

# ISOLATION OF QUERCETIN FROM FLOWER PETALS, ESTIMATION OF TOTAL PHENOLIC, TOTAL FLAVONOID AND ANTIOXIDANT ACTIVITY OF THE DIFFERENT PARTS OF *RHODODENDRON ARBOREUM* SMITH

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**Abstract:** The flowers of *Rhododendron arboreum* Smith is a source of polyphenolic compounds. A flavonol, quercetin, was isolated from ethyl acetate fraction of the methanol extract of flower petals of *R. arboreum* by repeated Sephadex LH-20 column chromatography. Isolated quercetin was characterized by comparing melting point,  $R_f$  values, UV and IR spectra with authentic quercetin. The isolated quercetin was used as a standard for the estimation of total flavonoids.

Total phenolic and total flavonoid content in different parts of *R. arboreum* was carried out spectrophotometrically using Folin-Ciocalteu reagent and Aluminium chloride reagent respectively. Gallic acid and quercetin were used as standard for the construction of calibration curve of phenolic and flavonoid respectively. The results showed that the highest total phenolic content was detected in the 70% acetone extract of the flowers (600 mg GAE/g extract) and petals (600 mg GAE/g extract) and the lowest amount was detected in methanol extract of stem (188 mg GAE/g extract). Similarly, the highest total flavonoid was detected in the 70% acetone extract of the twigs (170 mg QE/g extract) and the lowest amount was detected in the methanol extract of stem (45 mg QE/g extract).

The antioxidant activity of the methanol extracts obtained from different parts of *R. arboreum* was determined by 2, 2- diphenyl-1-picryl hydrazyl (DPPH) assay and the radical scavenging activity ( $IC_{50}$ ) was calculated. The highest free radical scavenging effect was observed in leaves with  $IC_{50}=8.34$   $\mu\text{g/ml}$  and lowest was observed in stem with  $IC_{50}=67.83$   $\mu\text{g/ml}$ . The  $IC_{50}$  values, total phenolic and total flavonoid content (correlation coefficient  $R^2=0.923$  for phenolic,  $R^2=0.965$  for flavonoid) were correlated which showed strong correlation indicating that the major components responsible for antioxidant activity is phenolics. The highest the phenolic content, the lowest the  $IC_{50}$  value observed. The result indicated that *R. arboreum* is a rich source of high value polyphenols as natural antioxidant to use in preventive medicine as well as in food and pharmaceutical industry.

**Keywords:** *R. arboreum*; Quercetin; Total phenol; Total flavonoid; Antioxidant activity.

## INTRODUCTION

*R. arboreum* Smith (Ericaceae), locally known as LALIGURAS is the national flower of Nepal. It is distributed throughout the Himalayan and hilly region at an altitude between 1500-3300 m. It has high medicinal, commercial and religious values. The flowers are offered in temples to gods and goddess. In Nepalese traditional medicine, the flowers are used in the treatment of checking diarrhea and bloody dysentery. The fresh and dried corolla is given when fish bones get struck in the throat. Flowers with sweet and sour taste are used in the preparation of squash, jam, jelly and local brew. Fresh petals are processed to prepare pickles, jelly and sharbat. The young leaves are considered as poisonous but used as medicine to alleviate headache

and applied on the forehead<sup>1</sup>. *R. arboreum* is the active constituents of Ayurvedic preparation "Asoka Arishta," which possesses oxytocic, estrogenic, and prostaglandin synthetase-inhibiting activity<sup>2</sup>.

Many bio-active phytochemical constituents are reported from the different parts of *R. arboreum*. The bark is reported to contain taraxerol, ursolic acid acetate, betulinic acid and leuco-pelargonidin<sup>3</sup>. Green leaves are reported to contain ericolin, ursolic acid,  $\alpha$ -amyrin, epifriedelinol, campanulin, quercetin and hyperoside<sup>4</sup>. Flowers are reported to contain quercetin, rutin, coumaric acid<sup>5</sup> and Quercetin-3-rhamnoside<sup>6</sup>. The plant extract also showed significant anti-inflammatory and anti-nociceptive<sup>7</sup>, hepactoprotective<sup>8</sup>, anti-diabetic<sup>9</sup>,

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anti-diarrheal<sup>10</sup>, anti-oxidant and adaptogenic<sup>11</sup> activities.

