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PHYTOCHEMICAL AND ANTIMICROBIAL CHARACTERIZATION OF *RHODODENDRON ANTHOPOGON* FROM HIGH NEPALESE HIMALAYA

Bikash Baral[1*], Geeta Shrestha Vaidya[1], Bijaya Laxmi Maharjan[1], Jaime A. Teixeira da Silva[2]**

[1]Biotechnology Unit, Nepal Academy of Science and Technology (NAST), GPO Box: 3323, Kathmandu, Nepal
[2]P. O. Box 7, Miki-cho post office, Ikenobe 3011-2, Kagawa-ken, 761-0799, Japan
Corresponding authors. E-mail: *bikubaral@yahoo.com, **jaimetex@yahoo.com

Abstract

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The biological and chemical properties of the medicinally important high altitudinal plant *Rhododendron anthopogon* D. Don were assayed. Extracts at a concentration of 100 mg·ml^{-1} from leaves and flowers were collectively obtained by employing hot extraction method with different solvents. The antibacterial and antifungal properties were assayed against different clinical bacteria (two Gram^{+} and six Gram^{-}) and six phytopathogenic fungi. *Klebsiella pneumoniae* and *Salmonella typhimurium* were the most inhibited bacteria, while *Fusarium eridiforme*, a fungus, was highly inhibited. However, all the tested bacteria were resistant to *n*-hexane and chloroform extracts. Of all the extracts obtained, the ethyl acetate fraction, followed by the hot methanolic extract, was effective against all the pathogenic strains tested. The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values of ethyl acetate and aqueous extracts on bacteria ranged from 1.562 mg·ml^{-1} to 25.0 mg·ml^{-1}, whereas MIC and MFC (minimum fungicidal concentration) values of *n*-hexane and ethyl acetate fractions on fungi varied from 1.562 mg·ml^{-1} to 12.50 mg·ml^{-1}, which are slightly higher than expected. The extracts showed no activity against *Exserohilum turticum* and *Stenophylum* sp. There was a highly significant difference in the zone of inhibition between different extract fractions and bacterial or fungal strains. A preliminary qualitative phytochemical assay revealed the presence of polyphenols, reducing compounds, quinones, sterol, triterpenes and fatty acids in leaves and flowers of the test plant. The observed antimicrobial effects are believed to be due to the presence of these compounds. The broad spectrum of activity of the extracts would apparently explain the widespread use of this plant for controlling human pathogenic bacteria and phytopathogenic fungi that widely destroy crops in Nepal. This research reveals *R. anthopogon* to be a highly promising source of potent antimicrobial drugs that could be used to design therapeutic drugs in the pharmaceutical industry.

Keywords: bacteria, fungi, microbes, phytochemical constituents, plant extract, zone of inhibition.

Abbreviations: ATCC: American type culture collection; CFU: colony-forming units; DMSO: dimethyl sulfoxide; MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration; MHA: Mueller Hinton Agar; MIC: minimum inhibitory concentration; NB: nutrient broth; PDA: potato dextrose agar; PDB: potato dextrose broth; SPSS: Statistical Package for Social Sciences; WHO: World Health Organization; ZOI: zone of inhibition.

INTRODUCTION

Rhododendron anthopogon D. Don., commonly called as Sunpati (Singh & Sundriyal, 2007; Chhetri et al., 2008) is a high altitudinal medicinal shrub belonging to the *Ericaceae* family and is native to Nepal, growing on open slopes at an altitude ranging from 3.300–5.100 m asl. The leaves and fresh flowers of this little bush are made into a tea by Himalayan healers and drunk to promote digestive

heat, treat sore throats and counteract water-earth illness, remove headaches and back pain, cure cold, blood disorders, bone disease, potato allergies, vomiting, stimulate appetite and relieve liver disorders, while the essential oil is calming and is used in skin care (Yonzon et al., 2005; Siwakoti, 2008; Kumar et al., 2009; Popescu & Kopp, 2013).

Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world (World Health Organization, 2002; Moshi, 2005). For centuries, plants have been continuously used as a source of medicines and plant extracts have great potential as antimicrobial compounds (Gislene et al., 2000). Also, the synergistic effect from the association of antibiotics with plant extracts against resistant bacteria has led to new choices for the treatment of infectious diseases, which enables plants to be used as potential candidates for drug development in the treatment of ailments caused by these pathogens (Toroglu, 2007; Farooqui, 2008; Baral et al., 2011). The curative potential of medicinal plants is attributable to their biologically active substances, which exist in different parts of the plant (Hena et al., 2010). These bioactive compounds render considerable protection against broad spectrum of human pathogenic bacteria and phytopathogenic fungi (Yonzon et al., 2005). However, the high rate of mortality caused by bacterial infections and diseases in human populations and its significance cannot be over-emphasized with ever-increasing multidrug resistance to current antibiotics (Adegoke & Komolafe, 2008, 2009).

