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Microscopic identification, GC-MS analysis of essential oil and antioxidant activity of *Artemisia roxburghiana*

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Abstract

Artemisia roxburghiana Wall. ex Besser, belongs to the family Compositae, is used as folk medicine against various ailments including diabetes mellitus. An essential oil found in its aerial parts was found effective as an insect repellant. The present study aims to establish the microscopic identification of *Artemisia roxburghiana*, characterise its essential oil and evaluate its antioxidative effect. The microscopic characterisation of the leaf, stem and root of the plant was carried out using a compound microscope under 4x, 10x, 40x and 100x magnification. The essential oil was isolated by the hydro-distillation method and characterised with the help of gas chromatography-mass spectrometry (GC-MS) analysis. The hydro-alcoholic extract and essential oil obtained from the aerial parts of the plant were evaluated for their antioxidant activity using the DPPH assay. The microscopic studies revealed that the leaf has a single-layered upper epidermis with multicellular trichomes whereas the stem has single-layered epidermal cells, compactly arranged without intercellular spaces. The epidermis of the root is double-layered, thin-walled and colourless without intercellular spaces. The GC-MS studies found α-eudesmole (6.52%), trans sabinene hydrate (6.02%), artemisia ketone (5.97%) and 1,8 cineole (4.92%) as the major volatile constituents present in the essential oil. The hydro-alcoholic extract of the aerial parts also showed DPPH scavenging activity with an IC_{50} value of 6.90±1.23 mg/mL when compared with rutin (IC_{50} = 0.65±0.11 mg/mL). The study has successfully established the methods for microscopic characterisation and essential oil analysis of *Artemisia roxburghiana*.

Keywords: *antimalarial activity; artemisia; essential oil; medicinal plants; microscopy; quality control.*

1. Introduction

The plants of the genus *Artemisia* (family Compositae) are widely distributed, except in the Antarctica continent (Vallès et al., 2003), mainly in temperate areas of mid to high latitudes of the northern hemisphere with a total of 474 accepted

species (The Plant List, 2013). Many of these species are well-known for their essential oil-bearing properties including *A. arborescens*, *A. campestris*, *A. lobelii*, *A. annua* and *A. absinthium* (Janaćković et al., 2019). Apart from being used in traditional medicine, many of their medicinal properties, like antimalarial, have been proven scientifically (Bora, & Sharma, 2011).

Artemisia roxburghiana Wall. ex Besser, a perennial wild herb found in South-Asia, is used in traditional medicine for malarial fever, intestinal worms, rheumatism, dysentery, hepatitis, diabetes, and skin disorders (Kumar, Aswal, Semwal, Chauhan, & Semwal, 2019). This plant contains a wide-range of volatile constituents together with other non-volatiles like coumarins, fatty acids, flavones and sterols. Various phytochemicals like betulin, betulinic acid, scopoletin, taraxerol acetate, artemisinin, humulene, β-caryophyllene, α-thujone, eugenol and palmitic acid have been reported from different parts of *A. roxburghiana* (Bicchi, Rubiolo, Marschall, Weyerstahl, & Laurent, 1998; Shah et al., 2016). This plant has potentially exhibited anti-inflammatory, antipyretic, anti-parasitic, anti-protozoal, anthelmintic, antidiabetic and anticancer activities in different experimental models (Kumar et al., 2019). Recently, the essential oil obtained from this plant was evaluated for its insect-repellant activity and found active in repelling mosquitos and ants (Semwal et al., 2019). Moreover, an aqueous-ethanolic extract of the aerial parts of this plant was found effective in inhibiting alpha-amylase and alpha-glucosidase in an *in vitro* assay whereas potentially reduced the glucose level of streptozotocin-nicotinamide-induced diabetic rats (Kumar et al., 2022).

Earlier phytochemical reports showed that *A. roxburghiana* is one of the least explored members of the genus *Artemisia* mainly for its volatile constituents. Moreover, many species of this genus are very similar in their morphology, and hence, it is challenging to identify them without using advanced tools and techniques. Therefore, in view of the limited phytochemical reports on the volatile constituents and the challenges in the identification of *A. roxburghiana*, the present study aims to establish the microscopic profile and identify the volatile constituents of this important plant.

2. Objectives

The main objectives of the present study are to establish the microscopic identification of *Artemisia roxburghiana*, characterise its essential oil and evaluate its antioxidative effect.

