




DNA barcoding, phytochemical screening and antimicrobial activity of *Rhododendron arboreum*, a high altitudinal medicinal plant from Nepal

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Abstract

DNA barcoding has been proposed as a powerful taxonomic tool for species identification genetically. The Consortium for the Barcode of Life (CBOL) Plant Working Group has recommended the combination of *rbcl* & *matK* as the core plant barcode. *Rhododendron* of Nepal shows genetic diversity due to which 31 different species were reported till date, found in Nepal based on morphological character only. For the genetic identification first time, we collected different samples from the central and western parts of Nepal and also based on its phytochemical too. Cold extraction was performed using methanol as solvents. The crude extracts were tested for the presence of phytochemicals such as alkaloids, Saponin, glycosides, tannins, flavonoids, and Coumarins. The extract was also used for antimicrobial assay into five different pathogenic micro-organisms. MIC was done for calculating the minimum effective concentration of the extract. Then TLC was done to separate the compound. The methanol extract was analyzed for core secondary metabolites by HPLC-QTOF-MS and found more than 20 important medicinal compounds. DPPH assay was also done with crude extracts for identifying antioxidant potency of the extracts. Among samples compared, Gulmi and Palpa showed the best result for antioxidant assay with minimum IC₅₀. Similarly, phenolic content was also checked and samples from Palpa showed high phenolic content. Finally with the *rbcl* and *MatK* gene loci was amplified and sequenced and based on alignment with NCBI-BLAST databased it show the 99.98% similarity for the *R.arboreum*. The final contig was submitted to NCBI and get the accession number LC456605.1 and were submitted to the BOLD database for the final authentication and succeed for the first time to publish DNA barcode of Nepalese plants.

Keywords: DNA Barcode, *rbcl* +*matK*, Antimicrobial, Antioxidant, and DPPH.

Introduction

Rhododendron is classified as the earliest of the flowering plants. It has been in existence for 800 million years ago (Vikas Kumar, 2019). The first *rhododendron* was observed in the temperate zone of northern hemisphere and the fossil of oldest *rhododendron* was found in the southern China, in a place where three Chinese province of Yunnan, Sichuan and Tibet meet (Wang et al., 2014) the area is situated around 1000 kilometers of Nepal). The first *rhododendron* was brought into Britain in 1656 from the European Alps, where the plant was classified and named as *Rhododendron hirsutum*. (Liu et al., 2012) Correspondingly, after Linnaeus introduced the

classification of plants under Species Plantarum, genus *Rhododendron* was included for the first time. However, at that time, only five species of *Rhododendron* were known. Coming to this time, 1024 species of rhododendron has been found (Meijón et al., 2010)

Nepal can be classified into several climatic conditions because of varying range of altitude i.e. from 67m in the lowly plain to 8848 m on the top of Mount Everest. It is known that temperature decreases by 6°C for every 1000 m rise in temperature, Nepal foster the growth of several vegetation that include various species (Liu et al., 2012) of *Rhododendron* is also termed as “Laliguras” in Nepal and is the national flower of Nepal.

Nowadays, this taxonomic system is generally accepted by *Rhododendron* specialists. (COX PA, 1997) However, the *Rhododendron* genus still has some problems at various systematic classification levels and there is no simple or universal manner to discriminate the various species within the genus. *Rhododendron arboreum* is a rapidly evolutionary genus within the angiosperms in recent years with many closely related species and there are many artificial and natural hybrids. (Kress et al. 2005)