Rhododendron anthopogon is collected and gathered by people in high Himalaya from many high altitudinal indigenous locations as a sacred fragrant substance to be burnt in offerings to please and harmonize the earthly divine, especially local earth spirits (Paul et al., 2010; Baral et al., 2011). It is one of the five common Himalayan incense herbs symbolizing the elements that are offered to sanctify and pacify the environment (Aarya Aroma, 2014). Experiments on the medicinal values of *R. anthopogon* revealed the frequent occurrence of metabolites with antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic properties (Sharma et al., 2004; Rajbhandari et al., 2009; Innocenti et al., 2010; Popescu & Kopp, 2013). Highly medicinal properties from the essential oil of this plant might be due to the presence of different monoterpenes (α-pinene, β-pinene, limonene), a sesquiterpene (δ-cadinene) and β-eudesmol (Yonzon et al., 2005; Innocenti et al., 2010).

The purpose of the present study was to expand the knowledge base on what is currently known about the biological and chemical potential of different extracts of this plant against different pathogenic microorganisms.

MATERIALS AND METHODS

Study site and sample collection

Plants were harvested in August and September 2010 (permission granted by the Department of National Parks and Wild-life Conservation, Babarmahal, Nepal) from the high altitudinal Himalayas of the Manaslu Conservation Area (27° 39' 15.7" N, 85° 18' 43.4" E; Elevation: 4.600 m asl). Aerial parts of the plant at the reproductive stage, i.e. leaves and flowers, were collectively used for the experiment. Plant specimens were identified with the help of available herbarium specimens and literature. Voucher samples were deposited at the NAST (Nepal Academy of Science and Technology) laboratory for future reference. The plant material was dried at room temperature (22°C) and was ground to a powder with a grinder. Phytochemical constituents were extracted according to their increasing polarity in an electrophile series.

Extraction

Each powdered sample was extracted successively in different solvents of analytical grade: *n*-hexane, chloroform, ethyl acetate, acetone, methanol and aqueous, each differing in polarity, using a Soxhlet apparatus (Borosil, Gujarat Borosil Ltd., India). The extracts obtained were concentrated to dryness using a rotary vacuum evaporator (Hahnvapor, Hahnshin Scientific Co., HS-2005V-N) (Tiwari et al., 1992).

The yield of each extract was calculated as:

percentage yield (%) = (dry weight of extract/dry weight of samples) × 100.

Phytochemical screening

The chemical compounds in the various extracts were screened by chemical tests, as outlined next (Trease & Evans, 1989; Harborne, 1998).

Test for volatile oils

The obtained *n*-hexane extract was concentrated to yield a residue to which methanol was added, shaken vigorously, filtered then spotted onto filter paper. The formation of a white precipitate indicated the presence of volatile oils.

Tests for alkaloids

The *n*-hexane extract was dissolved in 3 ml of 2% (v/v) HCl and divided equally for the Maeyer test and the Dragendorff test. Half of the solution was treated with three drops of Maeyer's reagent, and the presence of a white precipitate indicated the presence of alkaloids. The remaining half of the solution was treated similarly with Dragendroff's reagent (three drops), and the absence of a white precipitate indicated the absence of alkaloids.

Tests for sterols and triterpenes

The residue of the *n*-hexane extract was dissolved in 1 ml $(CH_3CO)_2O$ and 1 ml conc. H_2SO_4 added from the side of the tube. The presence of a violet ring at the junction of two liquids and the appearance of a green colour in the upper layer indicated the presence of sterols and triterpenes, respectively.

Test for carotenoids

The residue of the *n*-hexane extract was treated with 1 ml of conc. H_2SO_4. The presence of an orange yellow colour, initially similar to the extract then turning red, indicated the presence of carotenoids.

Test for coumarins

The ether extract was concentrated to yield the residue, which was dissolved in hot distilled water (4 ml) and was left to cool to room temperature. The tube was then treated drop-wise with 10% (v/v) NH_4OH solution until pH 8 and was observed under UV light (380 nm). A greenish-yellow fluorescence indicated the presence of coumarins.