3. Materials and methods

3.1. Plant Material

Artemisia roxburghiana was collected from Chaka, Gaumukh (Tehri Garhwal, Uttarakhand) (coordinates: latitude 30.2794° N, longitude 78.5271° E) in August 2018. The plant was authenticated from the Botanical Survey of India, Dehradun and a specimen of the plant is deposited in the herbarium (BSD) with accession No. 118189 for future records.

3.2. Microscopic characterization

The fresh leaf, stem and root were used to prepare and examine the transverse section. As per the standard protocols (Khandelwal, 2005; World Health Organization (WHO), 2011), the stem and root to be sectioned were directly held between the thumb and fingers of the left hand while the leaf was put between two potato pieces to have a firm grip. The thin sections of each part were cut across the object by the right hand using a sharp razor blade and collected into a watch glass containing purified water. Thereafter, the thin sections were transferred to another watch glass containing a mixture of 30% glycerol in ethanol. A single section of each part was put on the glass slide and stained with selected staining reagents. Separate slides were prepared for phloroglucinol-HCl, safranin and iodine reagents. After staining, the slides were mounted with glycerol and shielded with a thin cover glass. The slides were observed under 4x, 10x, 40x and 100x magnification in a compound microscope and the images were captured with the help of a specialised camera (Kumar et al., 2012).

3.3. Phytochemical analysis

Phytochemical screening of major classes of secondary metabolites in different extracts of aerial parts, prepared with methanol, water-ethanol, chloroform and hexane, was done by adopting the previously used method by Semwal and coworkers (Semwal et al., 2018).

3.4. Isolation of the essential oils

Freshly collected aerial parts (500 g) were subjected to hydro-distillation using a Clevenger apparatus (Badoni, Semwal, & Rawat, 2009). The apparatus consists of a round-bottom flask (1000 mL) into which the aerial parts of the plant with water were placed. The flask was connected to a Clevenger apparatus and a vertical condenser. The flask was heated on a rota mantle (REMI, India) and the temperature was set at 100 °C. The essential oil was collected, purified by adding anhydrous Na_2SO_4, and refrigerated in a sealed vial at 4°C until further analysis.

3.5. GC-MS analysis

The analysis was carried out by using an Agilent mass spectrometer (Model 5975C) coupled to an Agilent gas chromatograph DB5 column (60 m × 0.32 mm; film thickness 0.25 µm). Helium was used as carrier gas (flow rate 1 mL/min) with an ionization voltage of 70 eV. The mass spectrum was taken with a mass range of 40-600 amu. The oven temperature program was 60° to 220° with an increase in the rate of 3°/min and finally held isothermally for 5 min. the split ratio was 1:50 and the injection volume was 0.2µL.

3.6. Identification and quantification of essential oil

The essential oil (EO) sample was diluted with *n*-hexane (1 mg/mL) before injecting it into GCMS. A mixture of *n*-alkanes (C_9-C_{23}) diluted with *n*-hexane was also injected under similar GCMS-programmed conditions to calculate the linear retention indices (RIs) as described by Van den Dool and Kratz (1963). The mass spectra (MS), which were recorded in full scan mode, of EO constituents were compared with the internal reference MS libraries of NIST and WILEY. RI values of the EO components relative to *n*-alkanes were compared with those reported in the literature (Juteau, Masotti, Bessière, Dherbomez, & Viano, 2002; Baharum, Bunawan, Ghani, Mustapha, & Noor, 2010; Yang, Liu, Liu, & Du, 2012; Adams, 2017; Tundis, Leporini, Bonesi, Rovito, & Passalacqua, 2020). The quantification of individual components of EO was done by the percentage peak area method in which, the area of a peak was considered as a proportion of the total area of all peaks detected in GCMS (Ahmed, & Tavaszi-Sarosi, 2019).

3.7. Antioxidant Activity

The antioxidative effect of the water-ethanol extract and essential oil obtained from the aerial parts of *A. roxburghiana* was measured using DPPH radical scavenging assay by Stanković and coworkers (Stanković et al., 2016). Briefly, 1 mL of essential oil, 50 mg of the extract and 10 mg of rutin, used as standard, were separately dissolved in 1 mL of methanol to prepare stock solutions. Each stock solution was serially diluted to prepare volumes of different concentrations. Thereafter, 10 µL of each sample (all concentrations) was mixed with 1 mL of DPPH radical solution (prepared by dissolving 90 µM DPPH in 1 L methanol) and the final volume was made up to 4 mL with methanol. The mixtures were shaken vigorously and incubated in the dark for 60 min at room temperature. The methanolic solution of DPPH was used as a control. The absorbance of the resulting solutions was measured at 517 nm using Epoch Microplate Reader (BioTek, USA). The following equation was used to calculate the value of DPPH radical scavenging capacity.

