RESEARCH ARTICLE

Characterization and evaluation of antioxidant properties of leaf extracts from *Rhododendron arboreum*

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ABSTRACT
Phytochemicals have been known to suppress microbial activity and oxidative stress, indicating plant material's significance as an anti-microbial and antioxidant agent. In this light, the current study aims to screen the phytochemicals from the *Rhododendron arboretum* leaf extract and characterize it using HPLC. The alkaloids and flavonoids are the major secondary metabolites found during screening. Further, the anti-oxidant properties of leaf extract of *R. arboreum* prepared in different solvents (methanol, ethyl acetate, petroleum ether, and chloroform) were analyzed using DPPH and FRAP assay. Observation of the present study indicates that methanolic extracts showed significant anti-oxidant activity with the IC_{50} value of 241.2 µg/ml and 163.6 µg/ml in DPPH and FRAP assay respectively. In conclusion, the leaf extracts of *R. arboreum* can be one of the better natural antioxidant sources and could be used for therapeutic purposes in the treatment of different diseases.

Key words: phytochemical, secondary metabolites, *Rhodendron arboreum*, HPLC, antioxidant activity

Introduction

Oxidative stress plays a pivotal role in tissue damage that promotes disease progression and is linked to inducing the aging process. Oxidative stress mainly depends on the production of free radical molecules such as Reactive oxygen species (ROS) and Reactive nitrogen species (RNS). Aberrant production of free radicals induces oxidative stress at the cellular level (Uttara et al., 2009; Powers et al 2011; Liguori et al., 2018). Production of free radicals can be endogenous or exogenous, and their negative impact can be neutralized by antioxidant molecules (Liguori et al., 2018). The imbalance between antioxidant and free radical molecules is linked to pathophysiological conditions. A broad range of synthetic pharmaco-active compounds as antioxidant drugs have been identified to suppress the severity of various pathologies by suppressing free radical production. However, these synthetic drugs have undesirable side effects and promote negative impacts on health (Chaves et al., 2020; Shahinuzzaman et al., 2020). Therefore, pharmaco-active compounds obtained from plant extract have been required to identify that could be a better option as natural antioxidants with minimal side effects. In this process, numerous efforts have been conducted, previously, and better natural antioxidant sources are still being searched for. As per reference to the World Health Organization, the use of plant-derived components-based therapeutics provides health benefits via using raw or processed ingredients from different

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flora, described as herbal medicine (Palhares et al., 2015; Mehra et al., 2023; Hoenders et al., 2024). Therapeutic values of herbal medicine can depend on the parts of the medicinal plant (leaves, fruit, bark, root, and other parts), the type of extract preparation, and the presence of bioactive molecules (Kumar & Bhat, 2014).

Natural product-based traditional medications have been frequently used by 80% of the population in the region of Asian and African continents, mainly tribal and local residents (Shamran & Al-Jumaili, 2020). In traditional medications, raw materials or extracts prepared from medicinal plants are used that consist of a broad range of phytonutrients (phytochemicals), including essential oils, phenols, alkaloids, flavonoids, tannins, saponins, and others. The great 'Himalaya' is located in the Asian continent and its mountain flora range is highly diverse, and about 116 aromatic plant species (belonging to 26 families) have been reported from these regions (Joshi et al., 2016). From the same region, *R. arboreum* also known as Laligurans or Rhododendron belongs to the family *Ericaceae.* It is an evergreen bush or little tree with a garish presentation of brilliant red roses. Rhododendron is the national blossom of Nepal and is known as the state tree of Uttarakhand. A wide range of phytochemicals (such as alkaloids, coumaric acid, flavan-3-ols, flavones glycosides, gallic acid, hydroquinone, lavonoids isovitexin, proanthocyanidins, tannins, and other secondary metabolites) have been identified from the extract from bark, leaf, flower, and fruit of *Rhododendron* species (Kashyap et al., 2017; Painuli et al., 2018; Gautam et al., 2020; Sharma et al., 2021).