The DNA barcoding, based on a short DNA sequence to identify species (Hebert et al. 2003) has been proposed as a rapid, accurate, and convenient taxonomic tool. (Hebert et al. 2003) The Consortium for the Barcode of Life (CBOL) Plant Working Group recommended the *rbcL* + *matK* combination as a barcode sequence in the plant kingdom, and they also suggested that ITS (ITS2) and *psbA-trnH* were good candidates for plant DNA barcoding, because of their fast evolution rates. (Kress et al., 2005) One of the problems for plant DNA barcoding was that the previous studies were mainly carried out on a large scale and rarely on a specific genus, with many closely related species, so some studies suggested that species identification using standard DNA sequences should be carried out within a narrow taxon (such as the genus) (Li, Tong, & Xing, 2016). One of the challenges for any DNA barcode is its utility in discriminating closely related species. (Newmaster et al. 2008; Wong et al. 2013). One of the challenges for any DNA barcode was its ability in discriminating closely related species (i.e., sister-species). (Liu et al., 2012; Newmaster et al., 2008).

The stem and roots of *Rhododendron arboreum* have medicinal importance. They have been used to cure illness. Even though, there are some cases but there is substantial evidence to support.

Rhododendron arboreum is also a source of traditional medicine in the Himalayas. One method of preparation is making a powder from the flower which is mixed with the starch of the boiling rice and given to the patient suffered from dysentery. Another remedy is to make a paste from the leaves and apply it to the forehead in the treatment of headaches. It is also used to treat skin diseases. An extract of the bark is used in the treatment of coughs, diarrhea and dysentery. In Ayurvedic medicine rhododendron plants are used to treat jaundice, diabetes, piles, enlargement of the spleen, liver disorder and worms. According to common folklore in Nepal a sip of the juice of the Lali Guras (Garg, 2017) flower dissolves fish bones stuck in the throat. Some *Rhododendron* species in the high mountains are used to make herbal teas. According to herbalist Amchi Lhakpa the tea “clears your stomach and improves digestion. It is helpful with lung and stress disorders involving general weakness of the body. It helps reduce fever and swelling of the abdomen due to the indigestion of food, restores the natural balance after changes in climate or water, and relieves numbness of the extremities, swelling and itching of the throat, or a feeling of thirst”. (Scott, 2010) *Rhododendron arboreum* experience natural and anthropogenic pressures and the later factors are realized as the main cause of their population decline (Mainra et al. 2010; Pokhrel, 1999). The color of the flower ranges from blood red, or pink to white. *Rhododendron* bears large compact clusters of about 20 flowers. The corolla is tubular, bell-shaped, 4-5 cm long and wide, 5 lobed. The grained wood of *Rhododendron arboreum* is utilized in making handles of ‘Khukhri’, packsaddles, gift-boxes, gunstocks and posts

(Paul et al. 2005). Flowers and leaves are used as decorations around the houses and temples by adjusting into ropes made of munja grass (Shrivastav, 2012).

Phytochemical processing of raw plant materials is essentially required to optimize the concentration of known constituents and also to maintain their activities (Andriani et al., 2019) Extraction is an important step in the itinerary of phytochemical processing for the discovery of bioactive constituents from plant materials. Selection of a suitable extraction technique is also important for the standardization of herbal products as it is utilized in the removal of desirable soluble constituents, leaving out those not required with the aid of the solvents.

Medicinal plants are currently in considerable significance view due to their special attributes as a large source of therapeutic (Deepak et al. 2019) phytochemicals that may lead to the development of novel drugs. Most of the phytochemicals from plant sources such as phenolics and flavonoids have been reported to have positive impact on health and cancer prevention (Venugopal et al. 2012) as also found in *Rhododendron arboreum* too. Plants are able to produce a large number of diverse bioactive compounds (Sharma et al. 2016). High concentrations of phytochemicals, (Deepak and Lamichhane 2019) which may protect against free radical damage, accumulate in fruits and vegetables (Kathirvel and Sujatha 2016) .

Phytochemical consumption is associated with a decrease in risk of several types of chronic diseases due to in part to their antioxidant and free radical scavenging effects (Zhang et al., 2015). Recent research has also highlighted their potential role in improved endothelial function and increased vascular blood flow (Shrivastav 2012).