$$\% \text{ Radical scavenging capacity} = (A0 - A1)/A0 \times 100$$

Where, A0 = absorbance of control and A1 = absorbance of sample

4. Results

4.1. Microscopical characterization

4.1.1. Microscopy of the leaf

The microscopical observation of the leaf section revealed that the upper epidermis was single-layered with two types of multicellular trichomes. The stomata as tiny pores were clearly seen in the slide. The mesophyll consisted of single-layered loosely arranged palisade cells and multi-layered spongy parenchyma. The lower epidermis was similar to the upper epidermis whereas the midrib contained collenchyma beneath the upper and lower epidermis. The endodermis was present on the outer layer of the vascular bundles which contained both the xylem and phloem (Figure 1).

4.1.2. Microscopy of the stem

The stem microscopy showed a circular shape with numerous epidermal trichomes. The epidermal cells were single-layered and compactly arranged without having intercellular space. These cells were found with uppermost cuticles and multicellular glandular trichomes. The extrastelar ground tissue lying internal to the epidermis, known as cortex tissue, consisted of chlorenchymatous and parenchymatous cells. It differentiated into hypodermis lying below the epidermis compactly packed without any intercellular space. Next to the collenchymatous hypodermis, many layers of parenchyma cells with intercellular spaces were also seen. The endodermis, the last layer of the cortex, was single-layered with barrel-shaped closely fitted cells forming a wavy band, delimiting the central cylinder or stele. The vascular bundles were placed more towards the epidermis than towards the centre. The bundles were collateral, open and 8-14 in number. The xylem with its component parts, vessels, tracheids, fibres and parenchyma, was found towards the centre. The proto-xylem vessels, with smaller cavities and round-shaped, were placed towards the centre and the meta-xylem vessels, with wider cavities towards the epidermis. The phloem with sieve tubes, companion cells and phloem parenchyma were seen outside or towards the circumference. A strip of the lateral meristem, called the cambium, was found between the xylem and phloem. The pith or medulla, a large central portion of the stem, was located towards the inner side of the vascular bundles and occupied by thin-walled colourless parenchyma cells without intercellular spaces. The parenchymatous cells were also recorded between every two vascular bundles, called medullary rays (Figure 2).

4.1.3. Microscopy of the root

The epidermis was double-layered, thin-walled, and colourless without intercellular spaces. The root hairs were also seen on the epidermal layer whereas the stomata and cuticle were absent. The cortex was homogenous, thin-walled and multi-layered made up of round-shaped parenchymatous cells with intercellular spaces. The endodermis has consisted of compactly arranged barrel-shaped parenchymatous cells. It contained both casparian strips and passage cells. The pericycle seen in the slide was made up of a single lined layer of parenchyma cells. The vascular bundles were radial and arranged in a ring. The xylem and phloem bundles were found separated from each other by parenchymatous cells (Figure 3).

