Analysis of phytochemical constituent and biological significance of rhododendrons collected from capital of laliguras, laliguras municipality, Tehrathum district, Nepal

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Abstract: The largest genus of the Ericaceae family, Rhododendron includes an inspiring number of species. Some of which have been used in the treatment of various ailments such as diseases associated with heart, dysentery, diarrhea, detoxification, fever, constipation, bronchitis and asthma around the world including Nepal. However, systematic study on phytochemistry and pharmacology of Rhododendrons grown in the Capital of Laliguras, Laliguras Municipality, Tehrathum of Nepal is lacking. So this study was conducted to evaluate the antioxidant activity, α-amylase inhibition activity, total phenolic and flavonoid contents of *Rhododendron arboretum* and *Rhododendron campanulatum* from Laliguras Municipality, Tehrathum of Nepal. Plant extracts were prepared in methanol by cold percolation method. Phytochemical constituents were analyzed by following standard methods. Antioxidant activity was measured through 2, 2-Diphenyl-1-Picrylhydrazyl assay. Total phenolic and flavonoid content were estimated using Folin–Ciocalteau and aluminum chloride method respectively. Alpha amylase inhibition assay was carried out by starch iodine method. Phytochemical screening of methanolic extract of flower and leaf of *R. arboretum* and leaves of *R. campanulatum* displayed the presence of different chemical constituents such as flavonoids, polyphenols, terpenoids, saponins and quinins. Samples showed dose-dependent radical scavenging and α-amylase inhibition activity. Radical scavenging activity and α-amylase inhibition activity of the methanolic extracts of different parts of *R. arboretum* and *R. campanulatum* ranged from 19.46 to 95.88% and 13.23 to 68.44% respectively. Flower extracts of *R. arboreum* showing the strongest radical scavenging activity and α-amylase inhibition with IC_{50} values 33.61 and 443.44 µg/mL respectively. Among the tested samples, flower extract of *R. arboretum* showed the strongest antioxidant activity, alpha amylase inhibition activity and contained the highest amount of total phenolic and flavonoid content.

Keywords: Rhododendron arboretum; R. campanulatum; Pntioxidant; α-amylase; Phenolic; Flavonoid; Inhibition.

Introduction

Biological oxidation of oxygen is continuous process within living organism as it creates energy to carry on the functioning of cells. This results the formation of various reactive oxygen species (ROS) in regular basis. These highly reactive molecules such as, hydroxyl radical (˙OH), peroxide (ROO˙) and superoxide radicals ($O_2^{\cdot-}$), can have potential to damage the cells and its components like deoxyribonucleic acid, proteins, lipids, and carbohydrates, under oxidative stress conditions[1]. This either directly leads to various chronic and degenerative diseases including atherosclerosis, diabetes, neurological disorder, cancer, inflammatory diseases, and early aging[2,3]. Antioxidants are significant regarding reducing oxidative stress, by scavenging free radicals[4]. Researchers have proved that the oxidative stress and its consequences can be decreased by consuming foods rich in phytochemicals contents that have antioxidant properties such as polyphenols, flavonoids etc[5]. The synthetic antioxidants such as butylated hydroxyl anisole (BHA), propyl gallate

