Isolation, Characterization and Activity of the Flowers of *Rhododendron arboreum* (Ericaceae)

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Abstract: The flowers of *Rhododendron arboreum* have been reported to possess certain polyphenolic compounds. Thus, this study was aimed at the anti-microbial and phytochemical screening of the flowers. Important bioactive agents like steroids, saponins and flavonoids were detected in the flowers. Quercetin (a flavonoid) was isolated from the diethyl ether fraction of alcoholic extract by solvent-solvent extraction method. Isolated quercetin was identified and characterized by chemical tests, M.P., TLC, paper chromatography (with authentic marker) and spectroscopic methods like UV-Visible, FT-IR, ¹HNMR, ¹³CNMR and Mass spectroscopy. The anti-microbial activity of the alcoholic and aqueous extract and isolated quercetin were investigated against five bacterial and two fungal strains by agar well-diffusion method. The activity was found to be concentration dependent. Ethanolic extract was found to be more active in comparison to the aqueous extract. Hence, isolation was done with ethanolic extract. The lowest effective concentration of quercetin was found to be 12.5 mg/ml against *S.aureus* and *P.aeruginosa*. Both extracts and isolated quercetin were found ineffective against fungal strains. Quercetin may be one of the components responsible for the observed anti-microbial activity of the plant.

Keywords: *R. arboreum*, Quercetin, Antimicrobial activity, ERA, ARA.

Introduction

Rhododendron arboreum is a small tree with deep scarlet flowers, often associated with “banj” and “kharsu” plants¹, occurring in the high altitudes from 1500m to 6000m in Himalaya forests or Nilgiri Hills in South India. It is Nepal’s national flower, locally known as *Lali Guras* or ‘rose tree’ in English, belonging to family Ericaceae. In India, *Rhododendron* is the state flower of Nagaland and state tree of Sikkim².

Traditionally, dried flowers fried with ghee were considered highly efficacious in checking diarrhea and blood dysentery³ and squash for the treatment of mental retardation⁴. Flowers have also been reported for anti-inflammatory and cholinergic activity^{5,6}. Ayurvedic preparation “Ashoka Aristha” containing *R. arboreum* possesses oxytocic, oestrogenic and prostaglandin synthetase inhibiting activities⁷. In Homeopathic Materia Medica, the nature of dried leaves has been mentioned to be useful in gout and rheumatism⁸. Shaifulla *et al.* have isolated flavone glycoside 5, 2'-dihydroxy-7-methoxy-4'-O-glucoside and dimethyl ester of terphthalic acid from the leaves⁹. Dixit *et al.* have isolated β -sitosterol, ursolic acid, quercetin and friedelin from leaves and bark¹⁰. Harborne *et al.* identified uniform flavonoid pattern and quantitated various flavonoids such as Gossypetin, Kaempferol, Myricetin, Azaleatin, Caryatin, Dihydromyricetin, Dihydroquercetin, Dihydrokaempferol and Coumarins in the leaf survey of 206 *Rhododendron* species, subspecies and varieties¹¹. Harborne studied natural distribution of flavonol 5-methyl ethers in the leaves and petals of 50 species of *Rhododendron*¹².

Experimental

Flowers of *Rhododendron arboreum* were collected from the forests of “Tala” village of Ukhi Math hills, situated in Rudraprayag (Uttarakhand), India. The plant was identified and authenticated by the Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow (India). Plant herbarium was submitted to NBRI bearing Ref. No. NBRI/CIF/72/2009.

The microorganisms (*B. subtilis* MTCC 441, *E. coli* MTCC 1573, *S. aureus* MTCC 1430, *P. aeruginosa* MTCC 424, *A. tumefaciens* MTCC 3329, *C. albicans* MTCC 183 and *A. niger* MTCC 2546) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Ethanollic extract (ERA)

Flowers of *R. arboreum* were shade-dried and powdered. The powder (100 g.) was packed in a soxhlet apparatus and subjected to continuous hot percolation for about 8 hrs with ethanol (350 mL) as solvent. The extract was concentrated to a semi-solid mass under vacuum and dried in a desiccator (yield 38.25% w/w).

Aqueous extract (ARA)

Approximately 100 g. of shade-dried powder of *R. arboreum* flowers were taken in a 1 L beaker and chloroform: water (1:99) was added up to a sufficient level to immerse the drug completely. Chloroform was added as a preservative to prevent microbial growth. This set up was placed aside for 72 h. with stirring at alternate intervals. Finally, the contents of the beaker were filtered with vacuum to get a clear watery brown colored extract. The extract was concentrated under high vacuum and dried in a desiccator (yield 6.9% w/w).