Among the bioactive phytochemicals, polyphenols are the most widely distributed in plants; fruits, vegetables, cereals, dry legumes and beverages; coca, fruit juices, tea, coffee, red wine<sup>12,13</sup>. It is well known that polyphenols are natural anti-oxidants and play an important role in the prevention of degenerative diseases, particularly cardiovascular diseases and cancers. Quercetin and its derivatives have received considerable attention because of their overwhelming presence in foods and displayed the ability to prevent the oxidation of low-density lipoproteins (LDL) by scavenging free radicals and chelating transition metal ions and reduce the risk of the coronary heart disease and cancer<sup>14,15</sup>.

Nowadays, the world market for functional foods and nutraceuticals is large and growing. In our previous study, we have quantified the total flavonoids content in different parts of *Camellia* plants and total phenolics content in different medicinal herbs and also assessed the selected extracts for their antioxidant activity using DPPH free radicals<sup>16,17</sup>. *R. arboreum* can be used potential source of functional food and value added processed products in terms of bioactive polyphenolic compounds and characteristic flavor. The flower petals could be a potential source of nutraceuticals. However, no quantitative informations are available on the bioactive polyphenol content and their antioxidant activity. Hence, the present study was conducted with the aim to isolate quercetin from flower petals and to quantify the total phenolics and flavonoids content in different parts of *R. arboreum* extracts obtained under different extraction conditions using quercetin and gallic acid as standard compounds. The extracts were also assessed for antioxidant activity using DPPH free radical. The correlations between total phenolics content, total flavonoids content and antioxidant activity were established. This will help to find new sources of polyphenols as natural antioxidant to use in preventive medicine as well as in food and pharmaceutical industry.

## MATERIALS AND METHODS

### Chromatographic materials and chemicals

TLC foils (precoated) Silica gel 60 GF<sub>254</sub>, 0.2 mm were purchased from Merck, Darmstadt, Germany. Sephadex LH-20 was purchased from Pharmacia Biotech, Uppasala, Sweden. DPPH was purchased from Sigma Chemical Company, USA. Gallic acid was purchased from Merck, Darmstadt, Germany. Authentic quercetin was provided by Prof. S. M Tuladhar, RECAST, T.U. Aluminum chloride reagent and Folin Ciocalteu reagent were purchased from SD fine-chemicals. All other chemicals were of analytical grade.

### Plant materials

The bark, stem, flower, leaves and twigs of *R. arboreum* were collected from midhills on the northern fringe forest of Shivapuri Nagarjun National Park at an altitude of 2000 m in February 2013. The plant was authenticated by comparison with the voucher specimen deposited in the National Herbarium at Godawari, Lalitpur.

### Extraction of *R. arboreum* flower petals and isolation of quercetin

The powdered flower petals (300 g) were subjected to soxhlet extraction for 10 hours with methanol (2 lit). The extract was concentrated to a viscous solid mass under vacuum. The dried methanolic extract (104 g) was suspended in 100 ml distilled water and shaken in ultrasonic bath for 15 min. The viscous liquid was extracted successively with hexane (100 ml x 5) and ethylacetate (100 ml x10) in separatory funnel. The ethyl acetate layer was collected and the solvent was evaporated under reduced pressure in rotavapor to get crude ethyl acetate extract. The ethyl acetate extract (9 g) was chromatographed on a Sephadex LH-20 column filled with 100 g Sephadex and eluted with methanol. The sub-fractions were collected consisting of 10-12 ml. Each fraction was monitored by TLC (Silica gel GF<sub>254</sub>) using solvent system, butanol-acetic acid-water, BAW (4:1:5), ethylacetate-methanol-water, EMW (100:15:10), chloroform-acetic acid-water, CAW (10:9:1) and toluene-ethylacetate-formic acid, TEF (10:8:1). The sub-fractions were pooled into four major fractions on the basis of TLC character. The quercetin accumulated fraction F-4 (2.5 g) was further chromatographed on Sephadex LH-20 column and eluted with 75% methanol. sub-fractions were collected and pooled into four major fractions on the basis of TLC character. The fraction F-4-D which was found to contain quercetin was concentrated and recrystallized from hot water to get 37 mg yellow powder.

