Impact of elevation and soil characteristics on phytochemical constituents and antioxidant potential of *Berberis aristata* and *Berberis asiatica*

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Species of *Berberis* have been used for a long time in traditional medicine, especially in the treatment of diarrhea, fever, and ophthalmic problems. The therapeutic potential of these species is attributed to the presence of various phytochemicals. The present study aims to analyze the impact of elevation and soil properties on phytochemical constituents and antioxidant potential of methanolic bark extracts of *Berberis asiatica* and *Berberis aristata*. Quantitative estimation of total phenol content (34.13±4.3 to 59.96±1.2 mg GAE/g DW) was found to increase with increasing elevation for both species. In contrast, total flavonoid content (31.69±2.4 to 27.24±1.2 mg QE/g DW) and antioxidant potential (IC_{50}; 79.26±0.9 to 102.97±2.3 µg/mL) in both species of *Berberis* were found to decrease with increasing elevation. Semi-quantitative estimation of Berberine content (0.76±0.01 to 0.95±0.02 mg/g DW), also showed a similar trend. The soil parameters including moisture and total NPK content were studied for the samples collected from the same sites used for plant sample collection. All soil parameters also increased with increasing elevation. The increase in soil moisture and NPK content showed an increase in total phenol content but a decrease in berberine and total flavonoid content for both species of *Berberis.* Overall, the outcome of the present study reveals the impact of elevational gradients and soil characteristics on phytochemical content and antioxidant potential in two species of *Berberis.*

Key words: *Berberis*; Elevation; Soil characteristics; Flavonoids; Phenols; Antioxidant activity.

Berberis, the largest genus in the Berberidaceae, grows in most vegetation types throughout Nepal, from 1000-4600 m asl. It is an ideal group to study the effect of the Himalayan orogeny on plant diversification as it grows in a wide range of habitats including forest floor, forest margin, open pastures at higher elevations, semi-desert vegetation, and the margins of cultivated land. Some species of *Berberis* found in Nepal are widespread while others are much more geographically and elevationally restricted (Mouri et al., 2004). Among the 21 species, *B. asiatica* commonly occurs in the western and central Himalayas, and in Tibetan plateau at an elevational range from 1000-2700 m asl. Similarly, *B. aristata* is often found in small patches on the hilly slopes in the central, western, and eastern Himalayas at an elevation ranging from 1300-3400 m asl. (Adhikari et al., 2012).

Berberis species are known for their ability to treat different ailments. Every part of the plant including the root, bark, stem, and fruit, is used in different Ayurvedic preparations. Among these, the stem and root are the most extensively studied in *B. aristata* and *B. asiatica,* mainly for their medicinal properties (Bhardwaj & Kaushik, 2012). In Nepal, extracts from the stem and root of these species are used as ophthalmic medicine, and to treat jaundice, fever, diarrhea, and other conditions (Manandhar, 2002). Similar applications have also been reported for the bark extracts of *B. aristata* in the traditional Sowa Rigpa system of medicine (Ghimire et al.,

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2021). The medicinal effects of *Berberis* spp. are attributed to various compounds, mainly isoquinoline alkaloids like berberine present in different parts of the plant (Saied et al., 2007). Berberine has also been reported to exhibit antioxidant, anti-inûammatory, hepatoprotective, anti-hyperglycemic and hypotensive properties (Soffar et al., 2001; Semwal et al., 2009; Singh & Kakkar, 2009; Končić et al., 2010; Tiwari & Khosa, 2010).

The quantity and quality of alkaloids in plants are, to some extent, genetically controlled but are also greatly influenced by varying growth conditions arising from difference in elevation and soil properties including moisture and potassium content (Nautiyal, 2007; Andola et al., 2010). Although significant work has been conducted on various aspects of *Berberis* species, studies on Nepalese species have been confined merely to either ethnobotanical studies (Rawal et al., 2009) or taxonomic revision (Adhikari et al., 2012). Consequently, investigations into the influence of ecological variation on their phytochemical profiles remained limited. In this background, the present study aims to determine the effects of elevational gradients and soil characteristics on the phytochemical content and antioxidant activity of bark extracts from two species of *Berberis* namely, *Berberis aristata* and *Berberis asiatica* in Nepal.

