Phytochemical Screening and Evaluation of Antioxidant and Antibacterial Potential of Selected Species of Gentiana from Nepal Himalaya

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ISSN : 2382-5359(Online), 1994-1412(Print)

DOI:

https://doi.org/10.3126/njst.v21i1.49913

Date of Submission: 22/10/2020

Date of Acceptance: 29/11/2021

ABSTRACT

Various species of *Gentiana* find their use to treat various digestive and topical ailments in traditional medicine in Nepal. The present work reports the results of preliminary phytochemical analysis and biological activities of extracts of four different species of *Gentiana* (*G. depressa*, *G.ornata*, *G. urnula*, and *G. capitata*) from Nepal Himalaya. Furthermore, the study also reports a semi-quantitative estimation of major bioactive compounds in those extracts. The total flavonoid content was found to be highest (19.09±0.97mgQE/g) in methanol extracts of *G. capitata* and lowest (4.22±0.66 mgQE/g) in aqueous extracts of *G. urnula*. The highest amount (79.2±19.19 mgGAE/g) of total phenolic content was observed in methanolic extracts of *G. depressa*, while the lowest amount (37.11±2.18 mgGAE/g) was observed in aqueous extract of *G. urnula*. The methanol extract of *G. depressa* showed the best antioxidant activity among the *Gentiana* species tested. Extracts of all the species tested showed weak antibacterial activity even at the highest concentration of the extract. Semi-quantitative estimation showed that swertiamarin was in higher quantities than amarogentin and mangiferin. The highest concentration of swertiamarin and mangiferin (0.109±0.013 mg/g and 0.018±0.001 mg/g, respectively) was identified in *G. ornata*, while the highest concentration of amarogentin (0.075±0.005 mg/g) was observed in *G. capitata*. These results justify the folklore use of these species in traditional medicinal practices in Nepal.

Keywords: *Gentiana*, Bioactivity, Phytochemistry, Markers, Quantification

1. INTRODUCTION

Traditional medicine forms an integral part of ethnopharmacology that uses medicinal plants against many human ailments, from cough, cold and parasitic infections to various chronic diseases (Cragg *et al.* 1997). It utilizes biological resources and indigenous knowledge about traditional plant groups (Timmermans 2003). Traditional medicine has been practised in different forms in different parts of the world.

The family Gentianaceae consists of flowering plants comprising approximately 87 genera and over 1500 species worldwide except for the Antarctic (Struwe *et al.* 2002). The genus *Gentiana* comprises 400 species, which, like many other species of the gentian family, are distributed at higher altitudes around the Himalayan foothills. Nepal's geography harbors 44 species of *Gentiana*, with three species endemic to Nepal (Joshi *et al.* 2000).

Gentiana species are small herbs of higher elevations with ethnomedicinal values. The roots of great yellow gentian (*G. lutea*) are used to treat loss of appetite and digestive/dyspeptic problems (Anonymous 2018). Flowers of *G. algida* are used in lung disorders and skin diseases, while the flowers and leaves of *G. robusta* are used for diarrhea, food poisoning, TB, and various other ailments in Mustang district Nepal (Pandey 2006; Anonymous 2016). Similarly, rhizomes of *G. kurroo* are used in wounds, ulcers, skin diseases, and urinary infections (Anonymous, 2016). *G. depressa* is used ethnomedicinally as a digestive and appetizer. The same species is also used for cough, cold, and fever (Lamichhane *et al.* 2014). Pills made from a mixture of dried powders from roots of *G. depressa*, *G. ornata, G. phyllocalyx* and *G. tubiflora* have been reported to be used to treat colds and headaches in Arunachal Pradesh (Chakraborty *et al.* 2017). Young shoots of *G.urnula* are also vegetables in Bhutan (Yeshi *et al.* 2019). Similarly, flowers of *G. urnula* are used as one of the ingredients in Mustang Tea, one of the herbal teas made by following the 'Sowa Rigpa' art of traditional formulations (https://himalayasherpaherbs.net/en/teas.html). The flowers of *G. urnula* are also used in traditional Tibetan medicine to treat poisoning, the common cold, and dysentery (Gyatso & Hakim 2010).

Plants belonging to *Gentiana*ceae are famous for their pharmacological properties due to the presence of bitter glycosides (Hostettmann-Kaldas *et al.* 1981). The occurrences of iridoids, xanthones, mangiferin, and c-glucoflavones provide these plants with therapeutic properties. Secoiridoid glucosides such as Swertiamarin appear to be present in all species investigated. However, xanthones are not universally present in all Gentianaceae (Hostettmann-Kaldas *et al.* 1981; Jensen & Schripsema 2002; Singh 2008). They also possess phytochemicals like amarogentin, bellidifolin, gentianine, and swerchirin that show significant anti-inflammatory, analgesic, anti-asthmatic, antihistaminic, antimalarial, antiamoebic, hepatoprotective, and hypoglycemic properties (Jensen & Schripsema 2002; Singh 2008; Mirzaee *et al.* 2017).