Test for emodins (Borntrager's test)

25% (v/v) NH_4OH solution (1 ml) was treated with the ether extract (2 ml) and was shaken vigorously then left to stand for a few minutes. The decolorization of the upper etheric layer and the formation of a red colour by the lower alkaline layer indicated the absence of emodins.

Test for quinones

Freshly prepared ferrous sulphate solution (1 ml) was added to ether solution followed by a few crystals of NH_4SCN and the drop-wise addition of conc. H_2SO_4. The presence of a deep red colour indicated the presence of quinones.

Test for polyphenols

One ml of the methanolic extract was mixed with double distilled water (ddH_2O; 1 ml) followed by 1% (w/v) $FeCl_3$ solution (three drops). The presence of a greenish-blue colour indicated the presence of polyphenols.

Test for reducing compounds

Methanolic extract (1 ml) was mixed with ddH_2O (1 ml) followed by a few drops of Fehling's reagent and warmed (60°C) over a water bath for 30 min. The presence of a brick-red precipitate indicated the presence of reducing compounds.

Test for alkaloid salts

The residue of the methanolic extract was treated with 2% (v/v) HCl (4 ml) followed by vigorous shaking with a vortex and filtered through Whatman No. 1 filter paper. The filtrate obtained was treated with 10% (v/v) NH_4OH solution until pH 8.0 and was extracted with $CHCl_3$ (15 ml). The lower combined layer was concentrated over a water bath (85°C) in a fume hood. The upper layer was discarded while 2% (v/v) HCl (5 ml) was added to the tube and used as the test sample. Firstly, the test solution was treated with Maeyer's reagent (three drops) and then with Dragendorff's reagent (three drops). In both cases, the presence of a white precipitate indicated the presence of alkaloid salts.

Test for glycosides

The methanolic extract residue was first treated with 25% (v/v) NH_4OH solution (2 ml) and was shaken vigorously using a vortex. Secondly, the test solution was treated with Molisch's reagent (5 drops) and conc. H_2SO_4 was added drop-wise from the side of the tube without disturbing the solution. The presence of a cherry-red colour in the first tube and a violet ring at the junction of the two liquids in the second test solution indicated the presence of glycosides.

Preparation of standard culture inocula

Eight different clinical bacterial strains and six phytopathogenic fungal strains were employed to test the antimicrobial potential of *R. anthopogon*. Standard bacterial cultures were obtained from the Central Laboratory, Teku, Kathmandu, Nepal and the fungal cultures from Nepal Agricultural Research Council (NARC), Khumaltar, Lalitpur, Nepal. Bacterial strains used for the experiment were *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi*, *S. paratyphi*, *S. typhimurium*, *Staphylococcus aureus* (ATCC 25923), *Shigella dysentriae* and *Escherichia coli* (ATCC 25922). The fungal strains employed in the experiment were *Fusarium proliferatum*, *F. eridiforme*, *F. moniliforme*, *F. oxysporum*, *Exserohilum turticum* and *Stenophylum* sp. For bacterial inocula, colonies were selected from 18–20-h-old cultures and inoculated into nutrient broth (NB; tryptone 10 $g \cdot l^{-1}$, yeast extract 5 $g \cdot l^{-1}$, NaCl 10 $g \cdot l^{-1}$, pH 7.4 ± 0.2). Turbidity was adjusted to 0.5 McFarland standards (1.5×10^8 CFU/ml). Similarly, a standard culture inocula of each fungal strain was prepared in potato dextrose broth (PDB; potato 200 $g \cdot l^{-1}$, dextrose 20 $g \cdot l^{-1}$; pH 5.1 ± 0.2; HiMedia Laboratories Pvt. Ltd, Mumbai, India) adjusting to $1 \times 10^6 - 5 \times 10^6$ spores/ml (spores counted using a haemocytometer) (Precision Scientific Instruments Corp., Delhi, India; Aberkene et al., 2002).