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(PG), butylated hydroxyl toluene are associated with chronic toxicity and side effects on health. Therefore, the interest of researchers is growing in identifying and isolating the plant derived phytochemicals which have high antioxidant, and antidiabetics activities and low toxicity to mammals and possess medicinal properties to maintain human health. Especially the angiosperms plants are rich in polyphenols contents. Polyphenols are reported as a free-radical scavengers and powerful antioxidants, considered as non-toxic and have no side-effect[6]. *Rhododendron* (Laliguras), an angiosperm, belongs to family Ericaceae. It is native to Bhutan, China, India and Nepal. Worldwide, around 1200 species and in Nepal, 30 species of Rhododendron have been estimated, among which 26 species have been reported from Tinjure- Milke- Jaljale (TMJ), Tehrathum, Nepal[7,8]. Due to this reason, Tinjure- Milke- Jaljale is known as the capital of rhododendron in Nepal. *R. arboreum* flowers are consumed as wine against altitude sickness, as jellies, local brew and jams in hilly areas of Tehrathum in Nepal (TMJ) and Himachal Pradesh in India [9]. The fresh flowers of *R. arboreum* is reported to effective in treatment of dysentery and diarrhea whereas dried flowers are taken to cure blood dysentery[10]. Latter, the hidden chemistry of these reported traditional medicinal uses of Rhododendron have been proved by numerous authors [11,12,13] and also reported a wide range of its pharmacological activities[14,15]. *Rhododendron campanulatum* is a very important member of the genus Rhododendron and found at altitudes between 2500-4300 m. Flowers of *R. campanulatum* are reported to use for the treatment of different types of pain in Nepal[16,17,18.] Literature survey revealed that scientific studies on phytochemical constituents and therapeutic potential of *R. campanulatum* from different places had been carriedout[19,20,21,22]. Although several ethnobotanical studies were conducted in the country, many areas remain unexplored [23]. In this context, the Rhododendron is the national flower of Nepal. To our best knowledge, there have been no previous studies on biological activity of Rhododendron grown in Laliguras Municipality. The phytochemical constituents of plant are greatly influenced by genetic, geographical, and biotic and abiotic factors and altitude of particular place[24]. Therefore, the proposed study was designed to the screening of phytochemicals and antioxidant, α-amaylase inhibition properties of *R. arboretum* flowers and leaves, and leaves of *R. campanulatum* collected from Laliguras Municipality in scientific ways as this area is located in hilly region.

Materials and methods

Enzyme, chemicals, and reagent

The enzyme porcine pancreatic α-amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). The solvent used in this study was methanol (Merck, Germany). All other chemicals used in this study were of the commercially available analytical grade.

Plant collection and preparation of their extract

Rhododendron flowers, leaves were collected through questionnaire, interviews and discussions among the traditional practitioners from Tehrathum, Milke danda which is famous as the Capital of Laliguras (Laliguras Municipality). At the same time, the prepared herbarium of these Rhododendron plants were authenticated by Botanist, Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal. All the collected plants were washed, shade dried and powdered and stored in cool and dry place until used for further experiment. The methanol extract was prepared for each powdered sample by cold percolation method. Filtrate methanol extracts of each plant were concentrated using a rotary evaporator. The concentrated product was then dried in oven 35 °C until the solvent is completely evaporated. Finally, it was stored at 4 °C in the fridge in airtight container until future use.

Qualitative phytochemical analysis

The method used for phytochemical screening was based on the standard protocol with some modification [25]. The different phytochemicals present in the different plant extract were identified by the color reaction with different reagents.

Free radical scavenging ability assay

DPPH assay was carryout as adopted methods in our laboratory [26,27]. In short, different concentration of test sample and ascorbic acid range from 10- 100 µg/mL were made from stock solution. 2 mL of all concentration of test samples were mixed with 2 mL of DPPH solution and shaken vigorously for uniform mixing and then were kept in dark place for 30 min. The control was

prepared as above but without plant extract or ascorbic acid. Methanol was used as blank and absorbance was measured on spectrophotometer at 517 nm.
The radical scavenging activity was calculated by using the following formula:

Standard graph was plotted by taking the concentration on the x- axis and percentage free radical scavenging on the y-axis. Based on this graph, IC_{50} value of each sample was calculated and compared.

Total Phenolic content

Total phenolic content of different plant extract was estimated by Folin- Ciocalteus reagent involving gallic acid as standard based on oxidation reduction reaction[28,29]. Different concentration of plant extract or standard (Gallic acid) was prepared in methanol. Then 1 mL sample solution from different concentration of plant extract or standard (Gallic acid) was poured into test tubes. Then 5 mL of 10% Folin- Ciocalteus reagent and 7% Na_2CO_3 were added and mixture was shaken well and incubated for 30 min at 40. After incubation absorbance was measured at 750 nm in spectrophotometer. Intense blue colored was developed in active tested extract. The standard calibration curve of gallic acid was plotted using the concentration ranging from 10-120 µg/mL. Based on this standard graph, the concentration of the individual samples was calculated. The total polyphenol content was expressed in terms of the milligrams of the gallic acid equivalent per gram of the dry mass (mg GAE g-1).