### Extraction of different parts of *R. arboreum*

An amount of 20 g each of the different parts (leaves, twigs, bark, stem and flower parts such as petals, sepals, androecium and gynoecium) of dried and powdered plant samples were extracted with methanol in a soxhlet extraction apparatus. The residue was extracted with 50% aqueous methanol under reflux. Similarly, 20 g each of different parts of dried and powdered samples were percolated with 70% acetone and subjected to ultrasound-assisted extraction. The extracts were filtered and the solvent was evaporated in a rotary evaporator under reduced pressure.

### Determination of total phenolic content in different extracts

#### Preparation of standard

The total phenolic content in plant extracts was determined by using Folin-Ciocalteu colourimetric method<sup>18</sup> based on oxidation-reduction reaction. Various

concentrations of gallic acid solutions in methanol (100, 75, 50, 25 and 10 µg/ml) were prepared. In a 20 ml test tube, 1 ml gallic acid of each concentration was added and to that 5 mL Folin-Ciocalteu reagent (10%) and 4 mL 7% Na<sub>2</sub>CO<sub>3</sub> were added to get a total volume of 10 ml. The blue coloured mixture was shaken well and incubated for 30 minutes at 40 °C in a water bath. Then, the absorbance was measured at 760 nm against blank. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

#### Preparation of sample

Various concentrations of the extracts (200, 100, 50 and 25 µg/mL) were prepared. Following the procedure described for standard, absorbance for each concentration of the extract was recorded. Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram dry extract (mg/g). The total phenolic content in all samples were calculated using the formula:  $C = cV/m$  where, C = total phenolic content mg GAE/g dry extract, c = concentration of gallic acid obtained from calibration curve in mg/ml, V = volume of extract in ml, m = mass of extract in gram.

#### Determination of total flavonoid content in different extracts

##### Preparation of standard

The total flavonoid content was determined by aluminum chloride colorimetric assay<sup>19</sup>. Various concentrations of standard quercetin (2.0, 1.0, 0.5 and 0.25 mg/mL) were prepared. An aliquot of 1 mL quercetin of each concentration in methanol was added to 10 mL volumetric flask containing 4 mL double distilled water. At the zero time, 0.3 mL 5% sodium nitrite was added, after 5 min, 0.3 mL of 10% AlCl<sub>3</sub> was added and at 6 min, 2 mL of 1 M sodium hydroxide was added to the mixture. Immediately, the total volume of the mixture was made up to 10 mL by the addition of 2.4 mL double distilled water and mixed thoroughly. Absorbance of the pink color mixture was determined at 510 nm versus a blank containing all reagents except quercetin. The average absorbance values obtained at different concentrations of quercetin were used to plot the calibration curve.

##### Preparation of sample

Various concentrations of the extracts (2.0, 1.0, 0.5 and 0.25 mg/mL) were prepared. Following the procedure described for standard, absorbance for each concentration of extract was recorded. Total flavonoid content of the extracts was expressed as mg quercetin equivalents (QE) per gram of dry extract (mg/g). Total flavonoid content is calculated by using the formula:  $C = cV/m$  where, C = total flavonoid content mg QE/g dry extract, c = concentration of quercetin obtained from calibration curve in mg/mL, V = volume of extract in mL, m = mass of extract in gram.

