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Phytochemical Profiling and Antioxidant Potential of Three *Rhododendron* Species Collected from Mustang District, Nepal

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ABSTRACT

Geographical and climatic variability significantly influence the chemical composition and biological activities of plant species. This study investigates and compares the extraction yield, phytochemical profiles, and antioxidant potential of three *Rhododendron* species namely *R. arboreum* (pink and red flower varieties), *R. campanulatum*, and *R. anthopogon* collected from Mustang district of Nepal. Among the species studied, *R. campanulatum* exhibited the highest extraction yield, whereas *R. anthopogon* yielded the lowest. Phytochemical screening revealed similar profiles between *R. arboreum* (pink and red flower varieties) and *R. campanulatum*, while *R. anthopogon* demonstrated comparatively lower phytochemical profiles. Antioxidant activity, assessed via the DPPH radical scavenging assay, indicated that *R. arboreum* (red flower) possessed the strongest free radical scavenging activity (IC_{50} = 9.43 μg/mL), followed by *R. campanulatum* (IC_{50} = 12.94 μg/mL), *R. arboreum* (pink flower) (IC_{50} = 46.90 μg/mL), and *R. anthopogon* (IC_{50} = 265.29 μg/mL). Variations in extraction yield, phytochemical composition, and antioxidant capacity compared to previously reported data may be attributed to differences in extraction techniques, solvents, plant parts analyzed, and the distinct environmental conditions of the Mustang region. Notably, the detection of proteins in the floral extracts of *R. arboreum* and *R. campanulatum* highlights their potential dual role as nutraceutical and phytotherapeutic resources.

Keywords: Antioxidant, *Rhododendron anthopogon*, *Rhododendron arboreum*, *Rhododendron campanulatum*

INTRODUCTION

The phytochemical composition and biological activities of plant samples can vary significantly based on geographical and climatic conditions (Khattak, 2015; Sharma and Adhikari, 2023). Various *Rhododendron* species have been utilized for medicinal purposes in the Himalayan region since prehistoric times. Numerous studies have investigated the pharmacological properties of different *Rhododendron* species. However, to our knowledge, no previous research has explored the phytochemical constituents and antioxidant activity of the ethanolic extracts of *Rhododendron arboreum* (pink and red flower varieties) (Figure

1a and 1b), *Rhododendron campanulatum* (Figure 1c), and *Rhododendron anthopogon* (Figure 1d) collected from the Mustang district of Nepal. This study aims to analyze and compare the extraction yield, phytochemical profile, and antioxidant activity of these three *Rhododendron* species from Mustang, Nepal.

The genus *Rhododendron*, which comprises more than 1000 species globally, is the largest in the Ericaceae family. *Rhododendron* exhibit an enormous morphological diversity, varying from 4-40 inch tall to 100 ft tall. In Nepal, *R. arboreum* (Nepali: Laliguras) was declared as "National Flower" in 2019 B.S. by the former King Mahendra Bir Bikram Shah Dev. More than 30 species of Rhododendron are reported from over 40 districts of Nepal. TMJ (Tinjure-Milke-Jaljale) area of Nepal is known as "Capital of Rhododendron" because it is home to 28 species of Rhododendron (GoN, 2013).

Different parts of *Rhododendron* species have been utilized for medicinal, culinary, and other applications in Himalayas region since prehistoric times. *R. arboreum* flower and leaf juice are used to promote healthy digestion. The bark juice is used for cough & diarrhea remedies and flower juice is utilized for the treatment of dysentery, menstrual disorders and in removing fish bones stuck in the throat (Tiwari *et al*., 2020). In addition to their medicinal uses, wood and bark are utilized as reliable source of fuel, essential for cooking food and providing warmth in the harsh, cold climate of the Himalayan region. The flowers are often used to prepare local delicacies such as jams, juices, and chutneys (Kumari *et al*., 2024).

