Evaluation of Antioxidant and Antibacterial Properties of *Rhododendron arboreum, Acmella calva* and *Trifolium repens* of Nepal

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ABSTRACT

Objectives: To evaluate the antioxidant and antibacterial properties of *Rhododendron arboreum*, *Acmella calva* and *Trifolium repens.*

Methods: Different extracts of the *R. arboreum, A. calva* and *T. repens* were used for detection of total phenolic content and total flavonoid content. 2, 2-diphenyl-1-picryl hydrazyl (DPPH) assay was performed to evaluate the antioxidant activity. Antimicrobial activity was carried out by agar well diffusion method. Minimum Bactericidal Concentration (MBC) was determined by broth micro dilution assay.

Results: Ethyl acetate extract of *R. arboreum* had high total phenolic content (128.36±1.65) and total flavonoid content (107.3±0.92). The high antioxidant activity with IC_{50} value of 34.97µg/mL was shown by ethyl acetate extract of *R. arboreum*. The ethyl acetate extract of *R. arboreum* showed maximum zone of inhibition against *E. coli, K. pneumoniae, P. aeruginosa, A. baumanii, S. aureus* and *E. fecalis* with the lowest MBC values.

Conclusion: *R. arboreum*, *A. calva* and *T. repens* have antimicrobial activity and compounds with antioxidant activity and should be investigated further for potential medicinal use.

Keywords: *Rhododendron arboreum, Acmella calva, Trifolium repens*, antioxidant, antibacterial activity

INTRODUCTION

Human beings relied on the therapeutic properties of medicinal plants before the development of modern medicines (Aswal et al 2013). Because medicinal plants are the backbone of traditional medicine, more than 3.3 billion people in different countries regularly use them (Davidson-Hunt 2000). Around the world, medicinal plants have been integral to many traditional systems (Lalremsanga and Lalthanpuii 2018), as well as in the discovery and development of pharmaceutical drugs. Due to their antioxidant capabilities and related health-promoting effects, plant extracts have attracted interest for use in the food business (Tajner-Czopek et al 2020).

One of the top three issues affecting human health according to the World Health Organisation (WHO) is antibiotic resistance. The "ESKAPE," bacteria *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp are the most prevalent and dangerous MDR bacteria. This unfortunate circumstances has pushed us to study more effective antibacterial agents derived from plant resources for use as active therapeutic ingredients and as lead molecules in the manufacture of optimized new medicines (Nascimento et al 2000).

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Oxidative stress has a crucial role in the development and progression of cancer, diabetes mellitus, cardiovascular disease, neurodegenerative illness, and inflammatory disease (Arika et al 2019). The synthetic antioxidants have been linked to negative consequences. Plants produce phenolic chemicals, which are hydroxylated benzoic acid and cinnamic acid derivatives with antioxidant and anticarcinogenic properties (Moriasi et al 2020).

In Nepal, the bulk of the population relies on the usage of medicinal herbs in traditional medicine and for centuries, the majority of people have used and valued folk herbal treatments. This can be explained by reasons like the scarcity of medical facilities, doctors, medications, and transportation options, as well as the high costs of receiving such treatments (Taylor 2013). It is estimated that between 75 and 80 percent of Nepal's rural residents use these folk treatments. Even though Nepal has a large herbal medicine industry, only a small number of species have actually had their biological activity tested. Therefore, additional research into the function of Nepalese medicinal herbs is worthwhile.

METHODS

Collection of samples

Selected medicinal plants were collected from different places of Nepal. The plant samples were identified by the National Herbarium, Godavari. The extraction process was conducted at NAST while antioxidant and antimicrobial activities were assessed in the laboratory of Central Department of Microbiology.

Processing of samples

The flower part of the plants was cleaned to remove extraneous matter. The samples were spread under the shade at room temperature until completely dried.