The use of plant extracts, as well as other alternative forms of medical treatments, was of great popularity in the late 1990s. The reason for this is due to increase in antimicrobial resistance, and the need of treatments for new emerging pathogens. Antimicrobial susceptibility testing is carried out to screen whether the extracts are potential antimicrobial agents and measure the antimicrobial activity of the substance. An antimicrobial susceptibility test is a determination of the least amount of an antimicrobial chemotherapeutic agent that will inhibit the growth of a microorganism in vitro using a tube dilution method, agar cup or disc diffusion method. The test may function as an aid in the selection of chemotherapeutic agent by the physician (Prajapati et al., 2018).

The effectiveness of antibiotics can be assessed by their ability to suppress bacterial growth, described by the MIC, or by their ability to kill bacteria, characterized by the minimal lethal concentration (MLC). MIC is usually derived by means of tests in solid media.

It involves the application of antibiotic solutions of different concentrations to cups, wells or paper discs, placed on the surface of or punched into agar plates seeded with the test bacterial strain. Antibiotic diffusion from these sources into the agarose medium leads to inhibition of bacterial growth in the vicinity of the source and to the formation of clear 'zones' without bacterial lawn. The diameter of these zones increases with antibiotic concentration. (Contreras-Lynch et al., 2017).

Material and Methods:

Study Area : Sample were collected in the month of February 206-17 from Bagmati province and Province no 5 (Lumbini Zone i.e Palpa /Gulmi and Rolpa) at altitude of 1350m (Palpa) to 3400 (Rolpa) and 1200m (Kathmandu valley).

Sample Preparation: Sample were air dried after collection for a week in dark room and herbarium file was created and identified by Tirtha Maiya Shrestha and deposited to the Kathmandu University to get the voucher number.(KU_2017_Rho P5 and KU_2016_Rho P3).

Extract preparation: The shade dried whole plant sample was taken, crushed in Grinder to make it in powder form and mixed with methanol. The mixture was stirred for about half an hour. Then the mixture was kept for 48 hours at room temperature. After 48 hours, the solution was filtered and fresh methanol was added to crude extract. The filtrate extract was evaporated using shaker. The final extract was weighted to and yield of extract was calculated.

Phytochemical screening : Test for Sterols and Triterpenes (Leibermann- Burchard's Test).

Extract solution was concentrated to yield a residue. This residue was dissolved in 1ml of acetic anhydride followed by 1 ml of Chloroform. Appearance of brown ring between the junctions of the two liquids with upper layer green indicates the presence of sterols while the brown ring with upper layer violet indicates the presence of triterpenes.

Test for the Coumarins

Extract solution is concentrated to yield a residue. The residue was dissolved in hot water.

After cooling, the solution was divided into two test tubes. To one of the test tubes, 10% (v/v) Ammonium hydroxide (NH₄OH) was added until the solution becomes basic. The other test tube was used as control. Observation of fluorescence indicates the presence of Coumarins.

Test for Tannins and Polyphenols (or Ferric Chloride Test)

1 ml of the extract solution was mixed with 1 ml of water. To this solution, 3 drops of 1% (w/v) Ferric Chloride (FeCl₃) was added. Appearance of Blue-Black or Violet color indicates the presence of Tannins and/or Polyphenols.

Test for Reducing sugars (or Fehling's Test)

1 ml of the extract was mixed with 1 ml of water. To this solution, 1ml of Fehling's reagent (1:1 mixture of Fehling's reagent A and Fehling's reagent B) was added. The resulted mixture was warmed over a water bath for half an hour. Appearance of Brick-red precipitate indicates the presence of reducing compounds.

Test for Saponins

2 ml of the extract was shaken vigorously for 30 seconds in a test tube. Persistence of thick froth (about 1 cm) height even after 30 minutes indicates the presence of Saponins.

Test for Glycosides

4ml of the extract solution was dried till 2 ml. 1-2 ml of NH₄OH was added and shaken. Appearance of Cherish Red color indicated the presence of Glycosides.

