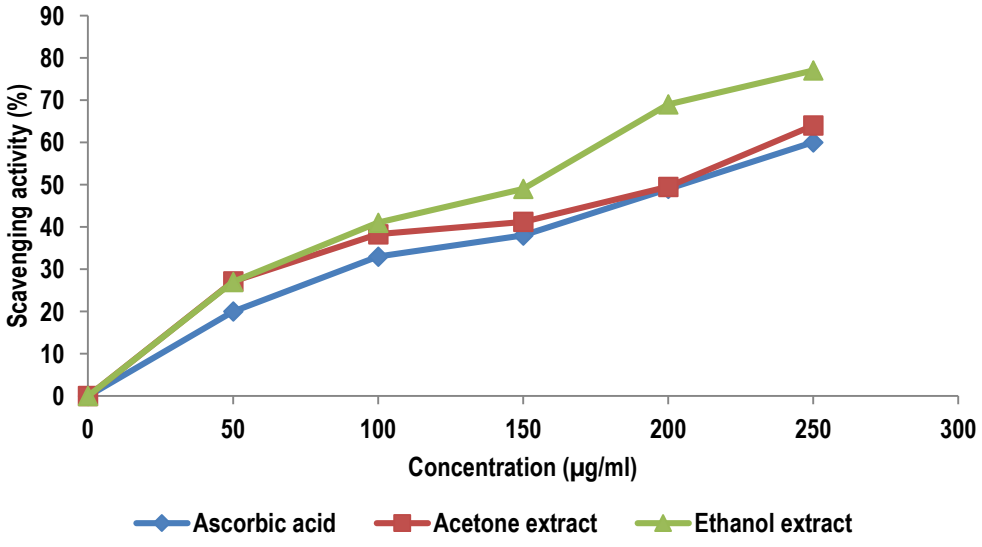

Fig 4. Superoxide radical scavenging assay of acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

Sanja *et al.* (2009) studied the superoxide radical scavenging activity of the methanol extract of *Portulaca oleracea* showed highest antioxidant activity (IC_{50} value is 182.02±9.64 µg/ml). In the present

study, IC_{50} value of acetone and ethanol extracts of *R. arboreum* ssp. *nilagiricum* was202.08µg/ml and 185.17µg/ml respectively.

5. Metal chelating ability of ferrous ions

Estimation of antioxidant potential and chelating activity *R. arboreum* ssp. *nilagiricum* extracts was evaluated against Fe^{2+}. Ferrozine quantitatively forms complexes with Fe^{2+}. The highest chelating effects of the ferrous ions in the acetone and ethanol extracts were 67% and 47% respectively and EDTA was 79% (Table 5; Figure 5). The IC_{50}value of acetone and ethanol extracts were found to be at the concentrations of 136.84µg/ml and 235.18µg/ml and standard EDTA concentration was 108.25 µg/ml (Table 8).

Table 5. Metal chelating activity of acetone and ethanol extractof *R.arboreum* ssp. *nilagiricum*

Concentration (µg/ml)	**Activity expressed as (%)±SE**		
	Acetone extract	**Ethanol extract**	**Standard (Ascorbic acid)**
50	35±0.28	26±0.51	39±0.40
100	47±0.11	38±0.23	48±0.11
150	52±0.75	43±0.11	56±0.51
200	59±0.17	50±0.28	71±0.38
250	67±0.80	47±0.86	79±0.51