Preparation of extraction of the plants

The plant extraction method was done according to (Kashyap et al 2017). The plant materials were collected and shade dried and used for solvent extraction. The dried plants were grinded into powder and soaked in methanol for 3 days. The methanolic extract of *R. arboreum*, *A. calva* and *T. repens* was concentrated to dryness using a rotary evaporator to obtain extract. The crude methanolic extracted was suspended in water and further fractionated by solvent-solvent extraction process to obtain hexane, dichloromethane, ethyl acetate and butanol extracts of *R. arboreum*, butanol and ethyl acetate extracts of *A. calva* and ethyl acetate, hexane and dichloromethane extracts of *T. repens*.

Calculation of yield

After extraction, the extract was concentrated to dryness using a rotary evaporator. The extract was then refrigerated at 4°C until further analysis.

The yield of respective extracts were calculated by the formula:

$$\text{Extract yield (\%)} = \frac{\text{weight of dried extract}}{\text{weight of dried flower}} \times 100$$

Determination of total phenolic content (TPC)

The quantification of TPC in different extracts were performed by the modified Folin–Ciocalteu method (Khanal et al 2022). A series of standard gallic acid solutions with concentrations of 5, 10, 20, 40, 50, and 60µg/mL were prepared. In triplicate, aliquots of 20 µL gallic acid and test solutions (5 mg/mL) were placed in the bores of a microtitre. 100 μL of Folin-Ciocalteu reagent (1:10 in DW) and 80 μL of 7.5% Na_2CO_3 solution were added to each solution. The mixture was incubated in the dark for 25 minutes at room temperature, and optical density was measured at 696 nm with a microtiter plate reader (HER 480). TPC was determined using the standard calibration curve and represented as milligrams gallic acid equivalents per gram of dry extract (mg GAE/g).

Determination of total flavonoid content (TFC)

The flavonoid content was determined based on the method according to Silva-Beltrán et al(2015), with slight modifications. 100 µL of a sample was combined with 430 µL of 5% $NaNO_2$ in a 2 mL Eppendorf tube, then incubated for 5 minutes. Following incubation, 30 µL of $AlCl_3$ (10%) and 440 µL of NaOH (1 mol/L) were added to the reaction mixture, and absorbance was measured at 496 nm using a microplate reader (HER 480) using quercetin as a standard. The results were given in milligrams of quercetin equivalents (QE) per grams of extract (mg QE/g).

Determination of Antioxidant activity

The antioxidant activity of several plant extracts was assessed using the modified DPPH free radical scavenging method (Khanal et al 2022). Test solutions of extracts and ascorbic acid at concentrations of 200, 100, 50, 25, and 12.5µg/mL were prepared. In triplicates, 100 µL of 0.1 mM DPPH and test solutions were placed into a 96-well

microtiter plate. Ascorbic acid was also added as a positive control. The mixture was incubated in the dark for 30 minutes at lab temperature, and the optical density at 496 nm was measured against a blank. The radical scavenging activity of various extracts and ascorbic acid was calculated as follows:

$$\% \text{ Inhibition} = \frac{A-B}{A} \times 100$$

Where, A is absorbance of control and B is absorbance of sample.

The half-maximal inhibitory concentration (IC_{50}) of the extracts and the positive controls were calculated by using the Graph Pad Prism 9 software.

Preparation of stock/working solution

100mg/mL of each crude extract was made by taking 100mg of the extract in 1 mL of DMSO in clean and capped test tubes. The extract was dissolved in DMSO by vortexing. After making stock/working solution the test tubes were capped, sealed and stored in refrigerator (2-8^{o}C) until use.

Preparation of standard culture inoculums

The inoculating loop was aseptically touched on two to three colonies from the NA plate that had similar colonies. Then it was put into a tube with 2 ml of sterile nutrient broth. Following that, the tube was compared to the turbidity standard (McFarland standard tube 0.5).