A broad range of phytochemicals (also known as phytonutrients) have been identified from different plant species that exert pharmacological value and are also used as remedies for pathological conditions. Additionally, these phytochemicals are the natural source of antioxidant molecules that can facilitate the reduction of free radical production and suppress oxidative stress-mediated pathological conditions (Zhang et al., 2015). Biological properties of *Rhododendron arboreum* have not been studied extensively, so far. Therefore, we aimed to elaborate on the pharmacological value of phytochemicals prepared from the leaves of the *R. arboreum* species. We also intended to characterize the profiling of different phytochemicals (such as alkaloids, flavonoids, tannins, glycosides, steroids, terpenoids, and saponins) derived from the *R. arboreum* leaves and explored antioxidant free radical scavenging properties of the methanol, petroleum ether, chloroform and ethyl acetate extract of *R. arboreum* leaves. Moreover, High-Performance Liquid Chromatography (HPLC) analysis for the identification of phytochemical components of methanol extract and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay studies were undertaken to characterize the antioxidant free radical scavenging activity of leaves extract of *R. arboreum*.

Materials and Methods

Chemicals & Solvents

A set of chemical reagents (analytical grade) were purchased from Sigma Aldrich (Missouri, United States), such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, ethanol, ethyl acetate, chloroform, methanol, and petroleum ether.

Collection and verification of plant species

R. arboreum is an Indigenous tree from the region of the Indian subcontinent, mainly from the Uttarakhand region. Therefore, the collection of fresh leaves of *R. arboreum* was performed in Bageshwar district (29.8404° N, 79.7694° E), Kumaun-Himalayan region, Uttarakhand state, India. The *R. arboreum* Sm (voucher number: 118761) was identified from the Northern Regional Centre (Dehradun, Uttarakhand), Botanical Survey of India.

Plant extract preparation

Freshly picked leaves were cleaned with tap water and dried at 40°C in a hot air oven for 72 hours. Dried leaves were homogenized to fine powder and stored in airtight bottles (Figure 1). Ten grams of air-dried powder was taken in four different conical flasks and placed in each 100 ml of solvents, such as ethyl acetate, chloroform, methanol, and petroleum ether. All conical flasks were sealed with cotton plugs and then kept on a rotary shaker at 190-220 rpm for 24 hours. Each prepared solvent was transferred in a centrifuge tube and performed centrifugation at 2700 g for 15 min. Supernatants were collected and allowed to evaporate under vacuum dry using a rotary evaporator. Each dried extracts were stored at 4°C in airtight bottles for further studies. The extraction method was conducted in triplicate (Olajuyigbe et al., 2012).

Figure 1. *Dried and powered material prepared from the leave of R. arboreum.*

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Quantitative screening of phytochemical

Screening of selected phytochemicals, such as alkaloids, flavonoids, steroids, tannins, glycosides, terpenoids, and saponins were performed by standardized biochemical assay (Usman et al., 2009; Aziz, 2015).

HPLC analysis

Reverse-phase HPLC was conducted for the identification of phytochemicals in leaf extracts of *R. arboretum,* applying the below-given conditions, and analysis of HPLC data was conducted according to previously published methodologies (Mushtaq et al., 2016; Shamran and Al-Jumaili, 2020). 1000 µg/ml stock solution of Berberine, Hydrastine, and Palmatine was prepared by dissolving 10 mg each in 10 ml of ethanol solvents and analyzed at 295 nm, 298 nm, and 242 nm wavelength, respectively. Similarly, different concentrations of phytochemical extracts were prepared in 10 µg/ml, 20 µg/ml, 30 µg/ml, and 40 µg/ml by adding ethanol and sonicated it at 20°C for 15 min. A 0.45-micron filter was used to filter the prepared samples before the reverse phase HPLC run. All experiments were accomplished in triplicate. Data were collected as the mean standard deviation of three independent measurements. The identification of individual compounds was carefully observed considering their respective retention time by comparing them with pure standard compounds.