Test for Flavonoids

To 4 ml of the extract solution, 1.5 ml of 50% Methanol solution was added and warmed.

Metal Magnesium was added to the solution. Then 5-6 ml of conc. HCl was added. Appearance of the following was sought: Black color – indication of Flavonoids .Orange color – indication of Flavones .Violet color – indication of Flavanones.

Test for Alkaloids:

5 ml of extract was concentrated to yield a residue. Residue was dissolved in 3 ml of 2% (v/v) HCL. Few drops of Mayer's reagent were added. Appearance of the dull white precipitate indicated the presence of basic alkaloids.

Microbial analyses

Bacillus subtilis, Pseudomonas, K. pneumonia, Streptococci, Candida albicans strains used for microbial analyses.

Sample Preparation

- 200 mg of each extracts was dissolved in 1000 μ l of DMSO separately i.e 200mg/ml
- This sample was used as crude to check for its antimicrobial activity

Preparation of microbial culture

- First respective strain of bacterial sample with ATCC (American Type Culture Collection) number was obtained. Nutrient Broth of desired volume were prepared in culture tubes and autoclaved at 121^o c at 15lbs pressure. With the help of cotton swab, a bacterial sample from standard sample was scrapped and it was dipped in broth. Then all culture tubes were tagged correspondingly and incubated at 37^oC for 12 hours

a) Disc Diffusion protocol

Filter paper disc of (6mm in diameter) are made .MH agar of desired volume was prepared and autoclaved at 121^oC and 15lbs pressure. Autoclaved media was then poured into sterilized petri plates under LAFH and allowed to set. The microbial sample from culture tube was swabbed in MH agar using sterilized L shaped spreader.15 μ l of each extracts of the preparation under test poured and dried in the paper disc with the help of a micropipette, It was then gently placed at the different sites of the Petri dish containing the inoculated medium .The plates were then incubated at a temperature of 37^oC. After 6 hours, the zones of inhibitions were measured with the help of a measuring ruler from the circumference of the disc

b) MIC (Minimum inhibitory Concentration)

After observation of crude extract, the sample showing antimicrobial activity was processed to its MIC.

- Various lowered concentration i.e. 100mg/ml, 50mg/ml, 20mg/ml, 10mg/ml, 5mg/ml from crude sample was prepared as per following calculation:

For 100mg/ml

Concentration of crude sample (s1) = 200mg/ml

Desired concentration (s2) = 100mg/ml

Final volume (v2) = 1ml

Volume to take (v1) = x

$$S1*v1=s2*v2$$

$$V1= (s2*v2)/s1$$

$$V1= 500\mu l$$

For 50mg/ml

Concentration of crude sample (s1) = 100mg/ml. Desired concentration (s2) = 50mg/ml

Final volume (v2) = 1ml .Volume to take (v1) = x

$$S1*v1=s2*v2$$

$$V1= (s2*v2)/s1$$

$$V1= 500\mu l$$

For 20mg/ml

Concentration of crude sample (s1) = 50mg/ml. Desired concentration (s2) = 20mg/ml

Final volume (v2) = 1ml. Volume to take (v1) = x

$$S1*v1=s2*v2$$

$$V1= (s2*v2)/s1$$

$$V1= 400\mu l$$

For 10mg/ml

Concentration of crude sample (s1) = 20mg/ml. Desired concentration (s2) = 10mg/ml

Final volume (v2) = 1ml. Volume to take (v1) = x

$$S1*v1=s2*v2$$

$$V1= (s2*v2)/s1$$

$$V1= 500\mu l$$

For 5mg/ml.