Determination of antibacterial activity by agar well diffusion method

Sterile MHA plates of approx. 4mm thickness were prepared. The plates were lawn cultured with standardized microbial culture broth using sterile cotton swab. The inoculated plates were left to dry for few minutes at room temperature with lid closed. Then four wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer (6 mm). Each well was filled with 50µl extracts of different concentrations of plant extracts, negative control (DMSO) and positive control (Ciprofloxacin 5 mcg). It was allowed to diffuse at room temperature for about 30 minutes before being incubated for 18-24 hours. After incubation, plates were examined for the formation of a clear zone around the well. The zone of inhibition (ZOI) was measured in mm (Manandhar et al 2019).

Determination of minimum bactericidal concentration (MBC)

To measure MBC, the crude extract of plants that demonstrated antibacterial activity was subjected to serial dilution. The MBC was determined by micro dilution method using serially diluted plant extracts. The extracts were diluted by two- fold dilution to get series of concentration from 0.39 to 50mg/ml in freshly prepared sterile MHB in 96 wells microtitre plate. To the 100µl diluted plant extract series in the microtitre plate, 20µl of broth containing microorganism suspension was added. On some wells of the same plate, positive growth having broth and microbial suspension and negative growth control containing only broth, were added. The plate was incubated at 37^{o}C for 24 hours. After incubation, wells of plant extracts with different dilutions were compared with positive and negative growth control wells. The results were interpreted based on the fact that growth occurs only in the positive control and in any well where the extract concentration is insufficient to limit growth. The minimum inhibitory concentration (MIC) is the lowest concentration of the extract that inhibits the growth of organisms as detected by a lack of visible turbidity. However, it was impossible to determine whether the turbidity was caused by bacterial growth or by the turbidity of the plant extract itself. So the series of dilutions were further sub-cultured on NA plates and incubated at 37^{o}C for 24 hours. The plates were then examined for the growth of microorganisms and MBC was determined by noting the minimum concentration of extract showing no growth of organisms (European Committee for Antimicrobial Susceptibility Testing 2000).

Data management and analysis

Data collected through log entry and the laboratory analysis were organized in Microsoft excel. The IC_{50} values were calculated using Graph Pad Prism 9 software. The experimental results of total phenolic and flavonoid were presented as mean ± standard error of the mean.

RESULTS

Percentage yield of plant extracts

The extraction yield was found to be methanol (9.44), hexane (0.82), dichloromethane (0.510), ethyl acetate (0.832) and butanol (1.08) of *R. arboreum*, methanol (11.73), butanol (1.30) and ethyl acetate (4.93) of *A. calva* and methanol (10.13) ethyl acetate (1.42), hexane (0.68) and dichloromethane (0.42) of *T. repens*.

Total phenolic content (TPC) and total flavonoid content (TFC) of plant extracts

The total phenolic content of plant extracts ranged from 8.66±0.820 to 128.36±1.65 mg GAE/gm. The results were derived from a calibration curve (y=0.007x + 0.102, R^2=0.989) of gallic acid (0-50µg/mL).

The total flavonoid content of plant extract ranged from 16.25±5.10 to 107.3±0.92 mg quercetin equivalent/gm dry weight. The results were derived from the calibration curve (y=0.002x + 0.026, R^2= 0.993) of quercetin (0-200µg/mL) (Figure 1).

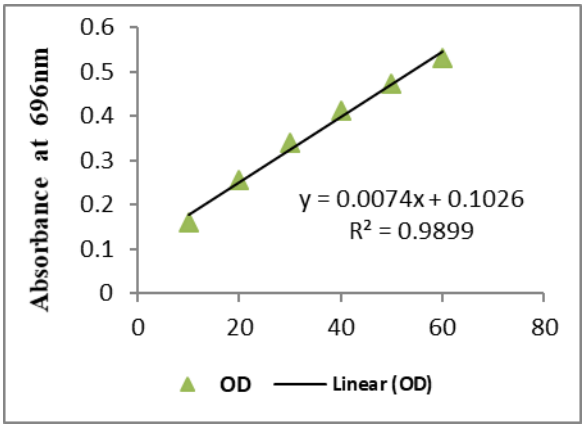

Figure 1: Calibration curve of gallic acid

Antioxidant activity of plant extracts

The IC_{50} value ranged from 34.97 to 902.4µg/mL of ethyl acetate extract of *R. arboreum* to DCM extract of *T. repens*. The IC_{50} value for ascorbic acid (control) was found to be 20.24µg/mL. In case of plant extracts, the lowest IC_{50} values were observed in *R. arboreum* ethyl acetate solvent.