Antioxidant activity assay using 1, 1-di-phenyl 2-picryl hydroxyl (DPPH) free radical scavenging method

DPPH assay was performed to determine the antioxidant properties of phytochemicals prepared from the prepared leaf extract, described previously by Toth and his colleagues in 2018 (Toth et al., 2018). For this purpose, different concentrations (50-250 µg/ml) prepared methanol leaf extract in 0.8 ml of tris HCl buffer (100 mM, pH 7.4). In each preparation, 1 ml DPPH (500 mM in 1.0 ml ethanol) was added, shaken vigorously, and kept at room temperature, for 30 min. Spectral absorbance was measured at 517 nm by spectrophotometer and for the reference, the solution taken was ascorbic acid. The inhibition curves were prepared and IC-50 values were calculated. The experiments were conducted in triplicate to achieve the mean values. Values of antioxidant activity were calculated using the following formula:

$$\text{Scavenging activity}(\%) = \frac{(A\ control\ -\ A\ extract)}{A\ control} \times\ 100$$

whereas $A_{control}$ and $A_{extract}$ determine the absorbance of control and extract, respectively.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP test was performed by the process described by Benzie and his colleagues (Benzie & Strain, 1996). The anti-oxidative property of extract to standard ratio was calculated by the linear calibration curve of $FeSO_4$ (10 to 80 µM).

Statistical analysis

All experiments were conducted in triplicate and results were expressed as mean ± SEM (standard error of the mean) and analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, California). Statistical significance would be defined at P-values less than 0.05 ($P<0.05$). Additionally, the following tests were decided to be performed for the analysis; Pearson's correlation test, One-Way ANOVA test Fisher's Exact Test, and Tukey's multiple comparisons test.

Results and Discussion

Quantitative screening of phytochemicals extracts from R. arboreum

Plants are the natural source of secondary metabolites such as phenols, flavonoids, alkaloids, tannins, and other phytonutrients, which consist of nutritional and pharmacological values. Therefore, our observations were primarily focused on determining the presence of phytochemicals in the leaf of *R. arboreum*. A biochemical assay-based quantitative screening was performed to demonstrate the presence of different bioactive compounds in leaf extracts of *R. arboreum*. Phytochemical profiling of alkaloids, flavonoids, glycosides, steroids, tannins, terpenoids, and saponins was investigated in different solvent extracts *R. arboreum* leaves, including methanol, petroleum ether, chloroform, and ethyl acetate (Table 1).

Table 1. *Phytochemical screening in different extraction solvents from R. arboreum plant.*

S. №	Tests	Methanol	Petrolium ether	Chloroform	Ethyl acetate
1	Phenols	+ + +	+	++	++
2	Alkaloids	+ + +	+++	+++	+++
3	Flavonoids	+ +	+	+	++
4	Tannins	+	-	-	-
5	Glycosides	-	-	+	-
6	Steroids	-	-	-	-
7	Terpenoids	-	++	+	-
8	Saponins	-	-	-	-

As demonstrated in Table 1, the methanolic solvent leave extract of *R. arboreum* contains alkaloids with a higher degree of precipitation (+++), flavonoids, and tannins with a lesser degree of precipitation (+) whereas, other phytochemical compounds like cardiac glycosides, steroids, terpenoids, and saponins were absent. In petroleum ether solvent extract alkaloids show a higher degree of precipitation (+++), while flavonoids and terpenoids show a moderate degree of precipitation (++) and tannins, cardiac glycosides, steroids, and saponins were absent. In chloroform

solvent extract alkaloids show a higher degree of precipitation (+++), while cardiac glycosides and terpenoids show a moderate degree of precipitation (+), and tannins, flavonoids, steroids, saponins were in ethyl acetate solvent extract alkaloids and flavonoids show higher degree of precipitation (+++), and cardiac glycosides, terpenoids, tannins, steroids, saponins were not present.

The excessive presence of alkaloids in all four solvents indicates that it might be a key factor in phytochemical-based antioxidant activity. Evidence indicates that alkaloids, flavonoids, saponins, glycosidase, betacyanin, phenols, and steroids are observed in methanolic and other extract. The existence of these selected phytochemicals in plants exhibits antimicrobial, antioxidant, and other biological properties (Zheng et al., 2001; Prabu et al., 2019). Furthermore, some of the phytochemicals such as phenols, flavonoids, and terpenoids have been identified as immune modulators (Idris et al., 2017). The difference shown in the results of alkaloids, flavonoids, glycosides, steroids, tannins, terpenoids, and saponins between the petroleum ether, methanol, chloroform, and ethyl acetate extracts may be added to the solvent of extraction and processing methodologies (Odeyemi et al., 2017; Prabu et al., 2019).