Antimicrobial assay

Antimicrobial activity was tested by the agar-well diffusion method (Holder & Boyce, 1994) for determining the susceptibility of different bacteria and fungi to *R. anthopogon* extracts. The assay was carried out in an aseptic zone under a laminar air flow cabinet (Horizontal air flow; Toshiba, India, New Delhi, India). The bacterial and fungal inocula were prepared in NB and PDB, respectively. *In-vitro* spreading techniques (in sterile Petri dishes; 90 mm diameter) of the respective strains were carried out in Mueller Hinton agar (MHA; beef dehydrated infusion 2 $g \cdot l^{-1}$; casein hydrolysate 17.5 $g \cdot l^{-1}$; starch 1.5 $g \cdot l^{-1}$; agar 17 $g \cdot l^{-1}$; pH at 25°C 7.4 ± 0.2), and potato dextrose agar (PDA; potato 200 $g \cdot l^{-1}$, dextrose 20 $g \cdot l^{-1}$, agar 20 $g \cdot l^{-1}$; pH 5.6 ± 0.2; HiMedia) medium. The working solutions of the extracts were prepared in dimethyl sulfoxide (DMSO). The extracts (concentration 100 $mg \cdot ml^{-1}$; 50 µl) were seeded into the wells (6 mm diameter) and incubated at 37 ± 1°C for bacteria (24 h) and 27 ± 1°C for fungi (7 days). The plates were refrigerated for 1.5 h in order to stop the organisms' growth and to facilitate diffusion of the substances. The zone of inhibition (ZOI), as indicated by a clear zone, i.e. without growth of microorganisms around the well, was measured. Tests were performed in triplicate and mean values together with standard deviations were assessed. For every sample tested, a set of positive (200 ppm Ampicillin for bacteria and 100 ppm Nystatin for fungi; Biomedicare Pvt. Ltd., New Delhi, India) and negative controls (DMSO used for preparing working solution of extracts) were run simultaneously. Plates were incubated in an incubator (Incubator Universal, NSW, India) in an upright position during the incubation period. The zones of the solvents, if observed, were deducted from the zones of inhibition created by the crude extracts.

A further assay of the most effective extracts was done by the two-fold broth dilution method to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC). The MIC and MBC for bacteria were evaluated in NB, while PDB was used to evaluate the MIC and MFC of fungi (Andrews, 2001; Maharjan et al., 2011). One ml of extract (100 $mg \cdot ml^{-1}$) was added to a test tube containing 1 ml of broth and a two-fold serial dilution of the extract was performed. Two drops of microbial standard culture broth (0.5 McFarland standards) was added to each test tube and incubated. The tube with the least concentration of the extract (ethyl acetate and water for bacteria; *n*-hexane and ethyl acetate extract for fungi) that showed no growth was determined as the 'MIC'. The tubes showing no growth of microbes were streaked onto the agar plates and incubated. The tube with the least concentration of the extract that showed no growth at that concentration was reported as the MBC of the extract for that particular microorganism.

Statistical analysis

The results of the yields percentage are expressed as the mean ± SE of three replicates (n = 3) in each test. A test for normality was performed on all data. Since normality was confirmed for all data, a parametric statistical test (one-way analysis of variance

(ANOVA)) was chosen for this study; the analysis of data between groups and pair-wise mean separations were carried out using Duncan's Multiple Range Test (DMRT) at α = 0.05 (Gomez & Gomez, 1984; Fig. 1) to assess the statistical significance using SPSS version 16.0 (SPSS, Inc., USA) and Excel 2007 (Microsoft Office).

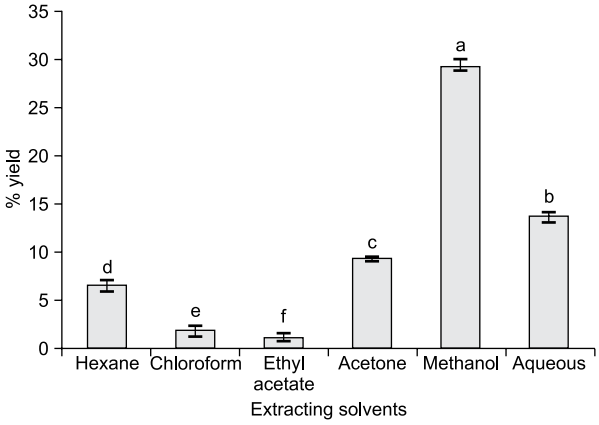

Fig. 1. Total yield percentage of *R. anthopogon* extract by different solvents. n = 3, mean ± SE. Different letters represent significant differences according to DMRT at α = 0.05. The error bar in the figure represents the standard error (±1 SE)

The results of the antimicrobial potentials (antibacterial and antifungal) are also expressed as the mean ± SE of three replicates in each test. The data of ZOI for the antimicrobial properties were evaluated by two-way ANOVA with Levene's test (Tables 1 and 2). Pair-wise mean separations were carried out using DMRT at α = 0.05 (Gomez & Gomez, 1984) and Levene's test was performed. A p value ≤ 0.05 represented the level of significance in all cases. Different letters were used to denote significantly different means ($p < 0.001$; Fig. 1).