Antibacterial activity of *R. arboreum*, *A. calva* and *T. repens* against gram positive and gram negative bacteria

The ethyl acetate extract of *R. arboreum* exhibited significant antibacterial activity against all four Gram negative bacteria. Ciprofloxacin (5 mcg), the antibiotic standard used, showed 24mm, 21mm, 20mm, 24mm,25mm and 25mm against *E. coli, K. pneumoniae, A. baumanii* and *P. aeruginosa*, *S. aureus* and *E. fecalis,* respectively. All three extract of *A. calva* showed significant zone of inhibition against *A. baumanii.* No extracts of *T. repens* showed zone of inhibition against *E. coli*. Also DCM showed no zone of inhibition against any of bacteria.

All extracts of *R. arboreum* showed significant zone of inhibition against *S. aureus*. The ethyl acetate and butanol extract of *A. calva* showed zone of inhibition against *S. aureus* while ethyl acetate and methanol extract showed against *E. fecalis.*

Minimum bactericidal concentration of *R. arboreum*, *A. calva* and *T. repens* against gram positive and gram negative bacteria

In case of gram negative bacteria, the lowest MBC value for ethyl acetate extract of *R. arboreum* was 1.56 mg/mL against *A. baumanii.* The lowest MBC value for ethyl extract of *A. calva* was 0.78 mg/mL against *A. baumanii*. The lowest MBC value for methanol extract of *T. repens* was 3.125 mg/mL against *A. baumanii*.

The lowest MBC value of *R. arboreum* of methanol extract was 3.125 mg/mL against *S. aureus.* Similarly, the lowest MBC for ethyl acetate extract of *A. calva* was 6.25 mg/mL for *E. fecalis*. The lowest MBC for ethyl acetate extract of *T. repens* was 6.25 mg/mL against *S. aureus* and *E. fecalis* and methanol extract was 6.25 mg/mL against *E. fecalis.*

Table 1: Total phenolic and flavonoid content of extracts of plant samples

Sample	Solvent	TPC (mg GAE/gm)	TFC (mg QE/gm)
R. arboreum	Ethyl acetate	128.36±1.65	107.3±0.92
	Butanol	86.80±5.01	63.86±0.23
	Hexane	77.13±1.52	96.31±0.25
	Methanol	48±0.49	53.31±0.30
	DCM	46.89±1.02	50.03±0.17
A. calva	Ethyl acetate	21.75±0.75	37.83±0.54
	Methanol	13.50±0.94	16.25±5.10
	Butanol	12.07±1.34	21.38±4.60

T. repens	Ethyl acetate	103.77±0.744	67.55±0.23
	DCM	59.58±0.82	56.55±0.33
	Methanol	44.04±1.65	38.86±0.32
	Hexane	8.66±0.82	20.01±0.24

Table 2: IC_{50} values of extracts of plant samples

Sample	**Solvent**	**IC_{50}(µg/mL)**
Ascorbic acid	(Control)	20.24
R. arboreum	Ethyl acetate	34.97
	Butanol	41.97
	DCM	166.8
	Methanol	197.9
	Hexane	506.4
A. calva	Ethyl acetate	69.77
	Methanol	298.3
	Butanol	184.8
T. repens	Ethyl acetate	94.15
	Methanol	106.5
	Hexane	170.7
	DCM	902.4

Table 3: Zone of inhibition of plant extracts of against gram positive and gram negative bacteria