Concentration of crude sample (s1) = 10mg/ml. Desired concentration (s2) = 5mg/ml

Final volume (v2) = 1ml .Volume to take (v1) = x

$$S1*v1=s2*v2$$

$$V1= (s2*v2)/s1$$

$$V1= 500\mu l$$

- As the calculation respective volume was taken and final volume was made to be 1ml by adding DMSO. Then Disc diffusion method was carried.

c) Antioxidant analysis

a) Free radical scavenging activity (DPPH Assay)

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm. When antioxidant reacts with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor and is reduced to the DPPH and as consequence the absorption is decreased from the DPPH. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenyl picrylhydrazine; non radical) with the loss of violet color.

b) Preparation of DPPH solution

DPPH solution of 100U μ m was prepared by dissolving 3.94mg of DPPH in 100ml of methanol. It was protected from light by covering the bottle with aluminum foil.

c) Preparation of standard solution

100mg/ml stock solution of Ascorbic acid was prepared by dissolving 10mg Ascorbic acid in 1ml methanol. Test solution of 1,2,4,6 and 8 μ g /ml of Ascorbic acid was prepared from stock by dilution.

d) Preparation of test sample

10mg of plant extract was dissolved in 1ml of methanol to prepare stock solution of 10mg/ml. Test solution of 1,2,4,6 and 8 μ g /ml was prepared from the stock by dilution.

Experiments were done in triplicate.

e) Estimation of DPPH scavenging activity

Estimation was done in two ratios (1:1 and 1:3)

a. For 1:1 ratio

- 1ml methanol and 1 ml of DPPH solution were mixed as control.
- 2ml methanol was taken in Eppendorf tube as blank.
- 1ml ascorbic acid was mixed with 1 ml DPPH as standard.
- 1ml plant extract was mixed with 1ml DPPH as sample.

b. For 1:3 ratio

- 0.5ml of methanol and 1.5ml of DPPH solution were mixed as control.
- 2ml of methanol was taken in Eppendorf tube as blank.
- 0.5 ml ascorbic acid was mixed with 1.5ml DPPH as standard.
- All of these mixtures were immediately kept in dark to prevent from light.
- After 30minutes, absorbance was noted in 517nm.

f) Calculation of IC50

IC50 was calculated from % inhibition. Absorbance at 517nm was determined after 30 minutes using UV-Visible Spectrophotometer and IC50 was determined. The capability to scavenge the DPPH radical was calculated using following equation.

$$\% \text{scavenging} = (A_0 - A_1) / A_0 * 100$$

Where, A_0 = absorbance of DPPH solution

A_1 = absorbance of DPPH along with different concentration of extract. IC50 was calculated from equation of line obtained by plotting a graph of concentration verses % scavenging of DPPH.

g) Determination of Total Phenol content.

Total phenol content estimation was done using Folin Ciocalteu's (folin) technique with little modification. Aliquots of 1ml and standard Gallic acid (20, 40, 60, 80 and 100) $\mu\text{g/ml}$ were placed in test tube. Then 5 ml of Folin Ciocalteu's reagent and 4.5ml of distilled water was mixed. 4 ml of 7% sodium carbonate was added after 5 minutes. Then after shaking it was incubated at 40^oc in water bath for few minutes. Then absorbance was measured at 760nm using UV-Visible spectrophotometer. The experiments were done in triplicates and the blank was made using reagent blank with solvent. Standard Gallic acid was used for calibration curve plotting. Total phenol content was expressed as milligrams of Gallic acid equivalents per gram of fresh weight ($\text{mg GAEg}^{-1}\text{FW}$).

h) Determination of Total Flavonoid content

Total flavonoid content was measured by using Aluminum chloride colorimetric assay. 1 ml of standard rutin solution (100,200,400,600 and 800) $\mu\text{g/ml}$ and 1 ml of aliquots was positioned into test tube. Then 200ul distilled water was added in each tube. 150ul of NaNO_2 was added and incubated for 5 minutes at room temperature. Then

150ul of AlCl₃ was added. After 6 minutes 2ml of sodium hydroxide was added and final the volume of each test tube was made to 5 ml by adding distilled water. The solution was shake well and incubated at room temperature for 15 minutes. Finally observance was measured at 510nm using UV-Visible spectrophotometer. Total flavonoid content was measured as mg rutin equivalent (mg RT/g).