Materials and methods

Plant material and soil samples

The samples (stem bark and soil) were collected from three different elevations (1400 m, 1900 m and 2400 m) of the Champadevi Hill, Kathmandu in February 2016. *B. aristata* samples were collected from 1900 m and 2400 m while those of *B. asiatica* were collected from all three elevations. Barks were taken from mature, thick twigs that were cut from the plants. Similarly, soil samples were collected from the depth of 5 cm beneath each *Berberis* plant. Voucher specimens were collected from each sampling site, and representative herbarium specimens have been deposited at Tribhuwan University Central Herbarium (TUCH). Table 1 summarizes the details of the *Berberis* samples collected from different elevations of Champadevi Hill, Kathmandu.

Preparation of methanolic extracts

The plant samples were extracted following Khanal et al. (2015) with minor modifications. Collected bark samples were cleaned to remove mosses, lichens, and dust particles. The samples were air-dried in the shade for three weeks to remove moisture and then ground. The powder was sieved to obtain a fine powder for the extraction procedure. Ten grams of the powder from each sample were mixed with 100 mL of methanol in a vial and subjected to sonication (UC-7240BDT E-Chrome Tech, Taiwan) for 2 hours for the first extraction, then filtered through Whatman No. 1 filter paper. The same process was repeated on the residue for another 1 hour and filtered as before. Both filtrates were combined and then evaporated using a rotary evaporator under reduced pressure. The crude extracts were allowed to dry on Petri plates under aseptic conditions. The dried extracts were scrapped off the Petri plates and stored in 2 mL polypropylene tubes at -20 °C.

Determination of total phenolic contents (TPC)

The total phenolic content (TPC) of the methanolic extracts was determined using the Folin–Ciocalteu method as described by Ainsworth and Gillespie (2007). 100 µL of the plant extract (2.5 mg/mL) was separately mixed with 1 mL of 10-fold diluted Folin-Ciocalteu phenol reagent (Fisher Scientific) and 0.8 mL of 1 M solution of Na_2CO_3 solution. The reaction mixture was incubated at room temperature for 15 minutes and absorbance was measured at 765nm using the UV-visible Spectrophotometer (CT-8200, E-Chrome Tech, Taiwan). A blank was prepared by substituting the plant extract with 100 µL of absolute methanol in the reaction mixture. For calibration, a standard curve was generated using gallic acid solution of different concentrations (25-250 µg/mL) prepared in a 50:50 (v/v) methanol–water mixture Total phenolic content in the plant extract were then quantified by using standard calibration curve. The TPC of the extracts was expressed as milligrams of

Table 1: Details of collected plant samples from Champadevi Hill, Kathmandu

SN	Species name	Plant part collected	Elevation (m asl.)	Latitude/Longitude
1	*Berberis aristata*	Stem bark	1900	27°38'17" N, 85°15'43" E
2	*Berberis aristata*	Stem bark	2400	27°38'58" N, 85°14'09" E
3	*Berberis asiatica*	Stem bark	1400	27°39'24" N, 85°15'51" E
4	*Berberis asiatica*	Stem bark	1900	27°38'17" N, 85°15'43" E
5	*Berberis asiatica*	Stem bark	2400	27°38'58" N, 85°14'09" E

gallic acid equivalent per gram of dry weight (mg GAE/g DW).

Determination of total flavonoid contents (TFC)

The total flavonoid content (TFC) was determined using the Aluminum chloride ($AlCl_3$) colorimetric method following Roy et al. (2011) with slight modifications. 250 µL of methanolic extract (10 mg/mL) of each sample was separately mixed with the 750 µL of methanol, 50 µL of 10% aqueous Aluminum chloride solution, 50 µL of 1 M potassium acetate solution, and 1.4 mL of distilled water. The resulting mixture was shaken and kept at room temperature for 30 minutes. The absorbance was then measured at 415 nm using the UV-visible spectrophotometer (CT-8200, E-Chrome Tech, Taiwan). A standard calibration curve was prepared using quercetin solutions (10–100 µg/mL in methanol), while the blank was prepared by replacing the plant extract with methanol. The TFC was expressed in terms of mg QE/g DW i.e. milligram of Quercetin per gram of dry matter by using the equation of linear curve.

Berberine content

Thin layer chromatography (TLC) was performed to detect and quantify the major alkaloid berberine present in the methanolic bark extract of two species of *Berberis* following Wagner and Bladt (1996). A stock solution of berberine (1 mg/mL; Sigma Aldrich, Germany) was prepared in methanol and subsequently diluted to prepare a series of of 0, 25, 50, 75, 100, 125, 150, 200 µg/mL. A 5 µL aliquot of each berberine standard solution and the plant extract (5mg/mL) were loaded on TLC Silica gel 60 F254 plates (Merck KGaA, Germany) making spot 2 cm above the bottom.