Estimation of total phenol, alkaloids, and flavonoids content

The quantitative analysis of the pharmacologically important phytochemicals in the plants shows that all the species contain different amounts of specified phytochemicals in the leaves. Therefore, we have investigated the total phenol, alkaloids, and flavonoids content in the leaf extract of *R. arboreum.* Results indicate that the total phenolic content of *R. arboreum* leaves was obtained from gallic acid (y=0.1355x+0.0388, and regression coefficient, R^2 = 0.9842), atropine (y=0.3042x+0.0058, and regression coefficient, R^2 = 0.9868) and rutin (y=0.031x + 0.0191, and regression coefficient, R^2 = 0.9872) determined from the regression equation of the calibration curve for the phenol, alkaloids, and flavonoids, respectively. The phenol content was observed higher in methanol (4.3% gallic acid equivalent/gm dry weight), ethyl acetate (1.6% gallic acid equivalent/gm dry weight), chloroform (0.6% gallic acid equivalent/gm dry weight) and lowest in petroleum ether (0.2% gallic acid equivalent/gm dry weight) solvent leaves extract of *R. arboreum* (Table 2).

As demonstrated in the figure, the alkaloid content was observed significantly highest ($p<0.0001$) in methanol (1% atropine equivalent/gm dry weight) and lowest in petroleum ether (0.2% atropine equivalent/gm dry weight) solvent extracts, with compare to other solvents. Interestingly, alkaloid contents were observed at basal level in the petroleum ether and chloroform solvent extracts of *R. arboreum* leaves. Furthermore, the contents of flavonoids were observed higher compared to the content of phenols and alkaloids in all solvents. However, the content of flavonoids was almost similar in all solvents and no significant changes were observed for the same. Evidences based on previous observations indicate flavonoids play a pivotal role in anti-oxidant activity, however, it is mainly dependent on the molecular structure and, orientation of hydroxyl (-OH) groups (Iqbal et al., 2015; Prabu et al., 2019). Therefore, abundant flavonoids can promote higher anti-oxidant activity of the leaf extract of *R. arboreum.*

Estimation of alkaloids from different prepared fractions using the RP-HPLC method

HPLC analysis of A1-A7 fraction of R. arboreum

HPLC was performed to separate the different components present in the prepared leaf extract of the *R. arboreum* plant. Observation based on HPLC chromatogram indicates that seven fractions A1-A7 were identified. Which, five fractions were positive from A2-A6, and negative fractions were in A1 and A7, demonstrated in Table 3 and Figure 2.

Table 2. *Total phenolic, alkaloids, and flavonoids content (µg/ml) of leave extracts from R. arboreum in different solvents.*

Phyto-chemical content	Methanol	Petroleum ether	Chloroform	Ethyl acetate
Phenol	4.333579	0.259779	0.685363	1.698893
Alkaloids	1.046053	0.226974	0.279605	0.532895
Flavonoids	3.749462	3.598925	3.448387	3.609677

Table 3. *Details of HPLC-based identification of separated compounds in plants of R. arboreum.*

Fraction	Reten. time 1 (min)	Area 1 (mAU)	Area %	Reten. time 2 (min)	Area 2 (mAU)	Area %	Reten. time 3 (min)	Area 3 (mAU)	Area %
A1	-	-	-	-	-	-	-	-	-
A2	-	-	-	3.57	7.84708	0.0870	4.230	25.06473	0.2780
A3	-	-	-	3.543	18.7992	0.1091	-	-	-
A4	1.906	93.29996	4.9815	-	-	-	4.225	11.34861	0.6059
A5	1.907	243.2059	5.8515	3.537	23.0436	0.5544	4.229	9.84294	0.2368
A6	-	-	-	3.513	17.49427	2.4393	-	-	-
A7	-	-	-	-	-	-	-	-	-