• **DNA Isolation and Sequence analysis**

Genomic DNA extraction. was done with Zymo mini prep kit as per manual .(Quick-DNA™ Plant/Seed Miniprep Kit Catalog No. D6020). (kit).

For PCR and sequencing with reference to I bold Manual (Hollingsworth et al., 2009; Kress, 2017; Kress et al., 2005) . We followed the same criteria to get plant authentication by using rbcL and matK gene loci amplification. We used Mega X (Stecher, Tamura, & Kumar, 2020) for phylogenic analysis and online tool of I bold Database (Bold, 2013) for identification. NCBI databased is used for identification and Blankt for sequence submission. EMBL-ebi tools were used for identification of 6 ORF and amino acid recognition site identification.

Results and Discussion

Extractive values of the extract from Rhododendron

The extractive values and the percentage yield of the leaves of various sample of Rhododendron arboreum. The highest yield was 6.8% from the Palpa. The total phytochemical extract yield is shown in table 1.

Table 1: Extract Percentage yield

Plants	Weight of leaf sample (gm)	Weight of crude extract (gm)	% yield
Dhu1	41.69	0.86	2.06
Dhu2	40.29	1.24	3.07
Gul1	30.96	0.68	2.20
Gul2	20.15	0.81	4.02
Pal1	45.91	1.67	3.63
Pal2	31.52	2.15	6.8
Rol1	31.51	1.48	4.70
Rol2	31.47	1.06	3.37

Phytochemical Screening

We have carried out qualitative test for phytochemicals present in Rhododendron arboreum extract. Analysis was carried out for methanol extract. Saponin, Glycosides, Alkaloid, Steroids and Flavonoids were present except Coumarins. The presence or absence of seven different phytochemical are shown in table 2.

Antibacterial Assay

From extract of each sample, 200 mg was taken and dissolved in 1ml DMSO to make concentration of 200mg/ml. After incubation zone of inhibition was observed and radius of zone of inhibition was measured in mm from center of disc. Finally it was found that the biggest zone of inhibition was found from sample of Gulmi1 *i.e.* 14 mm with respect to Bacillus subtilis.

The antioxidant activity of the extract was analyzed using the standard DPPH solution and compare with standard Ascorbic acid. The highest scavenging Activity was shown by Rhododendron arboreum species from Gulmi (gul1) sample and found to be 77.27%.

Table 2: Phytochemical screening of Rhododendron arboreum sample

Plants/ Properties	Coumarins	Saponin	Glycosides	Alkaloids	Steroids	Reducing Sugar	Flavonoids
Dhu1	--	++	++	++	++	++	++
Dhu2	--	++	++	++	++	++	++
Gul1	--	++	++	++	++	++	++
Gul2	--	++	++	++	++	++	++
Pal1	--	++	++	++	++	++	++
Pal2	--	++	++	++	++	++	++
Rol1	--	++	++	++	++	++	++
Rol2	--	++	++	++	++	++	++

DNA isolation and PCR: Genomic DNA was extracted from the raw explant of the sample by Zymo plant DNA extraction kit and was found to be 80ng/uL, by spectrophotometric method and for amplification and sequencing it was send to Barcode Bioscience Pvt.Ltd laboratory, and final sequence was assemble and developed the contig of rbcL gene and introduced as DNA barcode after bioinformatics analysis using online and offline tool.

Contig Sequence of rbcL gene

The pcr product of rbcL gene was sequenced by Sanger sequencing and the final contig prepared with compilation of reverse and forward region which is as show below.