The plates were then run in the mobile phase consisting of methanol: acetic acid: water (8:1:1, v/v/v) until the solvent front reached approximately three-quarters of the plate. Then, the plate was air dried and visualized under a UV chamber at 365 nm. The intensities of fluorescence quenching of the bands in the samples and reference standard in the photograph were estimated by using Gelquant, NET software version 1.8.2 (biochemlabsolutions.com). A standard calibration curve was generated by plotting the intensity of berberine against the concentration. The amount of berberine in different plant samples was then estimated semi-quantitatively using the standard curve.

Antioxidant activity assay

The antioxidant activity of the plant extract was determined using the DPPH (2, 2- Diphenyl-1-picrylhydrazyl) free radical scavenging activity with ascorbic acid as the standard (Singh et al., 2002). Various concentrations of the plant extracts (25-200 µg/mL) and ascorbic acid (10-100 µg/mL) were prepared in methanol in clean test tubes. Then, 0.5 mL of each sample was mixed with 0.5 ml of 0.2 mM DPPH solution. The mixture was shaken thoroughly and incubated in the dark for 30 minutes. A control solution was prepared in the same way, but with pure methanol instead of plant extract. After incubation, the absorbance was measured at 517nm using a UV-visible spectrophotometer (CT-8200, E-Chrome Tech, Taiwan). The free radical scavenging activity (RSA) of the plant samples was calculated as a percentage by using following formula:

$$\% \text{ Radical scavenging activity (RSA)} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100$$

The IC_{50} value of the DPPH radical scavenging activity of plant samples was calculated using the formula:

$$IC50 = EXP\ (LN\ (conc.> 50\%) - ((pi > 50\% - 50)/(pi > 50\% - pi < 50\%) * LN(conc. > 50\%/conc. < 50\%)))$$

Analysis of soil parameters

Moisture content

Soil moisture content was determined following the method described by O'Kelly (2004) with slight modifications. Fifty grams of soil were oven dried at 110 °C for 24 hours and then weighed. The soil moisture content (%) was calculated by using the formula:

Moisture content (%) = 100 × (W1-W2)/W1

Where, W1 is the initial weight of the soil, W2 is the final weight after oven drying

Soil pH

Twenty grams of finely ground air-dried soil were placed in a beaker and 40 mL of distilled water was added to it. This mixture was stirred thoroughly and allowed to stand undisturbed for 1 hour. The pH of the suspension soil was then measured by using a pH meter (Hanna HI 208 model).

Nitrogen, phosphorus, and potassium content

The total nitrogen (%) in the soil was analyzed by Kjeldahl method (Kjeldahl, 1883). Organic matter was oxidized by treating the soil with concentrated sulphuric acid (H_2SO_4). The digestion of the soil with sulphuric acid was facilitated by using sodium sulfate (Na_2SO_4). The digestion solution liberated ammonia, which was collected in a boric acid solution and titrated with standardized dilute acid using a mixed indicator. Similarly, total phosphorus content (Kg/ha) was determined following the modified Olsen method (Olsen et al., 1954). The extracting solution was sodium bicarbonate at pH 8.5 with a soil-to-solutin ratio of 1:20. Total potassium content (Kg/ha) was measured using a photometric method. Potassium content in the leaching extract was made with 1N ammonium-acetate at pH 7.0. The soil extract was then measured using a flame photometer (Labtronics 65).

Data analysis

All the experiments were performed in triplicates for each sample, and the values are reported as mean ± standard deviation (S.D.). All statistical analyses were conducted using Microsoft Excel 2013.

Results

Total phenolic content (TPC)

The standard graph obtained from the standard solution of gallic acid used to determine the total phenolic content (TPC) in the sample extracts is shown in Figure 1. In both species, TPC showed an increasing trend with elevation. In *B. aristata,* the highest TPC (58.04 ± 2.08 mg GAE/g DW) and the lowest TPC content (53.29 ± 5.5 mg GAE/g DW) were observed in extracts of samples from 2400 m and 1900 m, respectively. Similarly, for *B. asiatica* the highest TPC (59.96 ± 1.23 mg GAE/g DW) and the lowest (34.13 ± 4.27 mg GAE/g DW) were observed in extracts of samples from 2400 m and 1400 m, respectively (Figure 2).