#### Statistical analysis

All the experiments were carried out in triplicates and data reported are mean ± standard deviation. Calculation of linear correlation coefficient and correlation analysis were carried out using MS Office Excel 2007. The linear regression equation for a straight line is,  $Y = mx + c$  where, Y = absorbance of extract, m = slope of the calibration curve, x = concentration of extract, c = intercept. Using this regression equation, concentrations of extracts were calculated. From the calculated values of concentration of each extract, the total phenolics and flavonoid content were calculated.

#### Determination of antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl free radical

Antioxidant activity of the selected extracts was assessed<sup>20</sup> using DPPH free radical. DPPH solution (0.1 mM) was prepared by dissolving 3.9 mg of DPPH in 100 mL methanol and stirred overnight at 4°C. Thus, prepared purple colored DPPH free radical solution was stored at -20° C for further use.

Three different concentrations (5, 10 and 15 µg/mL) of methanolic solutions of each extract were prepared by the serial dilution of the stock solution of the respective extract. To each 0.5 mL extract solution, 2.5 mL 0.1 mM methanolic DPPH solution was added. A control was prepared by mixing 0.5 mL distilled water and 2.5 mL 0.1 mM methanolic DPPH solution. These samples were shaken well and kept in dark for 30 min at room temperature. The absorbance was measured at 517 nm against the blank solution consisting 2.5 ml MeOH and 0.5 mL distilled water. The radical scavenging activity was expressed as the radical scavenging percentage using the equation where; A<sub>s</sub> = absorbance of sample solution, A<sub>b</sub> = absorbance of blank and A<sub>c</sub> = absorbance of control

$$\% \text{ Scavenging} = \frac{[A_s - A_b]}{A_c} \times 100$$

IC<sub>50</sub> value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the graph of radical scavenging activity against the concentration of extracts. Statistically, the correlation between antioxidant activity and total phenolic and flavonoid content was determined by plotting IC<sub>50</sub> (µg/mL) against total phenolics and total flavonoids (mg/g).

## RESULTS AND DISCUSSION

### Isolation and characterization of quercetin from *R. arboreum*

Repeated column chromatograph of the ethylacetate phase of the methanol extract of *R. arboreum* over Sephadex LH 20 yielded quercetin which was purified by recrystallization with hot water. Thin layer chromatography (R<sub>f</sub> 0.79 in BAW; 0.88 in CAW; 0.87 in EMW; 0.45 in TEF) and the melting point (316-

318 °C) of the isolated quercetin is in good agreement with the authentic quercetin. The UV spectrum of the isolated and authentic quercetin showed absorption bands at 251 and 385 nm. The IR spectra of the isolated and authentic quercetin has a broad band around 3600-2400  $\text{cm}^{-1}$  region corresponding to the superimposed aliphatic and aromatic C-H and phenolic O-H stretching. Other stretching's were comparable with IR spectra of authentic quercetin.

#### Amount of different extracts obtained under different extraction conditions

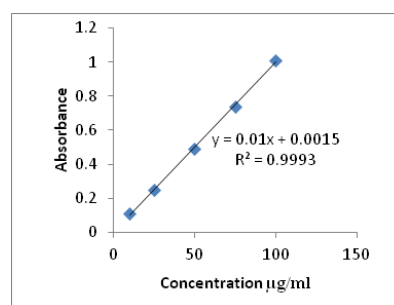
For the determination of total phenolics and total flavonoids in plant extracts, different extraction approaches such as soxhlet using methanol, reflux using 50% aqueous methanol and ultrasound assisted using 70% acetone were applied. The different parts of *R. arboretum* used for the extraction and the amount of different extracts obtained under different extraction conditions are given in Table 1.