Fractionation and isolation

About 20 g of ERA was suspended in 200 ml. of distilled water and extracted several times, by taking 10 mL. Each of solvents of decreasing polarity (such as *n*-hexane, chloroform, *n*-butanol and methanol), in a separating funnel. All fractions were concentrated under vacuum and placed in a desiccator at reduced pressure for complete drying.

Approximately 6 g of completely dried *n*-butanolic fraction (dark brown tarry mass) was dissolved in 200 mL of distilled water. The insoluble material was filtered out and the filtrate set aside at 4 °C for 48 h. No precipitation or crystallization was observed. The filtrate was then kept at room temperature for 1 h and further extracted several times with diethyl ether.

The ethereal fractions were pooled in a petri-dish and the solvent evaporated in air. A brownish-yellow amorphous powder was obtained, which was dissolved in 20 mL of ice-cold water and centrifuged at 2000 rpm for 5 min to remove the brown colored pigment. The supernatant was discarded and the remaining residue was extracted from ethanol. A yellowish - green amorphous powder was obtained (7.0 mg, 0.0167% w/w)¹³.

Anti-microbial screening

The stock solutions of ERA and ARA were prepared in concentrations of 100 mg/mL in water for injection; further five concentrations (50, 25, 12.5, 6.25 and 3.125 mg/mL) were prepared from stock solutions by serial dilution. The stock solution of isolated quercetin was prepared in concentrations of 50 mg/mL in DMSO; further five concentrations (25, 12.5, 6.25, 3.125 and 1.5625 mg/mL) were prepared from stock solutions by serial dilution.

Approximately 0.6 mL of nutrient broth suspensions of the test organisms were added to 60 ml of sterile molten nutrient agar, which had been cooled to 45 °C, mixed well and poured into sterile plates. Specific broth and agar media were used for *C. albicans* as per MTCC protocol. The agar was allowed to solidify, six wells (cups) were punched per plate using a six millimeters diameter sterile cork borer (separate borer for each organism), to ensure proper distribution of wells in the periphery with one well in the center. Agar plugs were removed and 50 µL test sample of each concentration was poured in the corresponding marked well by micropipettes. Triplicate plates of each organism were prepared. The plates were left at room temperature for 2 h to allow diffusion of the samples and incubated face upward, at corresponding temperatures of each organism, for 24 h. The diameters of the zones of inhibition were measured to the nearest millimeter (the cup size also being included)¹⁴.

Results and Discussion

Preliminary phytochemical analysis showed positive test for carbohydrates, steroids, saponins, phenolic compounds and flavonoids present in ERA and ARA¹⁵⁻¹⁷. Quercetin, a yellow amorphous powder (Melting point – 308 °C), was isolated from ERA using solvent-solvent extraction method. Shinoda and Pew's tests were found to be positive thus confirming the isolated compound as a flavonoid. TLC (precoated silica gel 60 F₂₅₄, MERCK, Darmstadt, Germany) and paper chromatography (Whatman No.-1) of isolated quercetin and an authentic marker (quercetin) were performed using solvent systems: n-Butanol: Acetic acid: Water (BAW- 4:1:5) and Chloroform: Acetic acid: Water (CAW-10:9:1) simultaneously. Chromatograms were observed under UV light at short and long wavelengths in a UV-chamber. Finally, these were developed with ammonia solution to confirm the compound to be quercetin (Figure 1). The R_f x 100 was measured for all chromatograms (Table 1).

Figure 1. TLC and Paper chromatography of isolated quercetin (IQ) and authentic marker (SQ).

Table 1. Chromatographic observations of isolated quercetin and authentic marker.

	Solvent system / R _f (x100 cm.)	
	BAW(4:1:5)	CAW(10:9:1)
TLC Analysis	2.7/5.4 x100 =50	3.9/4.4 x 100 =88.7
Paper chromatography	6.1/7.1 x 100 =85.9	Tailing
Conditions / Reagents	Color of spots	
Unaided	Dull brownish-yellow	Dull brownish-yellow
Short UV wavelength	Dull-brown	Dull-brown
Long UV wavelength	Black	Black
Ammonia solution	Intense-yellow color	Intense-yellow color