RESULTS

The highest yield was found in methanolic (29.30%) followed by aqueous (13.62%) and acetone extracts (9.21%) (Fig. 1). Some of the tested bacteria were not inhibited by different concentrations of the extracts (Table 1). Analysis of antifungal activity against different concentrations of hot ethyl acetate extract showed a good inhibitory effect, i.e. high ZOI (20.06 mm) against *F. eridiforme.* The extract was not effective against *Exserohilium* sp. and *Stenophylum* sp. (Table 2). Different solvent extracts showed good efficacy against *Fusarium* spp. (Table 2). *Klebsiella pneumoniae* (EtOAc; ZOI: 16.1 mm) and *Salmonella typhimurium* (EtOAc; ZOI: 15 mm) were greatly inhibited by the different extracts, with maximum ZOI displayed by the ethyl acetate fraction.

Plant extracts showed the presence of polyphenols, reducing compounds, quinones, sterol, triterpenes and fatty acids. These compounds (secondary metabolites) are biologically active and, therefore, might contribute towards the antimicrobial activities of *R. anthopogon* (Table 3). The *n*-hexane and the chloroform extracts did not show any ZOI for any of the tested bacterial strains, i.e. these extracts showed no antimicrobial properties (no potency). The MIC and MBC values for the bacterial strains (*S. typhimurium*, *S. paratyphi*, *K. pneumoniae* and *Staphylococcus aureus*) that were

Table 1. Antibacterial properties of *R. anthopogon* extracts against different pathogenic bacteria

Bacterial strains	Concentration (100 mg·ml^{-1})				*F*-value	Significance level
	EtOAc	Ace	Met	Aq		
	Zone of inhibition (ZOI, mm)*; (n = 3)					
Salmonella typhimurium	15.0 ± 0.36 b	14.2 ± 0.36 b	12.0 ± 0.36 b	13.5 ± 0.96 b	227.48	$p < 0.001$
Salmonella paratyphi	14.0 ± 1.15 c	12.0 ± 0.70 c	14.0 ± 2.22 c	9.06 ± 0.25 c	36.13	$p < 0.001$
Klebsiella pneumoniae	16.1 ± 0.75 a	9.20 ± 1.65 a	13.2 ± 2.32 a	11.1 ± 0.45 a	32.64	$p < 0.001$
Staphylococcus aureus	7.73 ± 0.35 h	11.13 ± 2.79 h	7.0 ± 0.78 h	8.23 ± 0.70 h	7.31	$p < 0.001$
Shigella sp.	12.0 ± 0.36 d	14.06 ± 0.70 d	11.9 ± 0.69 d	11.7 ± 0.78 d	106.09	$p < 0.001$
Pseudomonas aeruginosa	9.0 ± 0.50 f	13.46 ± 1.80 f	0 f (NI)	7.73 ± 1.05 f	33.5	$p < 0.01$
Salmonella typhi	11.0 ± 1.08 e	10.13 ± 1.55 e	13.66 ± 1.18 e	8.50 ± 0.95 e	27.45	$p < 0.001$
Escherichia coli	8.10 ± 0.36 g	10.16 ± 0.60 g	7.90 ± 0.62 g	9.83 ± 1.12 g	18.58	$p < 0.001$

EtOAc: ethyl acetate; Ace: acetone; Met: methanol; Aq: aqueous
Hexane and chloroform did not show any ZOI against any of the bacteria tested
*Mean ± SD (n = 3); NI: no inhibition
Same letters within a column represent significant differences according to DMRT at α = 0.05

Table 2. Antifungal properties of *R. anthopogon* extracts against different phytopathogenic fungi

Fungal strains	Concentration (100 mg·ml^{-1})						*F*-value	Significance level
	Hex	Chl	EtOAc	Ace	Met	Aq		
	Zone of inhibition (ZOI, mm)*; (n = 3)							
F. proliferatum	8.80 ± 0.98 a	7.96 ± 0.35 b	13.00 ± 0.65 b	11.3 ± 0.7 b	16.07 ± 0.55 a	13.13 ± 0.75 a	59.700	$p < 0.001$
F. oxysporum	7.73 ± 0.41 b	0 c (NI)	12.03 ± 0.60 c	0 c (NI)	0 c (NI)	0 b (NI)	502.337	$p < 0.001$
F. moniliforme	0 c (NI)	0 c (NI)	8.23 ± 0.70 d	0 c (NI)	0 c (NI)	0 b (NI)	30.331	$p < 0.001$
F. eridiforme	8.16 ± 0.60 a	10.56 ± 0.75 a	20.06 ± 1.33 a	12.37 ± 0.25 a	8.56 ± 0.87 b	0 b (NI)	125.759	$p < 0.001$