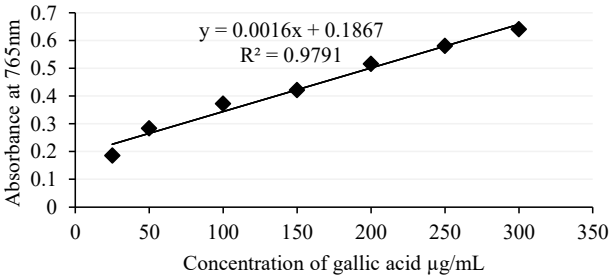

Figure 1: Standard curve of Gallic acid for calibration of total phenolic content

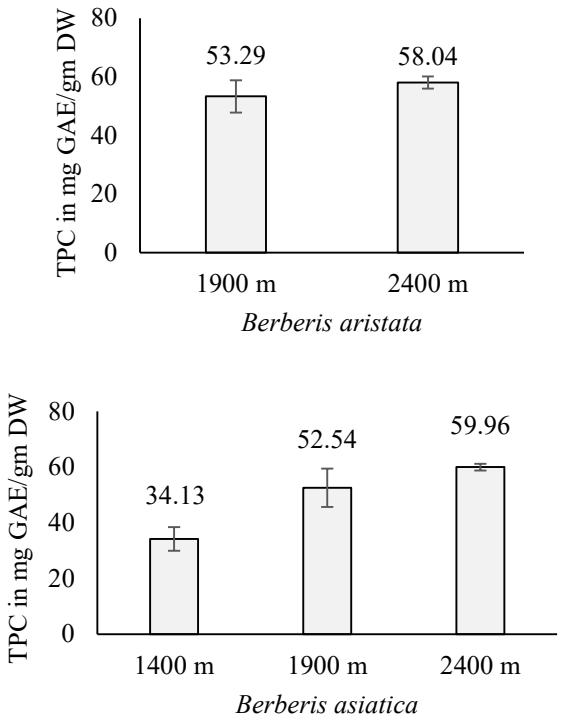

Figure 2: Total phenolic content present in the methanolic extract of two species of *Berberis* in different elevation. DW: Dry weight

Total flavonoid content (TFC)

A standard graph of quercetin used to determine total flavonoid content (TFC) is presented in Figure 3. In both species, TFC in the bark extracts showed a decreasing trend with increasing elevation. In *B. aristata,* the TFC highest value (29.85 ± 0.93 mg QE/g DW) was obtained in the extracts from 1900 m, while the lowest value (27.71 ± 1.08 mg QE/g DW) was from the extracts of samples from 2400 m. Similarly, in *B. asiatica* the highest TFC (31.7 ± 2.4 mg QE/g DW) was found in the sample from 1400 m, and the lowest (27.2 ± 1.2 mg QE/g DW) at 2400 m (Figure 4).

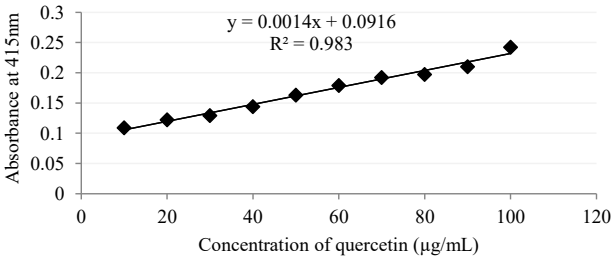

Figure 3: Standard curve Quercetin for calibration of the total flavonoid content