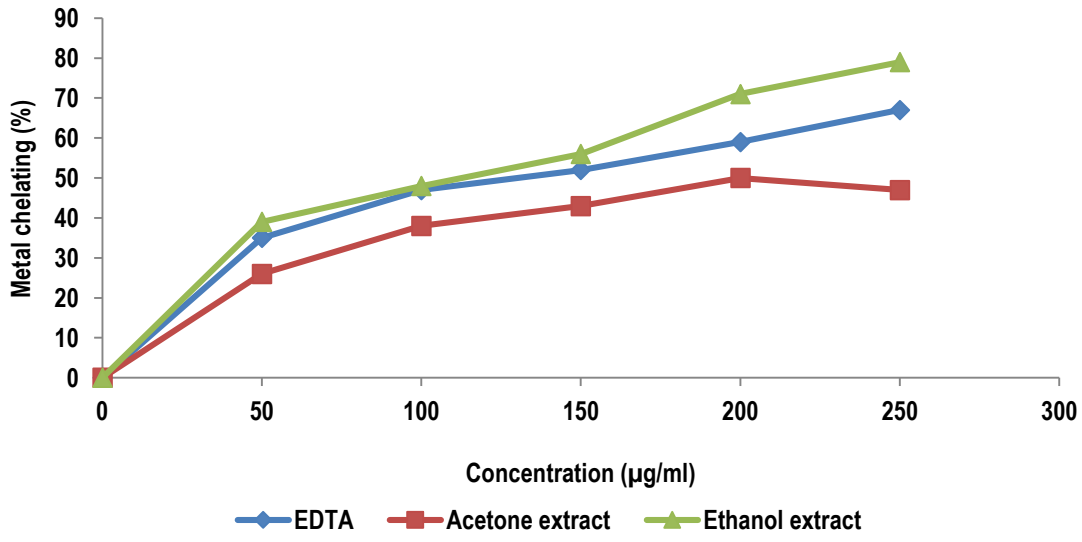

Fig 5. Metal chelating activityof acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

The ferrous ion chelating assay for *R. arboreum* ssp. *nilagiricum* was estimated using ferrozine. Acetone and ethanol extracts, the most active extracts interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. Previous report of Ebrahimzade *et al*., (2008) analysed the metal chelating activity in *Mentha arvensis* showed thatIC_{50} of the plant extract for chelating activity was 80 ± 0.01 µg/ml which is lower than the positive standard EDTA (IC_{50}= 17 µg/ml). The IC_{50} of chelating effect of other extracts on Fe^{2+} and ferrozine complex formation.Similar context was followed in the present study, that the IC_{50}value of acetone and ethanol extracts of *R. arboreum* ssp. *nilagiricum* were 136.84µg/ml and 235.18µg/ml and standard EDTA was 108.25 µg/ml.

6. Total antioxidant activity (Phosphomolybdate assay)

The antioxidant activity of acetone and ethanol extracts *R. Arboretum* ssp. *nilagiricum* was found to be dose dependent, i.e. 100 to 500 µg/ml (Table 6; Figure 6). The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyse and subsequent formation of a green phosphate Mo (V) complex at acidic pH.IC_{50} value of acetone and ethanol extract was showed 290 µg/ml and 208.47µg/ml while the standard ascorbic acid was 143.39 µg/ml (Table 8). The absorbance of the chromophore was measured at 765 nm in the presence of the extracts. The acetone and ethanol extracts recorded maximum percentage of TAC was 71 % and 83 % at the concentration 500 µg/ml. Strong antioxidants of the extracts could be attributable to the presence of phenolic compounds.

Table 6. Total antioxidant activity of acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

Concentration (µg/ml)	**Activity expressed as (%)±SE**		
	Acetone extract	**Ethanol Extract**	**Standard (Ascorbic acid)**
100	30±0.80	32±0.05	41±0.17
200	43±0.63	54±0.54	60±0.11
300	50±0.11	65±0.69	69±0.29
400	61±0.87	70±0.63	78±0.94
500	71±0.71	83±0.75	85±0.23