R. campanulatum, known as Chimal in Nepali, is a significant shrub species in the Himalayan region valued for its medicinal uses and role in the alpine ecosystem. The leaf juice of *R. campanulatum* is used for the treatment of joint pain and flower juice is utilized for the remedy of body ache and throat pain. The paste of leaves is used as a remedy for cuts, wounds, eczema, and rashes (Rana and Samant, 2011; Popescu and Kopp, 2013).

a) *R. arboreum* (Pink flower)

b) *R. arboreum* (Red flower)

c) *R. campanulatum*

d) *R. anthopogon*

Figure 1: Rhododendron species used in the study

R. anthopogon, known locally as "Sunpati" and "Polchiya" in Nepal, is a small, aromatic *Rhododendron* species found in the high-altitude regions of the Himalayas. Herbal tea made from the leaves and twigs of *R. anthopogon* is traditionally consumed to clear nasal and chest congestion, alleviate cough, boost the immunity, relief the cold symptoms and lower the blood pressure (Hussain *et al*., 2023; Popescu and Kopp, 2013). The leaves and twigs are often burned as incense in religious ceremonies and rituals. They are considered to purify the

environment and bring good fortune (Chhetri *et al*., 2020a).

MATERIALS AND METHODS

Collection site and Identification of Plants

Different species of *Rhododendron* (Table 1) were collected from Lete village of *Mustang* District of Gandaki Province, Nepal during May. Mustang district is one of the eleven districts of Gandaki province of Nepal covering an area of 3639 sq. Km and lies at latitude 28°57′35.28″ North, longitude 83°49′07.68″ East. Spectacularly beautiful Himalayan district is rich in trans-Himalayan biodiversity and characterized by semi-desert terrain, frigid climate, and high altitudes (1500-8000 m). About 121 plant species has been traditionally use as medicinal plant for the treatment of 116 ailments in Mustang district (Bhattarai *et al*., 2010).

Identification of plants was done with the help of National Herbarium and Plant Laboratories (KATH). The herbariums of plant samples were prepared, registered, and stored at Pharmacognosy Laboratory of Novel Academy, Purbanchal University, Pokhara, Nepal.

Table 1: List of plant used in the study

S. N.	Plant species	Local name	Parts collected	Herbarium registration number
1	*R. arboreum* Sm.	Laliguras	Flower (Pink)	NAH-2023-01
2	*R. arboreum* Sm.	Laliguras	Flower (Red)	NAH-2023-02
3	*R. campanulatum* D. Don	Nilo Chimal	Flower (White)	NAH-2023-03
4	*R. anthopogon* D. Don	Pol chiya	Flower	NAH-2023-04

Preparation of Crude Drug Sample

The collected plant samples were dedusted, washed thoroughly with clean water, and cut into small pieces. Then the plant samples were shade dried and stored in air-tight zipped locked bags and subsequently used for extraction.

Extraction

Extraction was carried out using double maceration method. The dried samples were macerated with ethanol at a 1:11 (w/v) ratio for 24 hours at room temperature with frequent agitation. After filtration, filtrate was collected. The remaining residue was macerated under same conditions for another 24 hours, followed by filtration. The filtrates from both maceration steps were combined and concentrated using a rotary evaporator at 40°C and 90 rpm under reduced pressure. The resulting extracts were then transferred into closed vials and stored at 4°C until further experiment was performed.

Extract Yield Percentage

The extract yield percentage of the plant sample is determined using the following formula:

$$\text{Extract Yield Percentage} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant sample}} \times 100\%$$

Phytochemical Profiling

Freshly prepared plant extracts were subjected to phytochemical screening to detect the presence of plant metabolites such as alkaloids, flavonoids, phenols, carbohydrates, glycosides, steroids, saponins, tannins, terpenoids, and proteins by following standard methods (Chhetri and Khatri, 2017; Yadav *et al*., 2014).

Assessment of Antioxidant Potential

DPPH radical scavenging assay was performed to assess the antioxidant potential of the plant extract (Chhetri *et al.,* 2020b). Two milliliters (2 mL) of each extract at varying concentrations (250 μg/mL, 125 μg/mL, 62.5 μg/mL, 31.25 μg/mL, and 15.625 μg/mL) was mixed with 2 mL of

60 μM DPPH solution. The reaction mixture was incubated for 30 minutes at room temperature in dark condition, and the absorbance was measured at 517 nm using an UV-Visible spectrophotometer. Upon reduction, the color of the solution faded from violet to pale yellow. The DPPH radical scavenging activity (%) was calculated as follows:

$$\%\ \text{DPPH Radical Scavenging Actvity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Control was the test solution without sample. Ascorbic acid was used as the standard. The antioxidant activity was expressed as IC_{50}, which is the concentration required to scavenge 50% of the DPPH radicals. The IC_{50} value was calculated by plotting the percentage of DPPH radical scavenging activity against the various concentration of the extract.