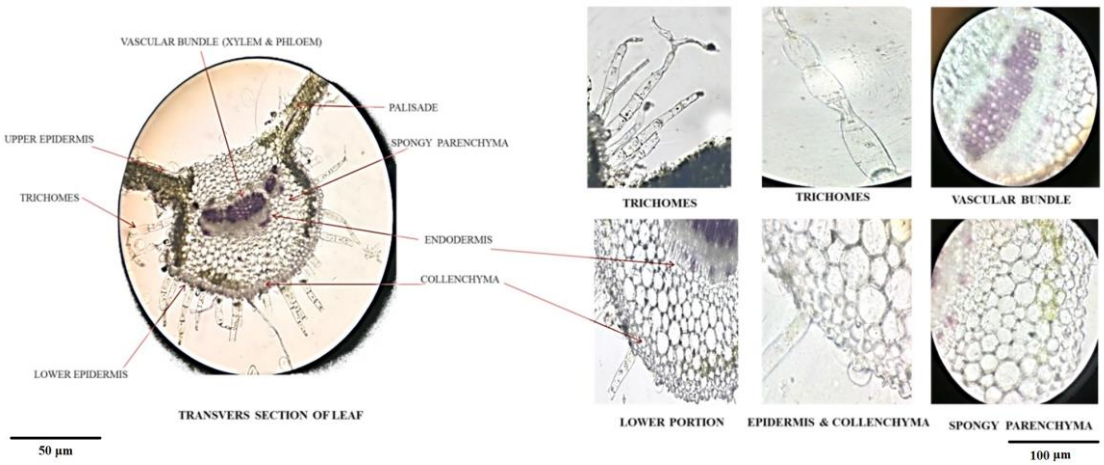

Figure 1 Microscopic characterisation of *Artemisia roxburghiana* leaf

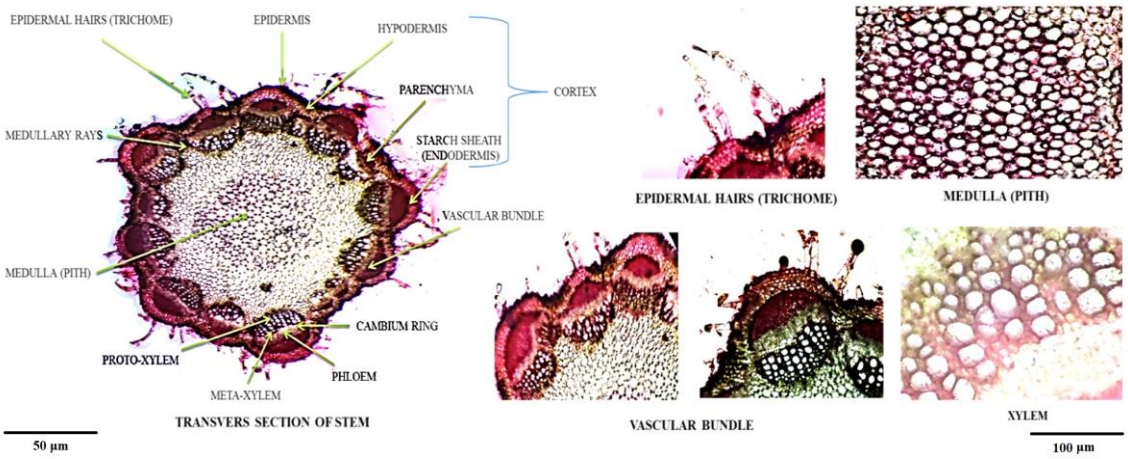

Figure 2 Microscopic characterisation of *Artemisia roxburghiana* stem

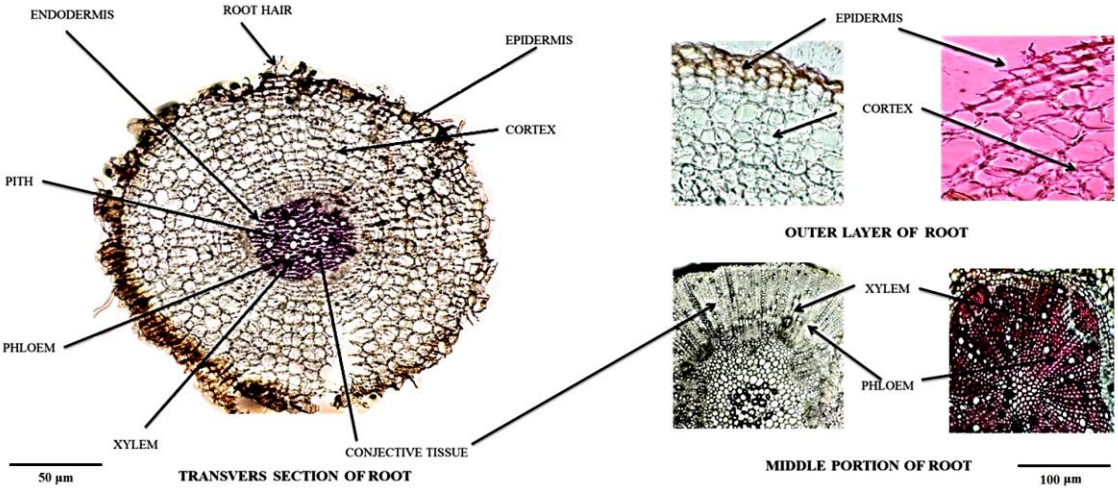

Figure 3 Microscopic characterisation of *Artemisia roxburghiana* root

4.2. Preliminary phytochemical screening

The results of the preliminary phytochemical analysis showed that *A. roxburghiana* contains phenols, tannins, terpenoids, flavonoids and volatile oil. Other constituents like alkaloids, steroids and saponins were not detected in the preliminary test which suggested that these classes of phytochemicals are either absent in the plant or they are present in trace amounts and thus, not detectable.