Hex: hexane; Chl: chloroform; EtOAc: ethyl acetate; Ace: acetone; Met: methanol; Aq: aqueous
Stenophylum sp. and *Exserohilum turticum* were not inhibited by any extract solution
* Mean ± SD (n = 3); NI: no inhibition
Same letters within a column represent significant differences according to DMRT at α = 0.05

Table 3. Phytochemical screening of *R. anthopogon* in different solvent systems

Solvent system	Compound tested	Results
Hexane	Volatile oils	–
	Fatty acids	–
	Coumarins	–
	Flavon aglycones	–
	Alkaloids	–
	Emodins	–
	Sterols	–
	Triterpenes	–
Methanol	Polyphenols	+
	Reducing compound	+
	Alkaloid salts	–
	Glycosides	–
	Quinones	+
	Sterols and triterpenes	+
	Anthocyanosides	–
	Anthracenosides	–
	Coumarin derivatives	–
	Fatty acids	+
	Flavonic glycosides	–
Aqueous	Polyoses	–
	Saponins	–
	Alkaloid salts	–

'+'= present; '-' = absent

greatly inhibited by the extracts (ethyl acetate and aqueous) were tested for their inhibitory concentrations and bactericidal concentrations. The ethyl acetate fraction showed good inhibition (ZOI: 16.1 mm) and a good bactericidal (at least concentration) value against *K. pneumoniae* (1.562 mg·ml^{-1} and 3.125 mg·ml^{-1}, respectively). However, the aqueous fraction showed good inhibition (ZOI: 13.5 mm) against *S. typhimurium* (MIC: 1.562 mg·ml^{-1}; MBC: 6.25 mg·ml^{-1}) (Table 4). Likewise, the three fungal strains that were greatly inhibited were tested for their MIC and MFC values. The *n*-hexane fraction showed good inhibition against *F. proliferatum* (ZOI: 8.8 mm; MIC: 3.125 mg·ml^{-1}; MFC: 6.25 mg·ml^{-1}) while the ethyl acetate fraction showed a good result against *F. eridiforme* (ZOI: 20.06 mm; MIC and MFC: 1.562 mg·ml^{-1}) (Table 5).

A two-way ANOVA between the effects of extracting solvent and different bacteria on ZOI was conducted. There was highly significant difference between the extracting solvents and bacterial strains used in terms of ZOI (*F*-value: 12.857; $p < 0.001$) with an insignificant difference based on DMRT (*F*-value: 0.768 and 1.00 for extracting solvents and bacterial strains, respectively). A highly significant difference for Levene's test (*F*-value: 3.846; $p < 0.001$) was obtained for the bacterial strains in terms of ZOI.

Similarly, a two-way ANOVA between the effects of extracting solvent and different fungal strains on ZOI was performed. There was highly significant difference between the extracting solvents and fungal strains used in terms of ZOI (*F*-value: 82.132; $p < 0.001$) based on DMRT (*F*-value: 3.792; $p < 0.05$ and *F*-value: 19.924; $p < 0.001$ for extracting solvents and fungal strains, respectively). A highly significant difference for Levene's test (*F*-value: 4.984; $p < 0.001$) was obtained for the fungal strains in terms of ZOI.

DISCUSSION AND CONCLUSIONS

Plants produce certain bioactive molecules, which react with other organisms in the environment, including bacteria or fungi, and the use of plant-derived drugs is a long-standing practice (Fennel et al., 2006; Bhattacharyya, 2011). In the present experiment, methanol extraction possessed the highest yield rela-

Table 4. MIC and MBC of ethyl acetate and aqueous extracts from *R. anthopogon* on different clinical bacterial strains

Test organisms	EtoAc extract		Aqueous extract	
	MIC ($mg \cdot ml^{-1}$)	MBC ($mg \cdot ml^{-1}$)	MIC ($mg \cdot ml^{-1}$)	MBC ($mg \cdot ml^{-1}$)
Salmonella typhimurium	3.125	6.25	1.562	6.25
Salmonella paratyphi	6.25	6.25	6.25	12.5
Klebsiella pneumoniae	1.562	3.125	3.125	3.125
Staphylococcus aureus	6.25	25.0	6.25	12.5

Table 5. MIC and MBC of hexane and ethyl acetate extracts from *R. anthopogon* on different phytopathogenic fungal strains