Data Analysis

Data are presented as mean ±standard deviation (SD) from three replicate determination. Statistical analysis was conducted using Statistical Package for the Social Science (SPSS) version 22. The significant differences between mean of various groups were compared using one way ANOVA followed by Tukey's post hoc test. A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Extraction and Extract Yield Percentage

The extraction of various *Rhododendron* species was conducted using ethanol as the solvent. Ethanol is particularly effective in extracting polar phytoconstituents, such as flavonoids, phenolic acids, and glycosides from plant materials due to its ability to dissolve a wide range of polar compounds. Ethanol's polarity arises from its hydroxyl group, which can form hydrogen bonds with other polar molecules (Altemimi *et al.*, 2017). In the current study (Table 2), it was observed that the extract yield was found highest in *R. campanulatum* and lowest in *R. anthopogon*. This finding suggests that *R. campanulatum* contains a greater amount of polar phytoconstituents than the other species examined. This result is further supported by preliminary phytochemical screening, which also indicated a higher abundance of phytoconstituents in *R. campanulatum*.

Table 2: Extract yield percentage of plant extracts

Plants names	Weight of dry extract	Yield %
R. arboreum (pink flower)	7.87 g	31.48
R. arboreum (red flower)	6.94 g	27.76
R. campanulatum	9.69 g	38.76
R. anthopogon	3.34 g	13.35

Phytochemical Profiling

The intensity of the color produced in response to specific reagents serves as an indicator of the presence and abundance of phytochemicals, facilitating a comparative analysis among species like *R. arboreum, R. campanulatum*, and *R. anthopogon*. Among the three examined species, *R. campanulatum* and *R. arboreum* (pink and red flower varieties) produced similar intensity of color which implies that these flower varieties possess comparable phytochemicals, while *R. anthopogon* exhibited the least intense color, suggesting a lowest abundance of phytochemicals (Table 3).

Table 3: Phytochemical profiling of plant extracts

S.N.	Phytochemicals	Tests	*R. arboreum* (Pink flower)	*R. arboreum* (Red flower)	*R. **campanulatum***	*R. **anthopogon***
1	Alkaloid	Mayer's test	-	-	-	-
		Wagner's test	-	-	-	-
		Hager's test	-	-	-	-
2	Carbohydrates	Molisch's test	+++	+++	+++	+++
		Benedict's test	++	++	++	++
		Fehling's test	++	++	+++	++
3	Flavonoids	Shinoda test	++	+++	+++	+
		Alkaline test	++	+++	+++	+++
4	Glycosides	Keller-kiliani test	+++	+++	+++	+++
5	Steroids		+++	+++	+++	+
6	Saponin	Froth test	+++	++	+	-
7	Tannin	Ferric chloride test	+++	+++	+++	+++
8	Phenols	Ferric chloride test	+++	++	+++	++
9	Terpenoids	Salkowski's test	+++	+++	+++	+++
10	Protein	Xanthoproteic test	++	++	+++	-

Note: (−) Absence, (+) Poor, (++) Moderate, (+++) Abundant

The ethanolic extract of *R. arboreum* (pink and red flowers varieties), *R. campanulatum,* and *R. anthopogon* contain phytochemicals like carbohydrates, flavonoids, glycosides, tannins, terpenoids, phenols, and steroids. Protein and saponins were found in *R. arboreum* (pink and red flowers varieties) and *R. campanulatum* but absent in *R. anthopogon*. Saponin was found abundantly in *R. arboreum* (pink flower), moderately in *R. arboreum* (red flower), poorly in *R. campanulatum* and absent in *R. anthopogon*. In the Shinoda test for flavonoids, *R. arboreum* (pink flower), *R. campanulatum*, and *R. anthopogon* exhibited a magenta color upon reaction, which indicates the presence of flavonols, whereas *R. arboreum* (red flower) showed an orange color appearance, signifying the presence of flavones. This differentiation in color change highlights the presence of diverse flavonoid compound in different species and even within different variety of the same species.