4.3. Essential oil analysis

The chemical constituents of the essential oil, isolated from the aerial parts of *A. roxburghiana*, were characterized with the help of gas chromatography-mass spectrometry (GC-MS). A total of 41 compounds composing 86.98% of total constituents were identified in the oil in which the most abundant constituents are given in Table 1. The oil contains α-eudesmole (6.52%), *trans*-sabinene hydrate (6.02%), artemisia ketone (5.97%), 1,8 cineole (4.92%), lavandulol (4.82%), 4-terpineol (4.14%), camphor (3.86%), β-thujone (3.72%), germacrene-D (3.65%), borneol (3.64%), and α-thujone (3.45%) as major components. However, 2-isopropyl-5-methyl-9-methylenebicyclo[4.4.0]dec-1-ene, *trans*-pinocarveol, carveol, fokienol, myrtenol,

1*R*,3*Z*,9*S*-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-3-ene, chrysanthenyl acetate, α-terpinene, α-humulene, β-ocimene, α-terpinolene, artemisia triene and curcumene were also identified as minor components with less than 1% concentration.

Table 1 Chemical constituents of *Artemisia roxburghiana* essential oil

S.No.	Compound name	Percentage (%)	RI (calculated)	RI (documented)
1	α-Eudesmol	6.52	1652	1616
2	*trans*-Sabinene hydrate	6.02	1058	1056
3	Artemisia ketone	5.97	1056	1040
4	1,8-Cineole	4.92	1026	1036
5	Lavandulol	4.82	1287	1287
6	4-Terpineol	4.14	1174	1175
7	Camphor	3.86	1141	1146
8	β-Thujone	3.72	1112	1116
9	Germacrene-D	3.65	1484	1486
10	Borneol	3.64	1165	1167
11	α-Thujone	3.45	1110	1114
12	β-Caryophyllene	2.94	1422	1423
13	Vulgarole	2.60	1480	1487
14	Chrysanthenone	2.28	1115	1114
15	Camphene	2.04	956	949
16	4-Terpinenyl acetate	1.99	1350	1350
17	β-Pinene	1.98	976	980
18	dl-Limonene	1.93	1033	1030
19	α-Pinene	1.72	932	938
20	Artemisia alcohol	1.71	1080	1081
21	Davanone	1.52	1587	1581
22	β-Himachalene	1.49	1480	1478
23	Zingiberene	1.34	1493	1489
24	β-Myrcene	1.32	948	949
25	γ-Terpinene	1.27	1068	1065
26	p-Cymene	1.24	1028	1030
27	Filifolene	1.20	1070	1074
28	Sabinene	1.09	939	937

4.4. Antioxidant activity

The results of the DPPH assay revealed that the water-ethanol extract of the aerial parts of *A. roxburghiana* showed free radical scavenging activity with an IC_{50} value of 6.90±1.23 mg/mL. The extract showed maximum inhibition of DPPH radicals by 81.49% at a concentration of 25±2.21 mg/mL. However, the essential oil was found poorly effective against DPPH radicals at an IC_{50} value of >500 µL/mL. On the other hand, rutin which was used as a positive control exhibited radical scavenging activity with an IC_{50} value of 0.65±0.11 mg/mL. A maximum scavenging effect of 86.36%

was shown by rutin at a concentration of 2.5±0.72 mg/mL.

5. Discussion

The present study was designed to explore the microscopic characterization of different parts of *A. roxburghiana*. It is evident from previous studies that microscopy plays a key role in the identification of medicinal plants and the quality control of herbal products (Veena, & Pracheta, 2013; Yu et al., 2019). In the present scenario when the demand for herbal products is much higher than the availability of their raw materials, a parallel market of adulterated products has been developed (Singh, Aeri, & Ananthanarayana, 2018). Hence, due to the distinct characteristics of each plant species, microscopy has been found one of the cheap and most authentic techniques for the quality control of herbal products. Recently, Kumar, Saxena, Goswami, Pant and Sameul, (2018) attempted to develop a microscopic method for the identification of the aerial parts of this plant. However, this study looks too preliminary with limited information on the characterization of the plant part by providing low-resolution images of the stem transverse section. However, in the present study, a complete microscopic characterisation of the stem, root and leaf has been done with high resolution for the first time.