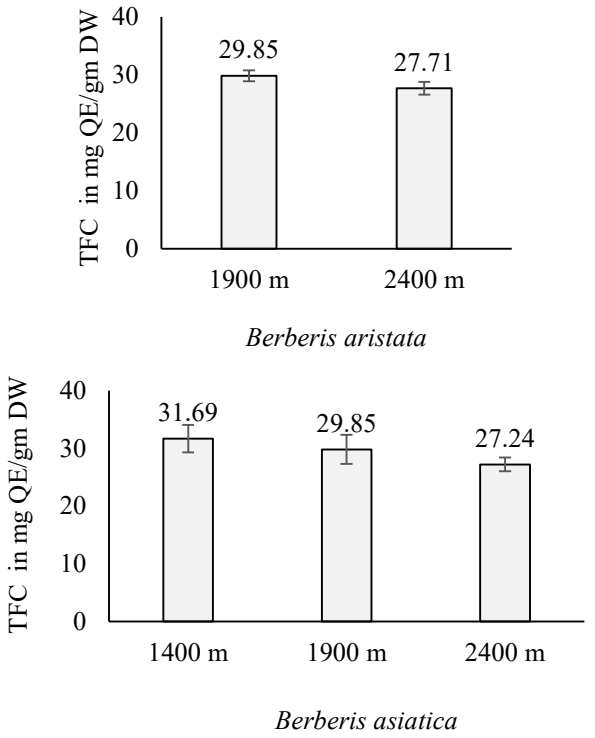

Figure 4: Total flavonoid content in methanolic bark extract of two species of *Berberis* from different elevations. DW: Dry weight

Berberine content

A standard graph of berberine was obtained from the standard marker compound, and the equation of the calibration curve used to quantify the amount of berberine in samples collected from different elevations for both *Berberis* species is shown in Figure 5.

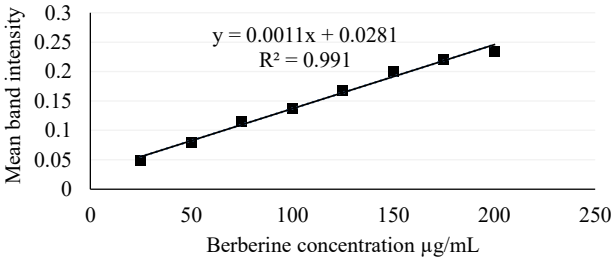

Figure 5: Standard curve of Berberine for calibration of the total berberine content

In both species, berberine content was highest in extracts from samples collected at lower elevations, and lowest in those of higher elevations. The berberine content in extracts of samples from corresponding elevations was comparable between the two *Berberis* species (Figure 6).

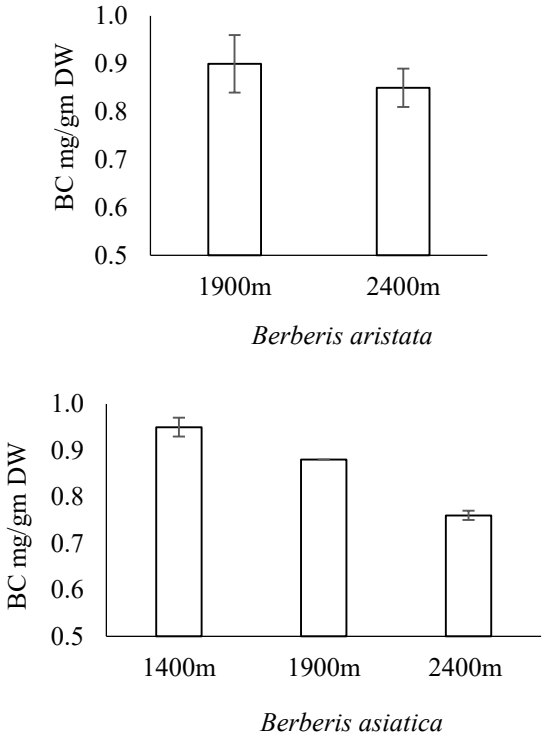

Figure 6: Berberine content (BC) in methanolic bark extract of two species of *Berberis* from different elevations

Antioxidant activity

IC_{50} value of DPPH radical scavenging activity for ascorbic acid was found to be 19.62 µg/mL, whereas the IC_{50} values for bark extracts of both *Berberis* species were significantly higher. In both species, the highest IC_{50} value was obtained in extracts from samples collected at the lowest elevation, and the lowest value was obtained in those from the highest elevation, indicating higher antioxidant potential in extracts of samples from higher elevation (Figure 7).