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ATCAAGCTGGTGTAAAGATTACAAATTGAATTATTATACTCCTCAGTATGAAACCAAAGATACTGATATC
TTGGCAGCATTCCGAGTAACTCCTCAACCGGGAGTTCACCTGAAGAAGCAGGGGCCGCGGTAGCTGCGG
AATCTTCTACTGGTACATGGACAACAGTGTGGACTGATGGACTTACTAGCCTTGATCGTTACAAAGGGCG
ATGCTACCACATCGAGCCTGTTGCTGGAGACGAAAATCAATATATTGCTTATGTAGCTTATCCTTTAGAC
CTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGCCC
TGCGCGCTCTACGTCTGGAAGATCTACGAATCCCTGTAGCGTATGTTAAAACGTTCCAAGGACCGCCTCA
TGGTATCCAAGTTGAAAGAGATAAATTGAACAAGTACGGTCGTCCCCTGTTGGGATGTACTATTAAACCT
AAATTGGGGTTATCTGCTAAAAA
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DNA Barcoding: Sequence analysis with NCBI BLAST OUTPUT

The above contig was submitted in NCBI for GI accession number and finally gets LC 456605.1 submitted to nBLAST mode in NCBI database for Rhododendron arboreum plant identification based on rbcL gene sequence alignment.

Phylogenetic Analysis: To find the close ancestor relationship among the available data source from NCBI , we compare rbcL gene of Rhododendron arboreum sequence with 10 closest similar sequence with Mega X software and found that the sequence similarity among them,which finally endorse for the development of rbcL gene DNA Barcode formation of Nepalese sample.

DNA Barcode of *rbcL* Gene: With due analysis of *rbcL* gene sequence with NCBI Blast and Mega X it is confirmed that the provide sequence belong to *Rhododendron arboreum*, since we develop the DNA barcode with of it.

The project has helped to reveal various biological activities of *Rhododendron arboreum* collected from different locations of the country. All the Samples showed presence of secondary metabolites (Table 2) such as Saponins, Glycosides, Alkaloids, Sterols, Reducing Sugars and flavonoids but not Coumarins which was further analyzed by different related tests. The antibiotic activity ranging from 2 mm to 14 mm in inhibition radii against *Bacillus subtilis*, 5 to 14 mm against *Pseudomonas*, 4 to 9 for *Klebsiella pneumonia*, and 4 to 10 mm for *Streptococcus* using 200 mg/ml solution of each sample. Sample 1 (Pal1) collected from Palpa region of Nepal showed the highest activity against all these bacteria. However, none of them showed any activity against *Candida albicans* showing the antibiotic tolerance that may be the key finding as it can be further implementation for new drug discovery. The same sample showed highest activity in DPPH bioassay very close to Ascorbic acid which can be stated from the regression analysis of DPPH for the IC50 value analysis of Ascorbic acid. All this might be due to the high content of Phenol in this sample. The extract yield of Palpa show the highest percentage among all the location of Nepal (Table 1). This detailed results show that *Rhododendron arboreum* has medicinal importance and the traditional healers use in specified community proves the facts. These results should be further validated scientifically by more rigorous scientific studies in days to come.

Also, this project highlights the necessity of assessing the right model for data evaluation and calculation which is justified by the results of MIC. As all the tests were performed using crude extract and so, the result cannot be validated. It is advised to test using isolated pure compound extracts for confirming the results presented here.

The promising result obtained from the research stimulates further research on the plant and its metabolites, and their safety concerns in human health. The great medicinal importance of this plants provokes the serious concerns for its preservation and conservation from the concerned authority. As evident from the above results and the PCR products of *matK* and *rbcL* gene) using specific primers of DNA demanded the barcoding of the genes in the given sample the genus of the sample is *Rhododendron arboreum*. The Phylogenetic analysis with Mega X also support the close relationship among the *Rhododendron* species available data bases. This is first time ever reported in Nepal for any DNA barcoding of Medicinal plant, among the all genes *rbcL* and *matK* barcoding used for the analysis, all showed similarity to *arboreum* species. Yet more to elaborate among the genetic diversity of *Rhododendron* species of Nepal as we get success only in one species out of 31 as registered in flora of Nepal.

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