The essential oil isolated from the aerial parts of *A. roxburghiana* was characterized by GC-MS by comparing the mass and compounds with NIST/ WILEY library. The identification was confirmed by calculating their Kovats retention indices (RI) and the same were compared with the reported literature. An initial study on the essential oil of Himalayan *A. roxburghiana* recorded β-thujone (65.3%) as the most abundant constituent (Mathela, Kharkwal, & Shah, 1994) whereas it was found to be 3.72% in the present study. The essential oil of this plant from Europe was earlier characterized by Bicchi et al. (1998), and reported 1,8-cineole (16.6%), camphor (15.2%) and α-thujone (10.0%) as major components, whereas in the present study, α-eudesmole (6.52%), *trans*-sabinene hydrate (6.02%) and artemisia ketone (5.97%) were the major constituents. On the other hand, Haider, Kumar, Banerjee, Naqvi and Bagchi (2009) analyzed the essential oil isolated from different altitudes of the Himalayan region and found that borneol (21.2%) and β-caryophyllene (18.4%) were the highest constituents of the oils isolated from the plants of higher and lower altitudes, respectively. However, in the present study, borneol (3.64%) and β-caryophyllene (2.94%) were found in a lesser concentration. A recent study by Fusani et al. (2022) revealed *cis*-thujone (23.05%), 1,8-cineole (21.56%) and camphor (13.82%) as major constituents of *A. roxburghiana* essential oil collected from Italy. However, 1,8-cineole (4.92%), camphor (3.86%) and β-thujone (3.72%) in the present case were detected in lower concentrations.

Various other species of *Artemisia* such as *A. arborescens*, *A. caerulescens*, *A. proceriformis* and *A. halodendron* were found also found to have similar volatile constituents but in different concentrations. The essential oil of *A. arborescens* (β-thujone/chamazulene chemotype) collected from Sardinia, Italy was found to contain 52% of chamazulene which was the highest concentration ever detected in the genus *Artemisia* (Ornano et al., 2013). The same group of researchers also analysed the essential oil of *A. caerulescens* subsp. *densiflora* from Sardinia, Italy and found davana ethers (17.5%), (*E*)-nerolidol (4.5%), β-oplopenone (3.3%), *cis*-sabinene hydrate (5.2%) and terpinen-4-ol (4.7%) as major constituents (Ornano et al., 2016). On the other hand, α-thujone (66.9%) was recorded to be a major component in *A. proceriformis* (Sampietro et al., 2016) whereas n-hexadecanoic acid (10.40%), biphenyl (7.867%) and 9,12-octadecadienoic acid (7.25%) were the main constituents in the essential oil of *A. halodendron* (Wang et al., 2018). The essential oil obtained from *A. marschalliana* was found rich in *trans*-phytol (29.22%), α-linolenic acid (13.47%) and n-hexadecanoic acid (9.28%) (Salehi et al., 2017).

As far as the antioxidant activity of *A. roxburghiana* extract and its essential oil is concerned, it has been found that the plant is least explored for its antioxidant activity. Bibi et al. (2021) revealed that the extract of *A. roxburghiana* showed significant activity upto 400 µg/mL using DPPH, TAC and TRP assays. In the present study, the extract was found to inhibit DPPH radicals by 81.49% at 25 mg/mL while the essential oil showed

poor DPPH radical scavenging activity with an IC_{50} value of >500 µL/mL.

6. Conclusion

Artemisia roxburghiana has been found effective against various ailments including malarial fever, arthritis and diabetes. The essential oil obtained from this plant was found effective in skin disorders and also showed remarkable insect-repellant activity. The present study successfully analyzed the essential oil with the help of GC-MS in which α-eudesmole and trans-sabinene hydrate were found in the highest concentration. Since the plant has similar morphology to many other species of *Artemisia*, hence, the microscopic profiles of the leaf, stem and root can be utilized for the identification of this species. Moreover, the present microscopic characterization would be useful for the quality control of its products in the near future.

7. Acknowledgment

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8. Conflict of interest statement

The authors declare that there is no conflict of interest.

9. Authors' contribution

DKS: conceptualized and supervised the work; AK, RS and AS: conducted the phytochemical and microscopic studies, and prepared the first draft of the manuscript; HCA: conducted GC-MS studies; RBS, SKJ and DKS: edited and reviewed the manuscript.

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