Sample	Extract	Concentration mg/mL	Zone of inhibition (mm)					
			E. coli (25922)	*K. pneumoniae (700603)*	*P. aeruginosa (7653355)*	*A. baumanii* (clinical)	*S. aureus (29213)*	*E. fecalis (51299)*
R. arboreum	Ethyl acetate	100	10	10	10	13	18	13
		50	6	9	0	10	14	10
		25	0	0	0	0	11	0
	Methanol	100	0	0	8	0	12	11
		50	0	0	0	0	10	0
		25	0	0	0	0	6	0
	Butanol	100	0	0	7	10	13	10
		50	0	0	0	0	9	8
		25	0	0	0	0	0	0
	Hexane	100	0	10	0	11	11	0
		50	0	8	0	9	9	0
		25	0	0	0	0	0	0
	DCM	100	0	0	0	0	15	0
		50	0	0	0	0	13	0
		25	0	0	0	0	9	0

A.calva	Ethyl acetate	100	11	0	9	13	11	10
		50	8	0	0	10	8	7
		25	0	0	0	8	0	0
	Methanol	100	0	11	9	12	0	9
		50	0	9	0	11	0	7
		25	0	7	0	0	0	0
	Butanol	100	10	10	0	14	10	0
		50	8	9	0	12	8	0
		25	0	0	0	0	0	0
T. repens	Ethyl acetate	100	0	0	9	11	11	14
		50	0	0	8	10	9	10
		25	0	0	0	9	0	0
	Methanol	100	0	10	8	14	8	11
		50	0	8	0	12	0	9
		25	0	0	0	8	0	0
	DCM	100	0	0	0	0	0	0
		50	0	0	0	0	0	0
		25	0	0	0	0	0	0
	Hexane	100	0	0	0	13	15	12
		50	0	0	0	11	13	11
		25	0	0	0	0	0	0

Table 4: Minimum bactericidal concentration (MBC) values of the plant extracts against gram positive and gram negative bacteria

Sample	**Extract**	**Minimum bactericidal concentration(mg/mL)**					
		E. coli (25922)	*K. pneumoniae* (700603)	*P. aeruginosa* (27853)	*A. baumanii* (Clinical sample)	*S. aureus (29213)*	*E. fecalis (51299)*
R. arboreum	Ethyl acetate	25	6.25	12.5	1.5625	6.25	6.25
	Methanol	0	0	25	0	3.125	6.25
	Butanol	0	0	25	3.125	6.25	6.25
	Hexane	0	12.5	0	6.25	12.5	0
	DCM	0	0	0	0	25	0
A. calva	Ethyl acetate	0	0	25	0.78125	12.5	6.25
	Methanol	25	25	12.5	12.5	25	25
	Butanol	25	25	0	12.5	50	0
T. repens	Ethyl acetate	0	0	12.5	6.25	6.25	6.25
	Methanol	0	25	25	3.125	25	6.25
	DCM	0	0	0	0	25	25
	Hexane	0	25	0	25	0	0

DISCUSSION

In this study, the ethyl acetate extract of *R. arboreum* was found to have high phenolic content, flavonoid content, antioxidant potential among all the three plant extracts.

The highest yield was found in methanol extract of *A. calva* (11.73%) and the lowest yield was found in the plant DCM extract of *T. repens* (0.42%). The extraction process, temperature, extraction duration, phytochemical makeup, and solvent used all have a substantial impact on the extraction's effectiveness (Ngo et al 2017). In this study, we used organic solvent (Ethyl acetate, Methanol, Butanol, Hexane, DCM for *R. arboreum)*, (Ethyl acetate, Methanol and Butanol for *A. calva)*, (Ethyl acetate, Methanol, Hexane and DCM for *T. repens*) for extraction. The findings demonstrated that different solvents had variable extraction yields. This is because differences in the polarity of the solvents used for extraction may result in a significant range in the concentration of bioactive compounds in the extract.