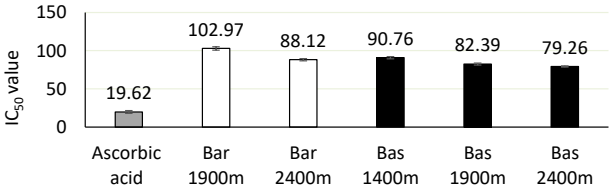

Figure 7: IC_{50} value for DPPH radical scavenging activity in methanolic extracts of two species of *Berberis* from different elevations. Bar: *Berberis aristata*, Bas: *Berberis asiatica*

Table: 2 Measurements of different parameters of soil samples of different elevation

SN	Soil characteristics	Elevation (m asl.)		
		1400	1900	2400
1.	Moisture content (%)	20.53±0.37	20.85±0.92	27.35±2.79
2.	Total Nitrogen content (%)	0.40±0.02	0.41±0.03	1.10±0.20
3.	Total Phosphorus content (P_2O5) (Kg/ha)	100.30±2.38	111.23±2.93	114.82±1.03
4.	Total Potassium content (K_2O) (Kg/ha)	174.20±3.54	294.80±4.85	710.20±0.93
5.	Soil pH	5.46±0.00	5.45±0.04	5.69±0.08

Impact of soil characteristics

The values of different soil parameters in the samples from different elevations are presented in Table 2. All tested parameters increased with increasing elevation.

Discussion

Plant phenols represent one of the major groups of compounds acting as primary antioxidants or free radical terminators (Sofidiya et al., 2012). These secondary metabolites are generally involved in defense mechanism against ultraviolet (UV) radiation and aggression by pathogens (Beckman, 2000). They are also responsible for antioxidant properties in cells (Velioglu et al., 1998)

The total phenolic content in aqueous extracts of *B. integerrima* and *B. vulgaris* was reported as 6.8 ± 0.1 and 2.3 ± 0.3 mg GAE/g dry mass (DM), respectively (Bayani et al*.,* 2016). Bhatt et al. (2018) reported phenol contents of 11.04 ± 2.2 and 13.73 ± 1.65 mg GAE/g in bark extracts of *B. aristata* and *B. thomsoniana*, respectively from Sagarmatha National Park, Nepal.

Similarly, Parajuli et al. (2012) reported total phenolic content of 80.2 ± 0.1 mg GAE/g in stem extracts of *B. aristata*. The phenolic contents in extracts of *B. aristata* in the present study is in the range between those reported by Bhatt et al. (2018) and Parajuli et al. (2012). Variation in phenolic content may be due to genetic variation, environmental conditions, timing of sample collection, and altitudinal factors (Ghimire et al., 2011). Furthermore, the present study also reveals an increase in total phenolic content with increased elevation in both the species of *Berberis*. Similar findings were also reported in *Thalictrum foliolosum* (Pandey et al., 2018).

Flavonoids are one of the most extensively studied groups of secondary metabolites in higher plants because they are the major constituents of plant pigments (Mol et al., 1998). Flavonoid variations are also considered a phytochemical adaptation to the abiotic and biotic environment (Dixon & Pavia, 1995). Flavonoids are potent antioxidants because of their free radical scavenging activity (Pal et al*.,* 2009). Total flavonoid content of *B. aristata* stem extract is reported to range from 3.03 ± 0.32 mg QE/g (Bhatt et al., 2018) to 122.2 ± 0.4 mg QE/g (Parajuli et al., 2012), while the bark extracts have shown values around 6.08 ± 0.50 mg QE/g (Bhatt et al., 2018). The TFC values in bark extracts of *B. aristata* in the present study are substantially higher than those reported by Bhatt et al. (2018). Furthermore, the present study found a negative correlation between TFC and elevation in both species of *Berberis*. Pandey et al. (2018) however, have reported a positive correlation between elevation and flavonoid content in *T. foliolosum.*

Antioxidants play a major role in protection against molecular oxidative damage (Evans, 2007). Precisely, antioxidants are compounds that delay or inhibit the oxidation of other molecules by interfering with the initiation or propagation of oxidizing chain reactions. Antioxidant compounds, such as phenolic acids, polyphenols, and flavonoids scavenge free radicals like peroxide, hydroperoxide, or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases. Antioxidant activity can be inferred based on the percentage of radical scavenging activity (%RSA) and the IC_{50} value. A higher %RSA corresponds to a lower IC_{50} value, indicating stronger antioxidant potential, and vice versa**.** The present study showed higher antioxidant activity from methanolic bark extracts of both species of *Berberis* in higher elevations and vice versa. A similar pattern was also reported for the methanolic fruit extract from *Elaeagnus angustifolia* (Sarirani et al*.,* 2017).