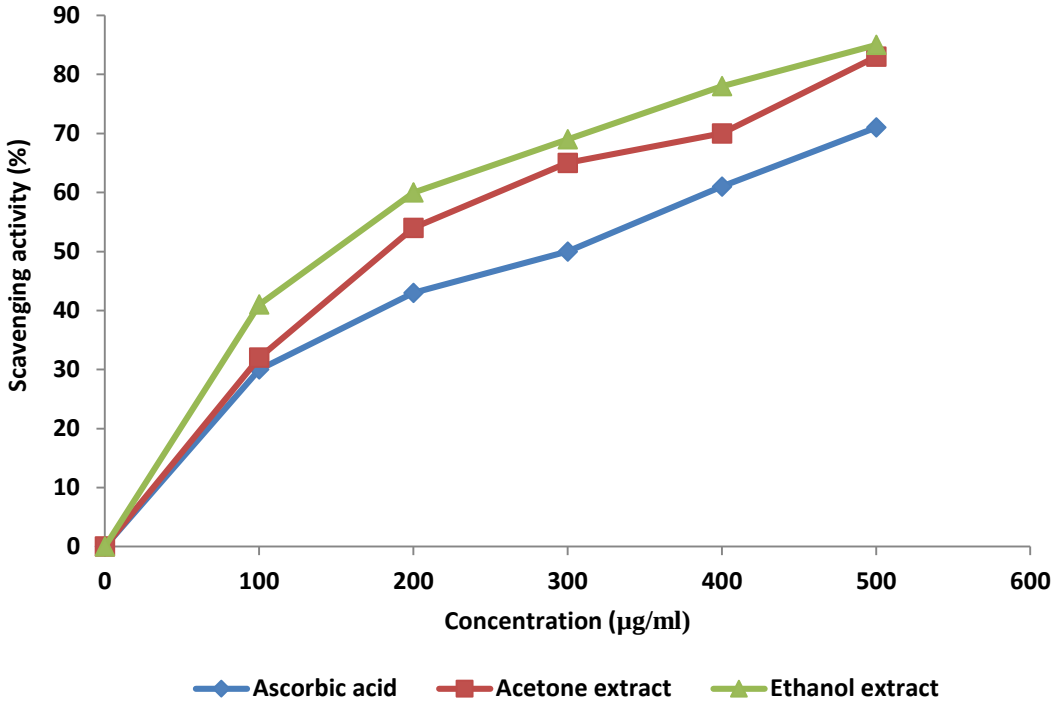

Fig. 6. Total antioxidant activity of acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

The present study, acetone and ethanol extracts of *R. arboreum* ssp. *nilagiricum* exhibited the highest antioxidant activity for phosphomolybdate reduction. Recent studies have shown that many flavonoid and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants (Krishnaiah *et al*., 2011; Sharififar *et al*., 2009). Gopakrishnan *et al*., (2011) analyzed the free radical scavenging potential of methanolic extract of *Coleus vettiveroides* shown maximum activity is 72% at 1000µg/ml; for as Standard (ascorbate) it was found to be 69% at 1000 µg/ml respectively. The acetone and ethanol extracts of *R. arboreum* ssp. *Nilagiricum* showed TAC content was71 % and 83 % and standard ascorbic acid was 85% at the concentration 500 µg/ml. Strong antioxidant activity of extracts statistically similar to ascorbic acid indicates strong antioxidants in this fraction and these could be attributable to the presence of phenolic compounds.

7. Reducing power assay

For the measurement of the reductive ability, the Fe^{+3} and Fe^{+2} transformations in the presence of *R.arboreum* ssp. *nilagiricum* acetone and ethanol extracts were investigated. In the present study, increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample (Table7; Figure 7). The reducing power of 500 µg/ml concentration of the acetone and ethanol extracts was found to be 0.927and 0.829 µg/ml respectively, which was relatively more pronounced than that of standard ascorbic acid (0.610µg/ml). IC_{50} value in reducing power of acetone and ethanol extracts were found to be 110.04 µg/ml

and 201.05 µg/ml respectively, while comparing with the standard ascorbic acid at the concentration of 358.22 µg/ml (Table 8).

Table 7. Reducing power assay of acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

Concentration (µg/ml)	Activity expressed as OD ±SE		
	Acetone extract	Ethanol extract	Standard (Ascorbic acid)
100	0.436±0.07	0.382±0.01	0.282±0.03
200	0.589±0.03	0.459±0.01	0.313±0.05
300	0.757±0.01	0.532±0.03	0.387±0.04
400	0.813±0.01	0.789±0.04	0.439±0.05
500	0.927±0.01	0.829±0.01	0.610±0.5