Total flavonoids content estimation

The TFC of the selected plant extracts were determined according to aluminum chloride colorimetric method involving quercetin as standard[30]. Different concentration of plant extract or standard (quercetin) was prepared in methanol from the stock solution 10 mg/mL. From the different concentration solution, 1 mL sample solution was added in test tube containing 4 mL distilled water and at zero time, 0.3 mL 5% $NaNO_2$ was added. Then, after 5 min 0.3 ml of 10% $AlCl_3$ and after 6 min, 2 mL of 1M NaOH were added. Immediately total volume was made up to 10 mL by adding 2.4 mL distilled water and mixed thoroughly. Finally, absorbance of pink colored mixture was measured at 510 nm using the UV – visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The blank solution contained all the reagents except plant extract or Quercetin. The calibration curve was plotted using standard Quercetin. The total flavonoid content was expressed in terms of the milligram of quercetin equivalent per gram of the dry mass (mg QE/g) [15].

Alpha-amylase inhibition assay

The anti-diabetic activities of each plant extract was calculated by using α-amylase inhibition assay using standard protocol with slight modification[31]. The concentration of undigested starch due to enzyme inhibition was determined through the blue starch iodine complex measured in spectrophotometer at 630 nm. Potato-starch was prepared by dissolving 200 mg of starch in 25 mL of NaOH (0.4 M) by heating at 100 ºC for 5 minutes. After cooling, pH was adjusted to 7.0 and the final volume made up to 100 mL using distilled water. Alpha amylase solution (50 µg/mL) was prepared in 100 mL of 20 mM phosphate buffer of pH 6.9 containing 6.7 mM sodium chloride by dissolving 5 mg of 3 U/mL α–amylase sigma. Acarbose was used as a standard inhibitor for alpha-amylase enzyme. The extracts and acarbose were dissolved in DMSO to give suitable concentrations for assay. To 200 µL of starch solution, 200 µL of acarbose or plant extract at varying concentrations (40, 80, 160, 320, 640, and 1000 µg/mL), was added and pre-incubated at 37 °C for 5 min. After this 200 µL of α–amylase solution was added to each of them and then again incubated further for 15 minutes at 37 °C. Then the reactions were terminated by adding 800 µL of HCl (0.1 M). Finally, 1000 µL of iodine reagent (2.5 mM) was added, and absorbance was measured at 630 nm.

Percentage of inhibition was calculated using the formula:% Inhibition=(1-[Abs2-Abs1/Abs4-Abs3]) ×10

Where, Abs1 is the absorbance of the reaction mixture containing plant sample, α-amylase, and starch, Abs2 is the absorbance of the reaction mixture of starch, and sample Abs3 is the absorbance of the reaction mixture of α-amylase and starch Abs4 is the absorbance of reaction mixture containing starch only.

Statistical Analysis

All the analysis was carried out in triplicate and the results are expressed as mean +SD.

Results and discussion

Table 1: Names of the plants, family, parts used and their uses.

Sample code	Scientific Name	Common Name	Family	Study part	Medicinal value
SC1	*R. arboreum*	Laligurans	Ericaceae	Leaves	Rheumatics, alleviate headache and fever, diuretics, fungal infection
SC2	*R. arboreum*	Laligurans	Ericaceae	Flower	To dissolve fish bone, diarrhea and blood dysentery
SC3	*R. campanulatum*	Chimal	Ericaceae	Leaves	Skin diseases, throat pain and body ache, antioxidant

Phytochemical screening: The results obtained from phytochemical screening for each plant is tabulated as follows table no. 2.

Table 2: The results from the phytochemical analysis of studied plants (methanol extract).