Berberine, a protoberberine alkaloid, is the major phytochemical responsible for many of the

pharmacological properties of *Berberis* species. A significant decrease in berberine content in all parts at high elevation areas as compared to those at lower elevation was reported for eight species of *Berberis* from western Himalaya (Chandra & Purohit, 1980). Similar results have been revealed for *Berberis* species from other parts of the Himalayas (Nautiyal, 2007; Maithani et al., 2014). High accumulation of alkaloids in low-elevation populations compared to higher elevations have been also reported for *Lupinus argenteus* (Carey & Wink, 1994) and *Thalictrum foliolosum* (Pandey et al., 2018). The findings of the present study are consistent with all these previous studies. The higher berberine content in extracts of plants from lower elevation may be attributed to their high requirement of defensive compounds to cope with greater abundance of microorganisms and herbivores in warmer climates in those areas.

Soil is the main source of water, and all the mineral nutrients required for the growth and development of plants. Its physical properties such as structure, texture, and depth determine the total capacity for storing available water for plant growth. Elevation strongly influences landscape topography, geology, and rainfall, consequently influencing soil moisture and texture, ground-water depth, hydrology, evaporation, soil type, and vegetation (Knoop & Walker, 1985). Usually, water deficit in soil has been suggested to increase the amount of secondary metabolites in different medicinal plants (Zobayed et al., 2005; Jaleel et al., 2008). The present study showed a decrease in both flavonoid and berberine contents with an increase in soil moisture. The decrease in berberine content with increasing soil moisture aligns with the findings of Andola et al. (2010). However, consequent decrease in phenol content with a decrease in soil moisture in this study suggests that moisture alone may not be the determining factor for phenol accumulation in plants.

Low nitrogen in soil induces flavonoid and isoflavonoid nod gene inducers and chemo-attractants for nitrogen-fixing symbionts resulting in increase in flavonoid content with decrease in nitrogen content in soil (Wojtaszek et al., 1993). However, in the present study, a gradual increase in total phenolic content (TPC) along with increasing levels of nitrogen, phosphorus, and potassium (NPK) was observed. This trend aligns with the findings from different previous studies. For instance, Liaqat et al. (2012) reported a decrease in total phenolics in blackberries with the decrease of potassium levels. Ibrahim et al. (2012) found that elevated potassium levels enhanced phenolic production in various plants. Regarding the correlation between amount of potassium and berberine content, Andola et al. (2010) observed positive correlation for the samples of both root and stem bark of *Berberis asiatica*. By contrast, the present study showed a negative correlation between potassium levels and berberine content.

Soil pH is governed by its mineral composition, especially nitrogen content. It strongly inûences abiotic factors, such as carbon availability (Andersson et al., 2000; Kemmitt et al., 2006), nutrient accessibility (Kemmitt et al., 2005; 2006; Pietri & Brookes, 2008), and the solubility of metals (Firestone et al., 1983; Flis et al., 1993). Soil pH decreases with increase in available nitrogen, thereby increasing soil acidity. In the present study, soil collected from 2400 was highly acidic compared to that of 1400 m and 1900 m, where there was only little variation in acidity. This difference in soil Ph may be due to higher nitrogen content in the soil from 2400 m and low varied nitrogen content in the soils from 1400 m and 1900 m.

Conclusion

The present study demonstrates that two species of *Berberis: B. aristata* and *B. asiatica,* collected from different elevations exhibit significant variations in their phytochemical and antioxidant profiles. Total flavonoid content (TFC) and berberine concentration decreased with the increase in elevation while total polyphenol content and antioxidant activity increased with rise in elevation. Furthermore, soil parameters like moisture content, pH, and NPK levels, varied along the elevation gradient, in the soil as the environmental factor also showed variation along the elevation gradient suggesting their role in influencing the biological activity of both *Berberis* species. As different phytochemicals respond to elevations in different manners, it is worth exploring similar types of biological activities in other medicinal plant species. In this study, the unavailability of certain chemicals has limited phytochemical screening to a limited number of compounds, which presents opportunities for future research. Furthermore, the semiquantitative estimation of berberine is only for reference purposes and may not give the exact quantity of the marker compound in the bark extracts. However, the approach taken in the present study may be useful for varietal or population-wise screening of medicinal plants rich in amount of specific marker compounds.

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Author's contribution statement

R. Awal: Sample collection, lab work, data collection, analysis, draft writing; **D. R. Pant:** Conception and design, manuscript revision, supervision; **G. P. Joshi:** Conception and design, result interpretation, manuscript revision, supervision.

Data Availability

The data used in this study are accessible upon request to the corresponding author.

Conflict of Interest

The authors declare no conflict of interest.

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