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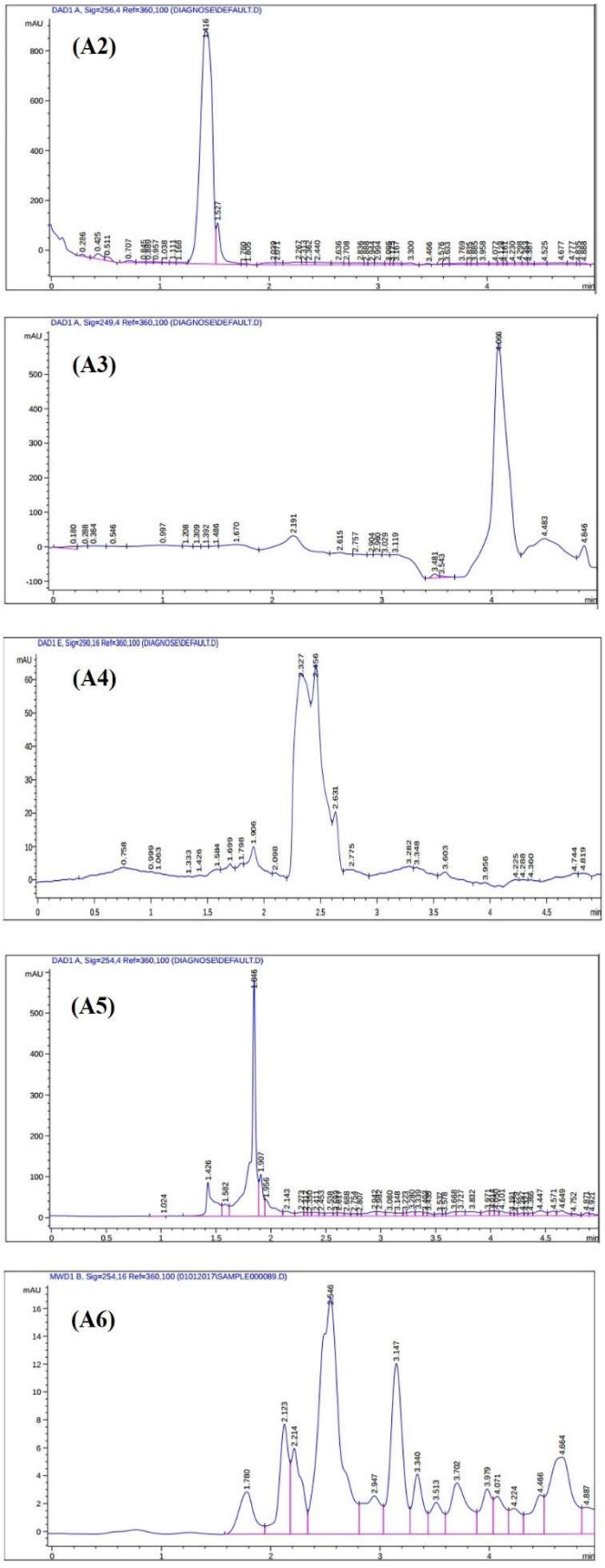

Figure 2. *HPLC analysis of A2-A6 fraction of R. arboreum.*

The spectral confirmation of the representative peak of alkaloids in the sample was performed with that of the standard after the analysis. The peaks of standard (Berberine, Hydrastine, and Palmatine) and samples were established in minutes with their corresponding Retention Times (RT). Also, the RT values were compared and an area value was obtained for the sample's representative peak of alkaloids. A2 fraction exhibited all three main alkaloids, in which Palmatine was mostly present in the analysis. Hydrastine was present in the A2 fraction at a much lower concentration. It is demonstrated in Table 4 that it is a Failure to detect fractions A1 and A7.

Antioxidant activity

DPPH free radical scavenging activity and FRAP scavenging activity of R. arboreum of different solvent extract

In this section, methanol, petroleum ether, ethyl acetate, and chloroform leaf extracts of *R. arboreum* were investigated for the scavenging activity of free radicals with DPPH assay, as well as FRAP assay. The DPPH radical contains an odd electron that is responsible for the absorbance at 517 nm and the visible deep purple color. When DPPH accepts a free electron released by a compound having antioxidant activity, the decolonization of the DPPH is noticed and evaluated quantitatively observing the changes in absorbance. DPPH assay-based observations indicated in Table 5 that all prepared extracts 50 - 250 µg/ml produced moderate to high DPPH scavenging activity (used ascorbic acid as a control reference). The % scavenging activity of DPPH was 53.41 ± 0.85, 47.61 ± 0.34, 46.03 ± 1.12, and 43.47 ± 0.56 observed in methanolic, chloroform, ethyl acetate, and petroleum ether extract of *R. arboreum*, respectively. Further, scavenging of free radicals was observed in a manner of dependent concentration ranging from 50 to 250 µg/ml. The anti-oxidant capability of the prepared extracts is because of the presence of different compounds, especially phenols and flavonoids which can release the hydrogen atoms in their hydroxyl groups (Iqbal et al., 2015; Sagbo et al., 2017; Prabu et al., 2019). In similar kind of methanolic flower extract of *R. arboreum* showed the lowest IC50 value which was comparable with the current study (Sharma et al., 2021).