**Table 1: Amount of different extracts obtained in gram from 20 g of different parts of dried *R. arboretum*.**

Plant parts	Methanol extract	50% Methanol extract	70% Acetone extract
Sepals	3.42	2.43	2.30
Petals	6.90	1.41	4.98
Androecium & Gynoecium	4.28	2.35	5.03
Flowers	7.15	1.17	6.79
Twigs	2.34	0.79	3.12
Leaves	2.98	1.31	4.28
Barks	2.33	3.21	3.10
Stem	0.93	0.73	1.67

#### Total phenolic content in different parts of *R. arboretum*

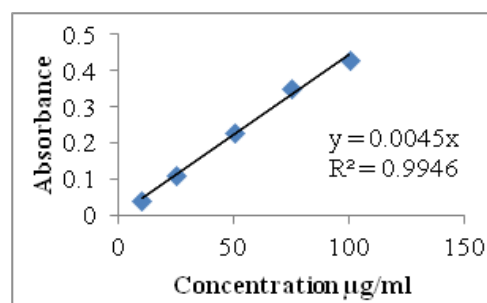
The total phenolic content in plant extract was determined by using Folin-Ciocalteu colourimetric method. Gallic acid was used as a standard compound. The absorbance values obtained at different concentrations of gallic acid was used for the construction of calibration curve (Fig 1). F-C method is based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdenic/phosphotungstic acid complexes to form blue coloured complexes,  $(\text{PMoW}_{11}\text{O}_{40})^{-4}$  that are determined spectrophotometrically at 760 nm. Total phenolic content of the extracts was calculated from the regression equation of calibration curve ( $Y = 0.01x + 0.001$ ;  $R^2=0.999$ ) and expressed as mg gallic acid equivalents (GE) per gram of dry extract (mg/g). The results are presented in Table 2.



**Figure 1: Calibration curve for gallic acid.**

#### Total flavonoid content in different parts of *R. arboretum*

Total flavonoid content in the extracts was determined by reaction with sodium nitrite followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which was monitored spectrophotometrically at maximum wavelength of 510 nm. Total Flavonoids content of the extracts was calculated from the regression equation of calibration curve ( $y=0.004x$ ;  $R^2=0.994$ ) and expressed as mg quercetin equivalent (QE) per gram of dry extract (mg/g). The results are presented in Table 2.



**Figure 2: Calibration curve for quercetin.**

#### Calculation of total phenolic content in different extracts

The absorbance values for different concentration of each extracts (200, 100, 50 and 25  $\mu\text{g/ml}$ ) were recorded at 760 nm for phenolic determination. The concentration of phenolic in each extract was calculated from the calibration curve using the regression equation  $Y = 0.01x + 0.001$ ,  $R^2=0.999$ . The total phenolic content was calculated using the formula  $C=cV/m$  and expressed as mg gallic acid equivalents (GAE) per g of extract in (mg/g).

#### Calculation of total flavonoid content in different extracts

The absorbance values for different concentrations of each extracts (2.0, 1.0, 0.5 and 0.25 mg/ml) were recorded at 510 nm. The concentrations of flavonoids in each test samples were calculated from the calibration curve using regression equation  $Y=0.004x$ ,  $R^2= 0.994$ . Total flavonoid content of the extracts were calculated using the formula,  $C=cV/m$  and expressed as mg quercetin equivalents (QE) per gram extract.

The total phenolic and flavonoid contents calculated in the methanol, 50% methanol and 70% acetone extracts of different parts of *R. arboretum* and the ratio of total flavonoid to total phenolic are given in Table 2.