Protein was found in abundant quantities in *R. campanulatum* in comparison to *R. arboreum* (pink and red flowers) and absent in *R. anthopogon*. In Mustang district of Nepal, the local population utilizes *Rhododendron* flowers to create a traditional beverage and jams. Presence of protein content and bioactive compounds establishes the *Rhododendron* as not only a source of nutrition but also a functional food that may offer therapeutic benefits. Animal protein has been associated with increased in plasma insulin-like growth factor 1 (IGF-1) level, which possesses the risk of several cancers (Holmes *et al*., 2002), whereas plant protein has been linked to lower blood pressure (He *et al*., 2005; Elliot *et al*., 2006), reduced low-density lipoprotein levels, and improved insulin sensitivity (Appel *et al*., 2005). Recently the demand of plant proteins is increasing due to high cost, limited supply of animal protein, and

cardiovascular related disease associated with consumption of animal protein. In the nearby future, animal protein may be replaced by plant protein, which is beneficial for healthy human lifestyle. Both *R. campanulatum* and *R. arboreum* contains significant amounts of protein, making them suitable alternatives or supplements to traditional protein sources.

All the studied *Rhododendron* species were found devoid of alkaloids. Several studies have also reported the similar findings (Kiruba *et al*., 2011; Paudel *et al*., 2011; Sonar *et al*., 2012; Winitcha *et al*., 2021; Venkatesan *et al*., 2024). However, there are several other studies which results varied with our present study. Sharma and Lamichhane, (2019) have reported the presence of alkaloid in methanolic extract of *R. arboreum* leaves collected from Tansen, Nepal. Kiruba *et al*. (2011) had previously reported the presence of phenols, proteins, steroids, tannins and carbohydrates and absence of alkaloids, flavonoids & saponins in the ethanolic extract of *R. arboreum* flowers collected from Palni Hills of Western Ghats, India. Similarly, Nisar *et al*. (2011) had revealed the presence the steroids, terpenoids, flavonoids & tannins and absence of alkaloids, saponins, glycosides & reducing sugars in the methanolic extract of *R. arboreum* flowers collected from Seran valley, Pakistan. The variation in phytoconstituents can be attributed to differences in the extraction solvent used, as well as the geographical location and climatic conditions where the plant samples were collected. Previous studies have also reported variations in phytoconstituents in different parts of the same plant, with different extraction solvents, and in the same plant species collected from different geographical locations (Ghasemzadeh *et al*., 2018; Giupponi *et al*., 2020; Gulzar *et al*., 2017).

The flower color is attributed to the presence of flavonoids and carotenoids, which have significantly free radical scavenging capacity. The type and concentration of anthocyanin are the key parameters that determine the colors of *Rhododendron* flower. Du *et al*. (2018) have reported the presences of higher total anthocyanin content in red colored flowers of *Rhododendron* species than purplish-pink color flowers. This study correlate with present findings where, flavonoid content was found higher in red flower than pink flower of *Rhododendron* species.

Antioxidant Activity

The antioxidant activity of the different *Rhododendron* species was assessed using the DPPH radical scavenging method. In the present study, all *Rhododendron* species and the standard showed a concentration-dependent increment in radical scavenging capacity, highlighting their potential as natural antioxidants (Table 4). Among the studied *Rhododendron* samples, *R. arboreum* (red flower) exhibited the highest antioxidant activity with an IC_{50} value 9.43 μg/mL, followed by *R. campanulatum* (IC_{50} value =12.94 μg/mL), *R. arboreum* (pink flower) (IC_{50} value = 46.9 μg/mL), and *R. anthopogon* (IC_{50} value = 265.29 μg/mL) (Figure 2). Although none of the *Rhododendron* species outperformed antioxidant potential of ascorbic acid (IC_{50} value = 3.24 μg/mL), *R. arboreum* (red flower) and *R. campanulatum* exhibited relatively strong radical scavenging potential, suggesting that they could serve as promising sources of natural antioxidants for pharmaceutical and nutraceutical applications.

Table 4: Percentage DPPH radical scavenging activity of different plant samples with reference to ascorbic acid

Plant Sample/ Standard→ Concentration↓	Percentage DPPH Radical Scavenging Activity				
	R. arboreum (Pink flower)	*R. arboreum* (Red flower)	*R. campanulatum*	*R. anthopogon*	Ascorbic acid
15.625 μg/mL	39.87 ± 3.29	57.22 ± 0.85	55.17 ± 4.8	10.61±0.93	75.3 ± 0.93
31.25 μg/mL	47.89 ± 1.37	67.69 ± 1.19	67.46 ± 2.04	14.44±1.11	83.45 ± 2.1
62.5 μg/mL	53.57± 0.9	76.67 ± 1.04	72.81 ± 3.25	16.41±1.66	93.33 ± 0.74
125 μg/mL	60.75 ± 1.37	79.29 ± 1.19	77.7 ± 1.86	27.27±1.18	97.4 ± 0.73
250 μg/mL	69.96 ± 2.14	83.39 ± 1.19	80.65 ± 1.65	48.02±2.6	98.38 ± 0.21