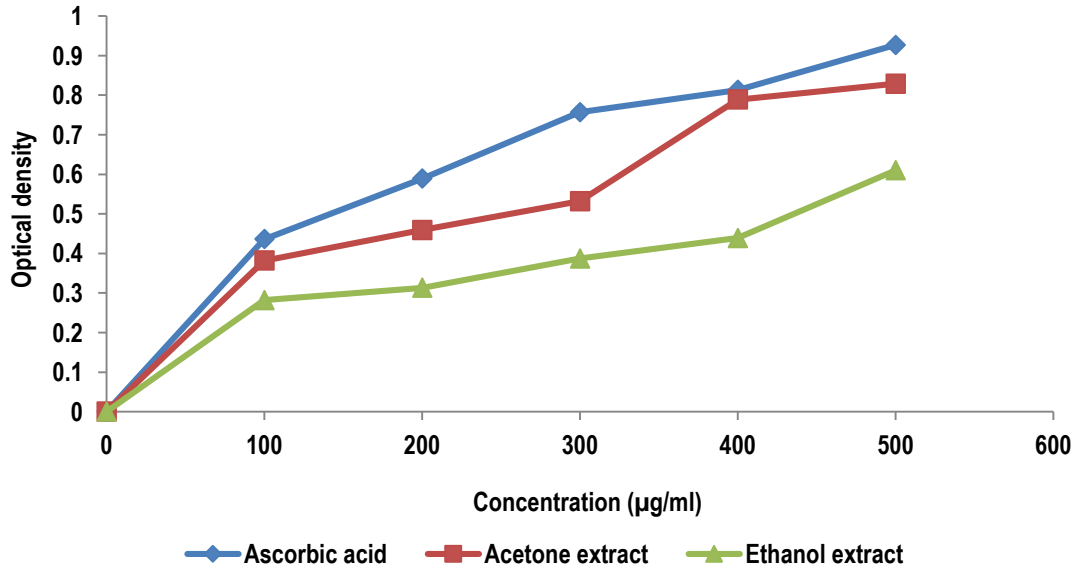

Fig 7. Reducing power assay of acetone and ethanol extractof *R. arboreum* ssp. *nilagiricum*

Benslama and Harrar (2016) revealed the methanol extract of *Zygophyllum album* have strong reducing capacity (2399.65±12.31µg/ml). In the present study acetone and ethanol extracts of *R. arboreum* ssp. *nilagiricum* showed reducing activity 0.927 and 0.829 µg/ml. Many studies demonstrated that the plants extract possess a strong reducing capacity.

Table 8. IC_{50} values of *R. arboreum*ssp. *nilagiricum*

S.No.	Antioxidant profile	Standard (µg/ml)	Acetone extract (µg/ml)	Ethanol (µg/ml)
1.	DPPH radical	(Ascorbic acid) 38.90	75.83	64.28
2.	Nitric oxide radical	(Ascorbic acid) 231.15	257.14	317.34
3.	Hydroxyl radical	(Ascorbic acid) 226.60	466.86	457.12
4.	Superoxide radical	(Ascorbic acid)139.84	202.08	185.17
5.	Metal chelating	(EDTA) 108.25	136.84	235.18
6.	Total antioxidant	Ascorbic acid 143.39	290.00	208.47
7.	Reducing power	Ascorbic acid 358.22	110.04	201.05

Different range of concentrations was used *R. arboreum ssp. nilagiricum* showed a dose-dependent percentage of inhibition on the scavenging assays. Therefore, the acetone and ethanol extracts showed significant antioxidant potential that may reveal its therapeutic potentials for several diseases. At a maximum concentration of 250 and 500µg/ml, the extracts showed radical scavenging activity. The radical scavenging activity of *R. arboreum* ssp. *nilagiricum* is reported to be higher comparing to other plant species (Prakash *et al.*, 2007; Lee *et al.*, 2011; Sanja *et al.*, 2009).

Conclusion:

The present study concluded that acetone and ethanolic extract of *R. arboreum* ssp. *nilagiricum* showed better antioxidant potential. The plant extracts could be explored for its highest therapeutic efficacy by pharmaceutical companies in order to develop safe drugs for various ailments.

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