**Table 2: Total Phenolic, TP and Total Flavonoid, TF content in different extracts and their ratio.**

Plant parts	Methanol TP mg/g	Methanol TF mg/g	Ratio TF/TP	50% Methanol TF mg/g	50% Methanol TF mg/g	Ratio TF/TP	70% Acetone TP mg/g	70% Acetone TF mg/g	Ratio TF/TP
Petals	485.0±5.00	126.8±3.24	0.26	482.0±4.33	30.0±0.00	0.062	600.0±0.00	98.1±2.07	0.163
Sepals	370.0±7.07	17.2±4.18	0.046	321.3±2.16	40.3±1.36	0.125	520.0±0.00	95.3±3.98	0.183
Andro & Gyno.	425.0±8.66	53.4±2.04	0.125	120.0±0.00	96.3±2.16	0.802	585.0±8.66	60.0±0.00	0.120
Flowers	440.0±0.00	103.8±2.16	0.235	520.0±0.00	62.8±2.84	0.120	600.0±0.00	61.3±5.44	0.102
Twigs	400.0±0.00	67.5±2.50	0.168	240.0±0.00	132.5±2.50	0.552	400.0±0.00	169.7±5.20	0.424
Leaves	495.0±8.66	150.0±0.00	0.303	440.0±0.00	63.8±2.16	0.145	485.0±5.00	70.0±0.00	0.144
Bark	240.0±0.00	51.3±6.49	0.213	360.0±0.00	55.6±3.69	0.154	396.2±10.80	20.0±0.00	0.050
Stem	187.0±8.29	45.0±2.54	0.240	215.0±8.66	80.0±0.00	0.372	369.0±0.00	10.7±0.75	0.028

The result indicated that the total phenolic content in the different parts of *R. arboreum* is directly depends on the method of extraction applied and the solvent used. The total phenolic content in methanol and 70% acetone extract were relatively high as compared with 50% aqueous methanol. The highest amount of phenolics were detected in 70% acetone extract of the flowers (600.0 mg/ GAE per gram extract) and petals (600.0 mg/ GAE per gram extract).

Similarly, in the methanol extract of leaves (495.0 mg GAE per gram extract) and petals (485.0 mg GAE/g extract) the highest amount of phenolics were detected. In the case of 50% aqueous methanol extract, the highest phenolic content was found in flowers (520.0 mg GAE/g extract) and petals (482.0 mg GAE/g extract). The lowest amount of phenolic was detected in 50% aqueous methanol extract of androecium and gynoecium (120.0 mg GAE/g extract) as well as methanolic extract of stem (187.0 mg GAE/g extract). The total flavonoid content (495.0 mg GAE per gram extract) in methanol and 70% acetone extracts were relatively high when compared with that of 50% aqueous methanol extract with few exceptions. The total flavonoid present in the methanol extract of *R. arboreum* is highest in leaves (150.0 mg QE/g extract) and petals (126.8 mg QE/g extract). The highest total flavonoid content was found in twigs of 50% aqueous methanol extract (132.5 mg QE/g extract) and 70% acetone extract (169.7 mg QE/g extract). The lowest amount of total flavonoid was found in stem (10.7 mg QE/g extract) and bark (20.0 mg QE/g extract) of 70% acetone extract. The result showed that the total flavonoid content is higher not only in case of methanol extract but also in 70% acetone and 50% methanol extracts. The ratio of flavonoids to phenolics content in methanol extracts were different. In leaves, the greatest (0.30) ratio and in the sepals, the lowest (0.04) ratio were observed which indicated that only 30% of total phenolics in the

leaves are flavonoids and only 4% of the total phenolics in the sepals are flavonoids respectively. Similarly, in the 50% aqueous methanol extracts, the greatest ratio (0.80) was observed in the sepals and the lowest ratio (0.06) was observed in the petals which indicated that 80% of the total phenolics in the sepals are flavonoids while 6% of total phenolics in the petals are flavonoids. On the other hand, in the 70% acetone extract, the highest ratio was found in the twigs (0.42) and the lowest in the stem (0.02) indicating 42% of the total phenolics in twigs are flavonoids while only 2% are flavonoids in stem.

#### DPPH assay for antioxidant activity

The methanol extracts of different parts of *R. arboreum* were assayed for free radical scavenging capacities using DPPH free radicals. The absorbance values were measured at wavelength 517 nm for different concentrations of extracts and the control. These values were used to calculate the percentage inhibitions of DPPH radicals against the samples. The IC<sub>50</sub> values of various extracts were calculated from the percentage inhibitions at various concentrations are given in Table 3.