S.N.	Phytochemical	*R. arboretum (Leaves)*	*R. arboretum (Flower)*	*R.campanulatum (Leaves)*
1.	Alkaloids	-	-	-
2.	Flavonoids	+	+	+
3.	Glycosides	-	+	+
4.	Polyphenols	+	+	+
5.	Terpenoids	+	+	+
6.	Steroids	+	+	-
7.	Carbohydrates	-	+	+
8.	Saponins	+	+	+
9.	Tannins	+	-	+
10.	Quinones	+	+	+

Where, + = Present; - = Absent

Preliminary phytochemical analysis of *R. arboretum* (leaves and flower) and *R. campanulatum* leaves showed positive results for the almost tested phytochemicals like flavonoids, polyphenols, terpenoids, saponins, quinones etc and its presence indicates that these plants possesses high profile medicinal value [32, 33]. Tannins were detected in the leaf extracts but not in the flower extract of *R. arboretum.* Steroids were detected in *R. arboretum* extract but not in *R. campanulatum* leaves. Alkaloids were absent in extract of both tested species of genus rhododendron but it is reported in the leaves sample of *R. arboretum* collected from Arghakhanchi District [34]. The result is supported by the previously report results in literature [35,36,37].

Antioxidant activity

The antioxidant ability and value of % free radical scavenging activity of plant is related with their medicinal potential values. Several techniques have been used to determine the antioxidant activity in vitro. In this study, the antioxidant activity of plant extracts was measured using DPPH assay. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant (plant extract) that decolorizes the DPPH solution.

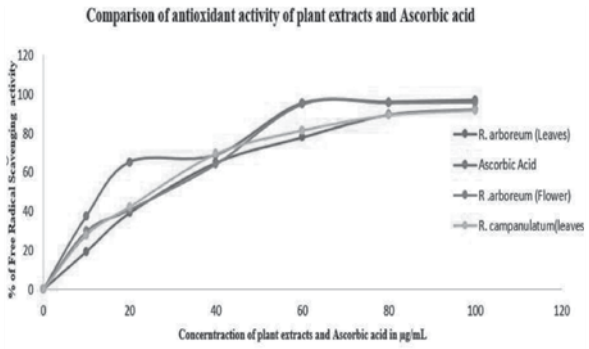

Figure 1: Comparison of percentage radical scavenging between ascorbic acid and Plant extracts.

The methanol extracts of *R. arboretum* (flower), *R. arboretum* (leaves), and *R. campanulatum* (leaves) revealed significant in vitro DPPH radical scavenging activities in a dose-dependent manner (Figure 1). The extracts concentrations of the studied plant required to scavenge 50% of the DPPH radicals (IC_{50}) were also determined. In this study IC_{50} values were found 33.61 µg/mL, 39.10 µg /mL and 35.75 µg /mL for *R. arboretum* (flower), *R. arboretum* (leaves), and *R. campanulatum* (leaves) respectively. On the other hand, the IC_{50} value of the standard (L-ascorbic acid) was 23.06 µg /mL. The standard (L-ascorbic acid) exhibited significantly higher DPPH radical scavenging activities than the DPPH radical scavenging activities of all the studied methanolic plant extracts (Table 3). At all the studied concentrations, the methanolic extract of *R. arboretum* (flower produced significantly higher DPPH radical scavenging activities than those recorded for the methanolic extracts of *R. arboretum* (leaves), and *R. campanulatum* (leaves) (Table 3).

Table 3: Comparison of IC_{50} values of different plant extract with Standard ascorbic acid.

S.N	Samples	Scientific Name	IC_{50}
1.	Ascorbic Acid		23.06
2.	SC1	*R. arboreum* (leaves)	39.10
3.	SC2	*R. arboretum* (flower)	33.61
4.	SC3	*R. campanulatum* (leaves)	35.75

It demonstrates that the phytochemical present in plants are responsible for antioxidant activity. It is reported that *R. arboreum* flowers extract is rich in content of antioxidant compounds like quercetin, rutin, coumaric acid, and other flavonoids[38]. Previous study showed that *R. arboreum* ethanolic flower extract possess antioxidant activity against hydroxyl radical, superoxide radical and lipid peroxidation[37, 39]. Flavonoids isolated from the leaves of *R. arboreum* was credited for potent antioxidant property[40]. The results obtained here are consistent with the findings of Subba et al and Gautum et al, where *R. arboretum* flower and leaves extract has shown significant free radical scavenging activity[15,34]. The fatty acid like palmitic acid and linoleic acid are reported from the extracts of the *R. arboreum* leaves and flowers by the GC-MS technique. Palmitic acid is reported as active free scavenger of free radicals[41]. Similarly, *R. campanulatum* flowers and leaves are reported to have diverse range of phenolics such as gallic acid, quercetin, and campanulin, along with pharmacological properties [42]. In a study, methanolic leaf extract of *R. campanulatum* has revealed good DPPH scavenging activity, which is comparable with the reported free radical scavenging activity of leaf extract of *R. campanulatum* [43].