The present work aims to discover the phytochemical properties and bioactivity of selected species of *Gentiana* L. from Nepal. It also estimates major phytochemicals like amarogentin, mangiferin, and swertiamarin semi-quantitatively usingthin-layer chromatography.

2. MATERIALS AND METHODS

2.1 Sample Collection and Extract Preparation

The whole plant samples of different species of *Gentiana*, namely *G. capitata* Buch.-Ham. ex D. Don, *G. depressa* D. Don, *G. ornata* (D. Don) Wall. Ex Grise*b.* and *G. urnula* Harry Sm.were collected from wild habitats in Langtang National Park, Central Nepal. The voucher specimens were collected and deposited as herbarium sheets at National Herbarium and Plants Laboratories (KATH) for future reference. The collected samples were cleaned and shade dried until constant weight. The dried samples were ground into a fine powder using an electric blender.

Ten grams of the powdered sample from each species was taken in a conical flask, and then 100 mL of solvent (methanol or water) was poured into it. The solvent was then left to percolate the powder with intermittent sonication for two hours. It was then filtered through Whatman No 1 filter paper, and extraction was repeated. The filtered extracts were allowed to evaporate separately at room temperature under aseptic conditions.

2.2 Phytochemical Screening

The crude methanol and aqueous extracts were used to screen for the presence of various secondary metabolites using the standard protocols described by Harborne (1998) with slight modifications; Mayer's Test for Alkaloids, Acetone Water Test for resins, Ferric Chloride Test for Phenols, Alkaline Reagent Test flavonoids, Modified Brontrager's Test for glycosides, Copper Acetate Test for diterpenes, Gelatin Test for tannins and Salkowski's Test for phytosterols.

2.3 Determination of Total Flavonoid Content (TFC)

The total flavonoid content in the plant extract was estimated using the Aluminium Chloride colorimetric method as described in Chang *et al.* (2002). Firstly, 0.25 mL of extract (10 mg/mL) was separately mixed with 0.75 mL of ethanol, 0.05 mL of the 10% aluminium chloride, 0.05 mL of the 1 M potassium acetate, and 1.4 mL of the distilled water. The reaction mixture was incubated at room temperature for 30 minutes. Then absorbance of the mixture was measured at 415 nm using the UV-spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). A standard calibration curve of a methanolic solution of quercetin (Sigma Aldrich, Germany) in the concentration range of 10 to 100 µg/mL was prepared. Using the standard curve. The total flavonoid content was expressed in milligrams of Quercetin equivalent per gram of the dry mass (mg QE/g).

2.4 Determination of Total Phenolic Content (TPC)

The total polyphenol content of *Gentiana* species was determined using the Folin–Ciocalteu phenol reagent (Singleton *et al.* 1999). For this, 0.1 mL of each extract (2.5 mg/mL) was separately mixed with the 1 mL of Folin–Ciocalteu phenol reagent (Merck Ltd, India) (1:10 dilution with the distilled water) and 0.8mL of aqueous 1.0 M Na2CO3 solution. The reaction mixture was allowed to stand for about 15 minutes, and then absorbance was measured at 765 nm using the UV-spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). A calibration curve was obtained using Gallic acid (Moly Chem, India) in methanol using a concentration ranging from 25-250 µg/mL as standard. Based on this standard graph, the concentration of the individual samples was calculated. Total polyphenol content was expressed in the milligrams of the Gallic acid equivalent per gram of the dry mass (mg GAE/g).

2.5 Determination of Antioxidant Activity

The antioxidant activity of the extract of *Gentiana* species and standard (Ascorbic acid) was assessed based on the radical scavenging effect of the stable 1, 1- diphenyl-2 picrylhydrazyl (DPPH) - free radical activity (Roy *et al.* 2010). For this, 0.5 mL of samples of plant extract and ascorbic acid of each concentration were taken separately in clean test tubes. To this sample, 0.5 mL of the 0.2 mM solution of DPPH in methanol was added. The tubes were shaken uniformly for proper mixing and incubated in the dark for 30 minutes. The control was prepared as above but without the plant extract or ascorbic acid, and methanol was taken as blank. The absorbance was taken on a spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5) at 517 nm. Different ascorbic acid concentrations (10-100 µg/mL) were prepared in methanol to generate a standard curve for ascorbic acid. Different concentrations for methanol (from 5.0 to 100.0 µg/mL) and aqueous (50.0 to 500.0 µg/mL) extracts were used and compared with ascorbic acid as standard. Then the radical scavenging activity was calculated using the following formula.