The solvent choice for each plant sample is different for our study. Various studies revealed that the Hexane and DCM extract of *Acmella* species were less effective than that of other solvent (Bahuguba and Dubey 2023; Borate et al 2013; Onoriode et al 2018). Also a study revealed that the butanol extract was less effective than that of other extracts (Feugap et al 2020).

The ethyl acetate extract of *R. arboreum*, *A. calva* and *T. repens* showed the highest phenolic content and the flavonoid content among solvents used. The extraction solvents, their polarity index (PI), and phenolic compound solubility in the extraction solvents appear to influence phenolic compound recovery (Nguyen et al 2022). Higher amounts of phenolic compounds are commonly extracted in polar solvents (Iloki-Assanga et al 2015). However, our study showed that methanol (Polarity Index= 6.6) extracted polyphenolic chemicals less efficiently than ethyl acetate (PI= 4.4). As a result of their varying gene expression, various regions of the same plant may create and store different chemicals or different amounts of the same component, which influences the antioxidant activity and other biological aspects of plant extracts (Kimia Sains dan Aplikasi et al 2020;Rafat et al 2010).

The ethyl acetate extract of *R. arboreum*, *A. calva* and *T. repens* had the highest antioxidant properties with lowest IC_{50} value. The most notable antioxidant phytochemicals, flavonoids and phenols, showed significant levels in the quantitative study, which may have contributed to the antioxidant efficacy of the examined plant extracts (Moriasi et al 2020). The antioxidant potential of these phytochemicals is assumed to be due to their reductive and oxidative capabilities, which facilitate absorption and neutralize the effects of free radicals (Elzaawely and Tawata 2012). Our research strongly suggests phenolics are important components of these plants, which is due to their biological impacts.

A significant difference in antibacterial activity was found when *R. arboreum* was extracted with different solvents (ethyl acetate, methanol, butanol, hexane and DCM). In this study, *R. arboreum* plant extract best showed antibacterial activity followed by *A. calva* and *T. repens*. The extract's antimicrobial properties could be attributed to the most common components, α-pinene, β-pinene, and limonene, which are particularly effective in inhibiting the growth of microbes. In case of solvents used for the extraction of plant parts, ethyl acetate was found to be the most appropriate solvent in terms of the antibacterial activity of the crude extracts followed by methanol, butanol, hexane and DCM. The phytochemicals analysis of tested plants showed the higher amount of TPC and TFC extracted by ethyl acetate, which could be the reason for the higher antibacterial activity of ethyl acetate extract. In general, the antimicrobial properties of plant extracts are influenced by a number of factors, including the environmental and climate circumstances under which the plant grew, the extraction method employed, test concentration, the extraction method used, antimicrobial activity determination method, and test microorganisms, the sensitivity of the bacterial strains used. In our study, we also observed that gram positive was more susceptible to the plant extract than gram negative. The cellular envelopes of both types of bacteria differ greatly, and the outer membrane of Gram-negative cells, with its lipopolysaccharide leaflet, may operate as an impenetrable barrier to extractable bioactive chemicals.

The plant extract having high antioxidant activity showed maximum antibacterial activity against gram positive and gram negative bacteria. Therefore, we expect correlation between antibacterial and antioxidant activities. However, this study did not investigate the active principle responsible for the antioxidant and antibacterial activity.

Conclusion

Different extract of different plants used in the study possess phenolic and flavonoid properties. Ethyl acetate extract of *R. arboreum* has highest phenolic and flavonoid

content and also has highest antioxidant value with minimum IC_{50} value indicating its applicability as the antioxidant agent. The ethyl acetate extract of *R. arboreum, A. calva* and *T. repens* demonstrated the greatest antibacterial efficacy against both gram positive and gram negative bacteria, with the lowest MIC values, showing its possible uses as the antimicrobial agent. The observed bioactivity of these plants could lead to a wide range of practical applications, including the design and production of new pharmaceutical medications

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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