Total Phenolic Content

The absorbance curve for standard Gallic acid is shown in figure below.

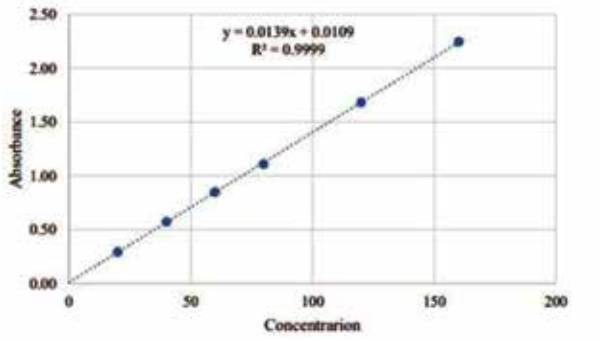

Figure 2: Variation of absorbance with concentration for standard Gallic acid.

The TPC in the different plant extract was calculated by using regression equation y=0.0139x+ 0.0109, R2 = 0.9999, obtained from above calibration curve of Gallic acid followed by formula cV/m (Figure 2). The TPC of studies plants were found are presented in table no. 4.

Table 4: Total Phenolic and Total Flavonoids Content of Different Plant Extracts.

S.N	Name of Plants	TPC (mg GAE/g)	TFC (mg QE/g)
1	*Rhododendron arboreum* (leaves)	166.85±3.7	64.4±2.08
2	*Rhododendron arboreum* (flowers)	178.20±1.8	52.4±5.45
3	*Rhododendron campanulatum* (leaves)	142.32±5.9	46.0±5.94

Phenolic compounds act as antioxidant due to their free radical scavenging properties. In this study, methanolic extract of the *R. arboreum* flower showed more TPC value 178.20 mg GAE/g among *R. arboreum* (leaves), and *R campanulatum* (leaves) with TPC values 166.85 mg GAE/g, and 142.32 mg GAE/g respectively. The previous study reveals that the total phenolic content was

found in the 70% acetone extract of *R. arboreun* flower (600 mg GAE/g) and 188 mg GAE/g in methanol extract[44].

Total Flavonoid Content

The total flavonoid content in the different plant test sample was calculated from the calibration curve and expressed as mg (quercetin equivalent)/g of dried plant material (Figure 3).

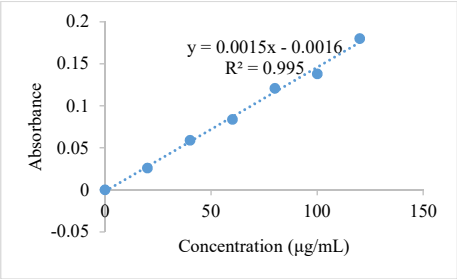

Figure 3: Variation of absorbance with concentration for standard Quercetin.

The TFC in different plant extract was calculated by using regression equation Y= 0.0015x - 0.0016, R^2 of the curve obtained from the calibration curve, followed by formula cV/m and expressed as mg QE per g of extract in dry weight. The TPC of different plant extract (mg quercetin equivalent per g dry extract) are tabulated below in table no. 4.

The obtained result reveal that TFC of methanolic extract of *R. arboreum* (leaves), *R. arboreum* (flower), and *R. campanulatum* (leaves) were found 64.4, 52.4, and 46.0 mg per gram quercetin equivalent (mg QE/g) respectively. This revealed that the TPC was highest in *R. arboreum* (leaves) among the all extracts.