Table 4. *Concentration of the alkaloids in the A2-A6 fraction of the R. arboreum using the HPLC technique.*

Fraction	Berberine (µg/ml)	Hydrastine (µg/ml)	Palmatine (µg/ml)
A2	-	0.011	1.63
A3	-	0.027	-
A4	0.027	-	0.74
A5	0.070	0.033	0.64
A6	-	0.025	-

Table 5 (a). *DPPH scavenging activities of R. arboreum of different solvent extract (values represent means ± SD, n=3).*

Conc (µg/ml)	% antioxidant activity				
	Ascorbic acid (µg/ml)	Methanolic extract	Ethyl acetate extract	Petroleum ether extract	Chloroform extract
50	14.94 ± 0.34	24.79 ± 1.38	24.54 ± 1.42	22.29 ± 0.81	23.24 ± 0.04
100	24.25 ± 2.59	29.85 ± 092	29.65 ± 0.70	29.05 ± 0.90	27.98 ± 0.40
150	34.65 ± 1.10	33.21 ± 1.06	32.35 ± 0.92	35.08 ± 0.90	34.54 ± 0.38
200	52.87 ± 0.92	37.15 ± 0.97	37.61 ± 0.88	38.33 ± 0.71	39.13 ± 0.37
250	58.27 ± 0.39	53.41 ± 0.85	46.03 ± 1.12	43.47 ± 0.56	47.61 ± 0.34
	IC-50 (µg/ml) 8.018	IC-50 (µg/ml) 241.2	IC-50 (µg/ml) 264.1	IC-50 (µg/ml) 266.8	IC-50 (µg/ml) 252.2

Table 5 (b). *FRAP scavenging activities of R. arboreum of different solvent extract (values represent means ± SD, n=3).*

Conc (µg/ml)	% antioxidant activity				
	Ascorbic acid (µg/ml)	Methanolic extract	Ethyl acetate extract	Petroleum ether extract	Chloroform extract
50	14.94 ± 0.34	22.47 ± 0.34	22.54 ± 0.45	18.79 ± 0.33	33.98 ± 0.43
100	24.25 ± 2.59	31.33 ± 0.72	67.65 ± 0.44	23.21 ± 0.67	32.13 ± 0.82
150	34.65 ± 1.10	44.08 ± 0.91	45.35 ± 0.81	31.15 ± 0.55	34.61 ± 0.56
200	52.87 ± 0.92	65.29 ± 0.18	89.61 ± 0.39	34.41 ± 0.43	41.54 ± 0.31
250	58.27 ± 0.39	71.05 ± 0.66	22.03 ± 0.56	39.85 ± 0.45	43.24 ± 0.34
	IC-50 (µg/ml) 8.018	IC-50 (µg/ml) 163.6	IC-50 (µg/ml) 178.2	IC-50 (µg/ml) 303.3	IC-50 (µg/ml) 263.4

An IC50 value concentration in the sample is required to scavenge 50% of the free radicals found in the system. IC50 value is inversely related to the antioxidant activity of crude extracts. The lowest IC50 value and highest activity were found in methanol extracts of *R. arboreum* compared to ascorbic acid at a concentration of 50 to 250 µg/ml whereas in FRAP assay showed the lowest IC-50 (µg/ml) i.e. 163.6 µg/ml in methanolic extract followed by ethyl acetate assay (Table 5b)

Conclusion

The current study supports the pharmacognostic importance of *R. arboreum* and the research was undertaken to establish anti-oxidant activity prepared methanolic, ethyl acetate, petroleum ether, and chloroform extract from the leaves of *R. arboreum*. Moreover, the screening and verification of phytochemicals were analyzed by established methodologies and the chemical content of specific solvent extracts was identified by chromatography techniques. The leaf extracts in different solutions aimed to find out the presence of phytochemicals. The current study provides a platform for further identification and separation of individual components of bioactive compounds and their anti-toxicity properties of *R. arboreum*.

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