Test organisms	Hexane extract		Ethyl acetate extract	
	MIC ($mg \cdot ml^{-1}$)	MFC ($mg \cdot ml^{-1}$)	MIC ($mg \cdot ml^{-1}$)	MFC ($mg \cdot ml^{-1}$)
F. proliferatum	3.125	6.25	1.562	3.125
F. oxysporum	6.25	12.50	3.125	12.50
F. eridiforme	6.25	6.25	1.562	1.562

tive to aqueous or acetone extracts, suggesting that more phytochemicals were extracted when methanol was used as an extracting solvent. This finding correlates with the results of Khan et al. (2005), who suggested that more bioactive compounds can be extracted in methanol – with a 1.8-fold higher inhibition value – than in water, or even the combination of methanol and water. A large number of plants possess antimicrobial activity and some of their active components have become a potential source of new anti-infective agents against different bacterial and fungal strains, the first being *in-vitro* antimicrobial activity (Maharjan et al., 2011). The ability of one or more extracts to inhibit the growth of several bacterial and fungal species is an indicator of the broad spectrum antimicrobial potential of *R. anthopogon*, which makes this plant a candidate for bioprospecting antibiotic and antifungal drugs. *R. anthopogon* may serve not only to mine biomedically active secondary metabolites (Ibegbulem et al., 2003) for the bio-medical and pharmaceutical industriers, thus providing potentially new financial alternatives for Nepalese farmers, but the identification of important compounds would also allow important germplasm to be preserved through *in-vitro* culture. Nepal, embellished with a plethora of plant genetic resources, is currently experiencing drastic environmental threats caused by various factors such as global warming and anthropogenic activities, which has drastically reduced species richness (Findlay & Houlahan, 2003; Pearson & Dawson, 2003) Thus, to protect and preserve *R. anthopogon* from extinction, long-term storage of germplasm and mass production of species using *in-vitro* techniques is essential.

As expected, the six *R. anthopogon* extracts yielded different levels of extract depending on the solvent, and each extract contained different secondary metabolites and, thus, different antimicrobial activity. The ethyl acetate fraction was more effective against *Klebsiella pneumoniae* followed by *Salmonella typhimurium*, similar to the result obtained by Chao et al. (2010), followed by the methanol extract. Among the fungal pathogens, the ethyl acetate extract was most effective against *Fusarium eridiforme*, followed by the aqueous extract against *Fusarium proliferatum*. Fungal toxicity is readily observed in antifungal compounds obtained through successive extraction in different organic solvents, suggesting that *R. anthopogon* may be used against different phytopathogens (Maharjan et al., 2011), although restricting their growth is difficult because of their complex and rigid cellular structure (hard chitin layer). Moreover, pathogens orchestrate both defensive and offensive arsenals to neutralize different compounds as in *Stenophylum* sp. and *Exserohilum turticum* (Devkota et al., 2011). When these fungi are grown in media with such compounds, a cascade of transcriptional responses essential for full protection against the toxicity afforded by the compounds is triggered. *F. oxysporum* and *F. moniliforme* were found to be more resistant to almost all the *R. anthopogon* extracts obtained by different solvents, which might indicate the presence of different cellular components and their defensive properties against different inhibitors.

The phytochemical properties of *R. anthopogon* in this study share similarities with previous reports

on *R. anthopogon* extracts (Innocenti et al., 2010; Gyawali et al., 2013). The detected range of antimicrobial properties of the extracts (ethyl acetate and acetone) could be due to the most abundant components α-pinene, β-pinene and limonene, which are highly effective in inhibiting the growth of microorganisms (Dorman & Deans, 2000; Innocenti et al., 2010). Volatile oils in this plant have been analysed in earlier studies (Yonzon et al., 2005; Innocenti et al., 2010; Olennikov et al., 2010), although in this study volatile oils were not detected. The presence of essential oil was tested with extracts in *n*-hexane, which showed no signs of its presence, although this may be a function of the extraction method, with steam distillation, hydrodistillation or supercritical CO_2 extraction likely to produce essential oils. This negative finding can also be justified by the fact that *n*-hexane with low polarity might not be able to extract the essential oil present in the plant, at least not at room temperature. Moreover, in our present study, the MIC, MBC and MFC values are found to be more than expected (for instance, 15-fold higher for *S. typhimurium*) (Innocenti et al., 2010), which might be due to poor extraction procedure of not extracting all the compounds or the selection of the solvent during the extraction. However, the values (MIC, MBC and MFC) of crude extract, isolated pure compounds and essential oils may differ and should not be directly compared. Thus, obtaining extracts in different organic solvents with varying polarities in an electrophile series may provide a wider range of alternatives to test for bioactivities of extracts from this plant. Compounds could not be quantified or detected in *n*-hexane and aqueous extracts despite showing bioactivity against the tested pathogens. More robust analytical techniques to detect compounds such as HPLC and GC-MS are needed to validate these results.