Alpha amylase inhibition activity

The anti-diabetic activity of methanolic extract of different plants were determined by α-amylase inhibition assay using starch iodine method. Percentage inhibition of acarbose and different plant extract was calculated by using standard formula and tabulated in table 6. The relative comparison of % α-amylase inhibition by different plant extracts and acarbose at different concentration is represented in figure 4. Percentage inhibition was found dose dependent. Also the IC_{50} values of each extracts were calculated (table 7).

Table 6: α- amylase % inhibition by different concentration of plant extract.

Sample	S-1	S-2	S-3	Acarbose
Concentration (µg/mL)				
1000	68.44	74.0	59.68	95.59
640	53.01	60.0	47.85	94.35
320	33.98	54.6	35.97	88.66
160	24.89	48.5	24.68	74.68
80	18.01	41.0	19.73	62.1
40	13.23	25.4	11.94	45.43

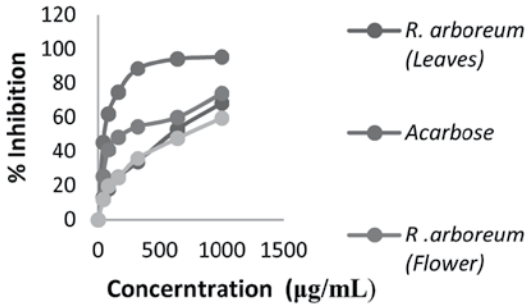

Figure 4: Comparison of percentage α-amyalse inhibition between acarbose and methanolic extract of Plants.

The IC_{50} values of the plant extracts along with the standard acarbose was calculated and tabulated (table no.7).

Table no.7: Comparison of IC_{50} values of different plant extract with standard acarbose.

S.N.	Samples	Botanical name	IC_{50} (µg/mL)
1.	Acrbose		84.12
2.	SC-1	*R. arboreum* (leaves)	638.63
3.	SC-2	*R. arboreum* (flower)	443.44
4.	SC-3	*R. campanulatum* (leaves)	725.68

Concentration of extract containing α-amylase inhibitor that inhibited 50% of amylase activity denotes IC_{50} value. Lower the IC_{50} value, higher the α-amylase inhibitory activity. From table no. 7, among the studied plant *R. arboreum* (flower) shows relatively lower IC_{50} value of 443.44 µg/mL than *R. arboreum* (leaves), and *R. campanulatum* (leaves) shows IC_{50} values, 638.63, and 725.68 respectively. The results revealed that *R. arboreum* (flower) able to inhibit the α-amylase to control the blood glucose level. Which was supported

by previous study showed that *R. arboreum* was found to be effective toward α-amylase inhibition with IC_{50} values 298.52 µg/mL[34]. Study revealed that flower contains anti-diabetic potential which property might be helpful for diabetes and its complications. But there was slightly lower IC_{50} value in present study may be due environmental factor, location, handling of equipment, and instrumental error. Present study showed these plants are active in antioxidant property but they are less active towards α- amylase inhibition. In antioxidant activity all selected plants showed lower IC_{50} values this indicates that they are good antioxidants and also phytochemicals like flavonoids, polyphenols, terpenoids are present in all selected plants which supports their higher antioxidant property. From previous study also showed they are good antioxidants.

Conclusions

Phytochemical screening of methanol extract of all tested plants revealed the presence of different chemical component such as flavonoids, polyphenols tannins, saponins. From antioxidant activity *R. arboreum* (flower) and *R. campanulatum* showed higher IC_{50} values 33.61 µg/mL, 35.75 µg/mL followed by *R. arboreum* (leaves) 39.10 µg/mL. Thus it is concluded that all the studied plants act as highly potential antioxidants. The results revealed that the total phenolic content was highest in *R. arboreum* (flower) 178.20 mg GAE/g, and total flavonoid was higher in *R. arboreum* leaves (63.61 mg QE/g) among studied plants extracts. *R. arboreum* (flower) and *R. campanulatum* contained TFC values 52.43 mg QE/g and 45.36 mg QE/g respectively. From α-amylase inhibitory assay *R. arboreum* (flower) showed the lowest IC_{50} 443.44 µg/mL and highest % inhibition towards enzyme thus able to control the blood glucose level.

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