Note: Data are expressed as mean ± standard deviation (n=3)

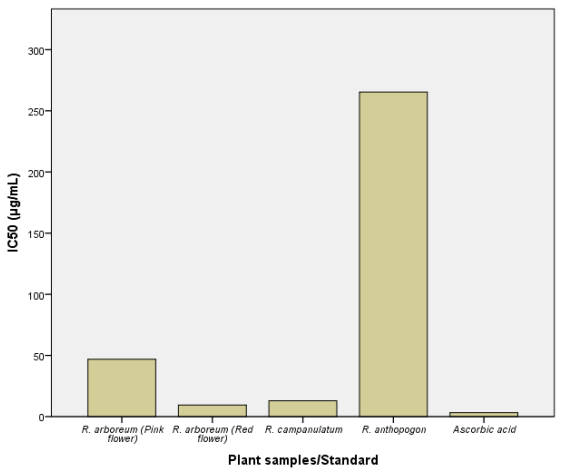

Figure 2: IC_{50} values of DPPH radical scavenging activity of plant samples and standard ascorbic acid

One-way ANOVA followed by Tukey's post hoc test was performed to evaluate differences in antioxidant activity among the studied *Rhododendron* species and ascorbic acid across various concentrations (15.625–250 μg/mL). The analysis revealed statistically significant differences (p<0.05) in antioxidant activity between each *Rhododendron* extract and ascorbic acid, as well as among most of the *Rhododendron* species at all tested concentrations. However, no significant difference (p>0.05) was observed between *R. arboreum* (red flower) and *R. campanulatum* at concentrations of 15.625, 31.25, 125, and 250 μg/mL, indicating comparable antioxidant potential between these two species. In a previous study, it was reported that ethanol extract of *R. arboreum* leaves collected from Himanchal Pradesh, India showed DPPH radical scavenging activity with an IC_{50} value 70.58 μg/mL (Madhvi *et al*., 2020). Bhandari and Rajbhandari (2014) have reported that bark, stem, twigs, petals, flowers, and leaves of methanol extract of *R. arboreum* collected from Shivapuri National Park, Nepal exhibited antioxidant activity with an IC_{50} value 65.45 μg/mL, 67.83 μg/mL, 46.02 μg/mL, 16.83 μg/mL, 25.15 μg/mL, and 8.34 μg/mL, respectively. Sharma *et al*. (2021) have revealed that hexane, methanolic, and water extract of *R. arboreum* flower collected from Kullu, Himanchal Pradesh, India, possesses antioxidant activity with an IC_{50} value of 184.01 μg/mL, 121.55 μg/mL, and 141.98 μg/mL, respectively. Kalauni *et al*. (2021) have shown that methanol extract of *R. arboreum* flowers exhibited the antioxidant activity with an IC_{50} value 45.55 μg/mL. The difference in the results of our findings with the previous studies might be due to the variations in the choice of extraction solvent, the specific plant part utilized, and geographical location of the plant samples collected. These

variations impact the phytochemical profile of the plant, thereby contributing to differences in antioxidant activity (Chatepa *et al*., 2024; Adhikari *et al*., 2020).

CONCLUSION

Among the *Rhododendron* species investigated, *R. campanulatum* demonstrated the highest extract yield, whereas *R. anthopogon* yielded the least. The phytochemical profiles of *R. arboreum* (pink and red flower varieties) and *R. campanulatum* were comparable and richer than that of *R. anthopogon*. In terms of antioxidant assay, *R. arboreum* (red flower) demonstrated the highest percentage of DPPH free radical scavenging activity, followed by *R. campanulatum*, *R. arboreum* (pink flower), and *R. anthopogon*. Furthermore, qualitative phytochemical analysis confirmed the presence of proteins in *R. arboreum* and *R. campanulatum*, suggesting potential nutritional benefits. These findings underscore the pharmaceutical and nutraceutical significance of these species, highlighting their potential as valuable sources of bioactive compounds for therapeutic applications.

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