The difference in sensitivity between bacteria and fungi may lie in the transparency of the cell wall (Knobloch et al., 1989). Due to the variable diffusability of extracts on the agar media, the ZOI may not be commensurate with the exact efficacy of the extracts, therefore, the MIC, MBC and MFC were also calculated in this study.

In general, $Gram^-$ bacteria are more resistant than $Gram^+$ bacteria, although this study showed that some of the $Gram^-$ bacteria were sensitive to a high concentration of the extract (ethyl acetate, acetone, methanol and aqueous fractions). Nevertheless, *E. coli*, which is a $Gram^-$ bacterium, was also inhibited by *R. anthopogon* extract, with acetone extract being the most inhibitory (ZOI: 10.16 mm). The ethyl acetate fraction was highly effective against different bacteria (*S. typhimurium*, *S. paratyphi*, *K. pneumoniae*) and a fungus (*F. eridiforme*), confirming that bioactive compounds are present in this fraction. Additional research to extract the corresponding chemical compounds in such solvents and to elucidate their therapeutic potential is necessary.

The observed bioactivity of this plant against human pathogens and phytopathogens may open a wide array of practical uses, including the design and production of potential pharmaceutical drugs. Moreover, the identification of the chemical compounds by GC-MS, HPLC and other analytical strategies may assist in better utilizing the plant for improving human health, providing protection against agricultural pests or identifying other useful bioactive properties.

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AUKŠTŲJŲ NEPALO HIMALAJŲ *RHODODENDRON ANTHOPOGON* FITOCHEMINĖS IR ANTIMIKROBINĖS SAVYBĖS

Bikash Baral, Geeta Shrestha Vaidya, Bijaya Laxmi Maharjan, Jaime A. Teixeira da Silva

Santrauka

Buvo tiriamos biologinės ir cheminės mediciniškai svarbaus aukštuminio augalo *Rhododendron anthopogon* D. Don savybės. Bendri lapų ir žiedų 100 mg/ml koncentracijos ekstraktai buvo gauti panaudojant įvairius tirpiklius karšto ekstrahavimo metodu. Jų antibakterinės ir priešgrybinės savybės buvo tiriamos prieš įvairias klinikines bakterijas (dvi Gram + ir šešias Gram –) ir šešis fitopatogeninius grybus. Labiausiai inhibuojamos bakterijos buvo *Klebsiella pneumoniae* ir *Salmonella typhimurium*, o labai slopinamas grybas – *Fusarium eridiforme*. Tačiau visos bandytos bakterijos buvo atsparios *n*-heksano ir chloroformo ekstraktams. Iš visų gautųjų ekstraktų, etilo acetato frakcija ir ekstrahavimas karštu metanoliu buvo veiksmingiausias prieš visus išbandytus patogeninius kamienus. Etilo acetato ir vandeninių ekstraktų MIC (minimali slopinamoji koncentracija) ir MBC (minimali baktericidinė koncentracija) dydžiai bakterijoms svyravo nuo 1,562 mg/ml iki 25,0 mg/ml, o *n*-heksano ir etilo acetato frakcijos MIC ir LMDP (minimali fungicidinė koncentracija) vertės grybams svyravo nuo 1,562 mg/ml iki 12,50 mg/ml, ir buvo šiek tiek didesnės nei tikėtasi. Ištraukos buvo neveiklios prieš *Exserohilum turticum* ir *Stenophylum* sp. Labai ryškus skirtumas pasireiškė tarp skirtingų ekstraktų frakcijų ir bakterinių ar grybelinių kamienų inhibavimo zonų. Preliminarus kokybinis fitocheminis tyrimas atskleidė, kad tiriamo augalo lapuose ir žieduose yra polifenolių, redukuojančių junginių, kvinonų, sterolių, triterpenų ir riebalų rūgščių. Manoma, kad antimikrobinis poveikis yra stebimas dėl šių junginių. Platus ištraukų poveikio spektras akivaizdžiai paaiškina šio augalo platų naudojimą prieš žmogaus patogenines bakterijas ir fitopatogeninius grybus, stipriai naikinančius pasėlius Nepale. Šis tyrimas atskleidžia, kad *R. anthopogon* yra labai perspektyvus stiprių antimikrobinių vaistų šaltinis, kuris gali būti naudojamas siekiant sukurti terapinius vaistus farmacijos pramonėje.