The UV spectra of isolated quercetin and authentic marker (quercetin) were recorded on a Double beam UV-Visible Spectrophotometer 1700 (SHIMADZU Japan) using ethanol as solvent, and by adding aluminium chloride, hydrochloric acid, sodium acetate and boric acid as diagnostic and shifting reagents. The interpretation of functional groups is given in Table 2. FT-IR (KBr): ν (cm^{-1}) 3284 (bonded -OH), 1664 (-C=O), 1610 (aromatic), 1560, 1521, 1448 (C=C Stretch aromatic). The mass spectra obtained by Electro Spray Ionization (ESI-MS), depicted the presence of (M+H) peak at m/z 303.2. ^1H NMR (DMSO): δ 6.187(s, H), 6.408(s, H), 7.524-7.673 (d, 2H), 9.322-9.378(d, 2H), 9.616 (s, H), 9.616 (s, H), 10.812 (s, H) and 12.496 (s, H). ^{13}C -NMR (DMSO): δ 93.71 (-CH of aromatic ring A), 98.54 (-CH of aromatic ring A), 103.36 (-C of ring A-C fusion), 115.41 (-CH of aromatic ring B), 115.95 (-CH of aromatic ring B), 120.34 (-CH of aromatic ring B), 122.31 (-C of aromatic ring B), 136.07 (-C of ring C), 145.40 (-C-OH of aromatic ring B), 147.16 (-C-O of ring C), 148.04 (-C-OH of aromatic ring B), 156.49 (-C of ring A-C fusion), 161.07 (-C-OH of aromatic ring A), 164.22 (-C-OH of aromatic ring A), 176.18 (-C=O of ring C).

Table 2. Comparison of the UV spectral effects of isolated quercetin with the authentic marker¹⁸.

S. No.	Shifting reagents	Spectral maxima (nm)		Spectral effect (Bathochromic shift)	Inference
		Band (I)	Band (II)		
1	Unaided	370* 372 [#]	255* 254 [#]	-----	Flavonoids
2	Aluminium Chloride (5% in Ethanol)	437* 443 [#]	267* 268 [#]	67 nm band (I) and 12 nm in (II) 71 nm band (I) and 13 nm in (II)	5 & 3 OH present
3	Aluminium Chloride + Hydrochloric Acid	431* 431 [#]	266* 264 [#]	No significant shift In band (I) and (II) in comparison to AlCl_3	Free 3 OH is confirmed.
4	Sodium Acetate	456* 448 [#]	270* 269 [#]	86 nm band (I) and 15 nm in (II) 76 nm band (I) and 15 nm in (II) in comparison to Unaided	7 OH present.
5	Sodium Acetate + Boric Acid	440* 452 [#]	269* 270 [#]	70 nm band (I) and 14 nm in (II) 82 nm band (I) and 16 nm in (II) in comparison to Unaided.	B ring has o-dihydroxyl group (3', 4' OH)

* = Isolated quercetin # = Authentic marker.

Antimicrobial activity (Table 3) of ERA, ARA (3.125-100 mg/mL) and isolated quercetin (1.5625-50 mg/mL) were tested against seven microorganisms. All of them were found active against *S.aureus* and *E.coli*. No activity was observed against *B.subtilis*, *P.aeruginosa*, *A.tumifaciens*, *C.albicans* and *A.niger*.

Table 3. Mean diameter of zone of inhibition (mm) ERA, ARA and isolated quercetin at various concentrations against *S. aureus* and *E. coli*.

S.No.	Compounds	Concentrations mg/mL	Microorganisms/Zone of Inhibition (mm)	
			<i>S. aureus</i>	<i>E. coli</i>
1	ERA	100	13	15
		50	13	12
		25	11	8
		12.5	-	8
		6.25	-	-
		3.125	-	-
2	ARA	100	13	13
		50	11	9
		25	-	-
		12.5	-	-
		6.25	-	-
		3.125	-	-
3	Isolated quercetin	50	18	14
		25	15	11
		12.5	11	8
		6.25	-	-
		3.125	-	-
		1.5625	-	-
4	Norfloxacin	10 µg/mL	28	27
5	DMSO (Control)	-	-	-

ERA= Ethanolic *R. arboreum* Extract; ARA= Alcoholic *R. arboreum* Extract.

The lowest effective concentration of ERA against *S. aureus* and *E. coli* were 25 and 12.5 mg/mL respectively and for ARA, it was 50 mg/mL. ERA exhibited a higher degree of activity as compared to ARA. Isolated quercetin was found effective up to 12.5 mg/mL against both strains but ineffective against other five strains.

Conclusion

In the present study, effective concentrations of alcoholic extract (ERA) against *S. aureus* were found to be 100, 50 and 25 mg/mL and against *E. coli* were 100, 50, 25 and 12.5 mg/mL respectively. Similarly, effective concentrations of aqueous extract (ARA) against *S. aureus* and *E. coli* were 100 and 50-mg/mL. The activities were directly proportional to the concentration. Thus, the extracts of flowers of *R. arboreum* could be promising antimicrobial agent of natural origin. Quercetin is a polyphenolic compound, and its antimicrobial action has been reported earlier¹⁹, thereby indicating that it may be one of the components responsible for the observed anti-microbial activity of the above extracts. This is the first report on antimicrobial activity of extract of *R. arboreum* flowers.

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