**Table 3: DPPH radical scavenging activity and TP and TF content in different parts of *R. arboreum* extract.**

Plant extract	IC <sub>50</sub> µg/ml	TPC (mg GAE/g)	TFC (mg QE/g)
Bark	65.45	240.0±0.00	51.3±6.49
Stem	67.83	187.0±8.29	45.0±2.54
Twigs	46.02	400.0±0.00	67.5±2.50
Petals	16.83	485.0±5.00	126.8±3.24
Flowers	25.15	440.0±0.00	103.8±2.16
Leaves	8.34	495.0±8.66	150.0±0.00

The calculated percentage of inhibition showed that all the six extract showed antioxidant activity. However, the highest radical scavenging activity was shown by leaves (8.34 µg/ml) and the lowest was shown by stem (67.83 µg/ml). The extract with the highest phenolic content showed the greatest radical scavenging activity.

### Correlation between total phenolic content, total flavonoid content and antioxidant activity:

The correlations between antioxidant activity and total phenolics content and total flavonoids content were determined by plotting  $IC_{50}$  ( $\mu\text{g/ml}$ ) against total phenolics and total flavonoids content (Fig 3&4). A direct correlation between radical scavenging activity ( $IC_{50}$ ) and TPC of the extracts was demonstrated by linear regression analysis. This relationship between radical scavenging activity and TPC revealed coefficient of determination  $R^2 = 0.923$ . The relationship between radical scavenging activity and TFC revealed coefficient of determination  $R^2 = 0.965$

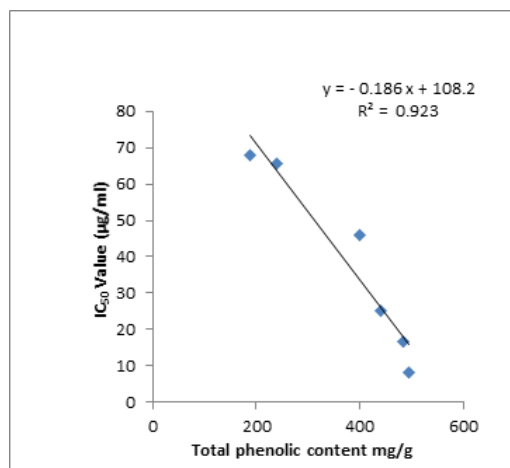


Figure 3: Correlation between  $IC_{50}$  value and total phenolic content.

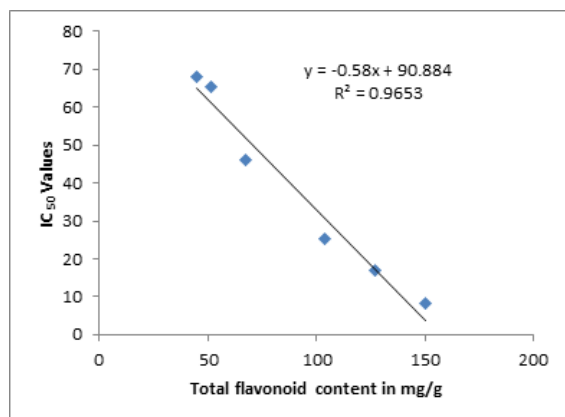


Figure 4: Correlation between DPPH radical scavenging activity ( $IC_{50}$ ) and total flavonoid.

### CONCLUSIONS

Many plant materials from Nepal have not yet received much attention as sources of antioxidant polyphenols due to limited popularity or lack of commercial application. However, the present research indicated that different part of *R. arboreum* is a source of high value phenolics and flavonoids that could be used in preventive medicines and nutraceuticals. The utilization of underexploited resources and the evaluation of traditional knowledge may offer many benefits in the promotion of human health. Thus, the intake of *Rhododendron* flower is beneficial for the prevention

of cancer, cardiovascular and other diseases. Since no pharmacological reports of the use of other parts of *R. arboreum* are known, *in vivo* toxicity evaluation studies must be performed prior to any kind of commercial use.

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