% Radical scavenging activity = [(Control abs-sample abs)/Control abs)] x100

2.6 Determination of Antibacterial Activity in Crude Extracts

ATCC cultures of *Escherichia coli* (25922), *Salmonella typhimurium* (14028), *Klebsiella pneumonia* (700603), *Staphylococcus aureus* (25923) and *Enterococcus faecalis* (29212) were provided from the microbiology laboratory of Central Department of Biotechnology, Tribhuvan University. The antibacterial test was performed by the modified agar well diffusion method. Concentration-dependent antibacterial activity was tested on varying concentrations of methanol and aqueous plant extracts (100 mg/mL, 50 mg /mL, 25 mg /mL and 12.5 mg /

mL). DMSO was used as the negative control, while Streptomycin (25 mg/mL) was taken as the positive control. The plates were incubated on the microbial incubator overnight at 37°C, and the zone of inhibition was observed for individual plant extracts for individual bacteria at different concentrations. Mean±SD values for the zone of inhibition in mm tested against three Gram-negative (*E. coli, S. typhiimurium* & *K. pneumoniae*) and two Gram-positive (*S. aureus* & *E. faecalis*) bacteria of their methanol and aqueous extracts were reported.

2.7 Detection and Estimation of Amarogentin, Mangiferin and Swertiamarin

We used silica TLC plates to detect the presence of three bioactive compounds (amarogentin, mangiferin and swertiamarin) in methanol extracts. The pre-coated TLC plates (Merck-Millipore, Germany) of 250 microns thickness were used for the quantitation from the crude methanol extracts and their standard compounds. 2.0 mg/mL stock of each standard compound (amarogentin, mangiferin and swertiamarin) was prepared, and 5.0 µL of methanol extracts of each sample was carefully run in suitable solvent systems. Elution was done using ethyl acetate:methanol:water (77:15:8 v/v/v) for amarogentin and swertiamarin, and ethyl acetate:methanol:formic acid:water (67:17:8:8v/v/v) for mangiferin. All chromatographic TLC separations were performed at room temperature, and detection was carried out by UV light at 354 nm (Wagner *et al.* 1984). The visualized compounds from TLC were quantified using respective standards. First, calibration curves were prepared from standard compounds and peak areas in terms of intensity (pixels) were plotted against concentration. The pixel ratio was calculated from visualized methanol extracts against their reference compounds using GelQuant.NET software (Biochemlab Solutions 2014).

2.8 Statistical Analysis

All the experiments were performed in triplicates, and the data are reported as mean±SD. Linear regression analysis was used to calculate total phenol content, flavonoid content and DPPH radical scavenging values. All the statistical analyses were performed using GraphPad Prism7 and Microsoft Excel 2013. We used linear regression and one-way analysis of variance (ANOVA) test for TFC, TPC, IC50 and Tukey's test for quantification of compounds among different species. Differences at $P<0.05$ were considered significant.

3. RESULTS AND DISCUSSION

3.1 Extract Yield

The total amount of plant extracts isolated and their characteristics varied with species. The percentage yield of extract in methanol ranged from7.34 % (in *G. depressa*) to 11.9% (in *G. capitata*) while that in water ranged from 7.8% (in *G. ornata*) to 10.40% (in *G. urnula*). Lamichhane *et al.* (2014) also reported comparable percentage yield values (6.11%) for methanolic extracts in *G. depressa*. In general, alcohols are the preferred solvents in the extraction of secondary metabolites from gentians as the secoiriods and xanthones are more soluble in alcohols (Jensen & Schripsema 2002), however, the results of the present investigation (Table 1) indicate that extract yield may be higher in water in some species like *G. depressa* and *G. urnula*. Since the extract yield in any solvent depends on the polarity of the solvent and that of metabolites, the opposite trend in extract yield in two solvents in selected species of *Gentiana* from Nepal may reflect the differences in the polarity and relative proportions of different kinds of secondary metabolites in those species.

Table 1: Percentage yield and physical characteristics of plant extracts obtained from selected species of *Gentiana*

Plant Extract	Yield	Extract Colour	Consistency
GCA-Aq	8.4	Green	Sticky
GCA-Met	11.9	Deep Green	Oily
GDE-Aq	8.8	Grey	Greasy
GDE-Met	7.3	Grey	Greasy
GOR-Aq	7.8	Brown Grey	Greasy
GOR-Met	8.1	Grey	Sticky
GUR-Aq	10.4	Light Brown	Greasy
GUR-Met	8.7	Brown	Greasy

Legend: GCA - *G. capitata*, GDE - *G. depressa*, GOR - *G. ornata*, GUR - *G. urnula*; Aq- Aqueous extract; Met- methanol extract

3.2 Qualitative Phytochemical Screening

The preliminary phytochemical screening revealed the presence of most of the tested phytochemicals like alkaloids, flavonoids, terpenoids, steroids, and glycosides) in methanolic extracts of all the species tested, many phytochemicals were absent from aqueous extracts (Table 2). The occurrences of phytoconstituents are similar to those reported for *G. kurroo* (Wani *et al.* 2011) and *G. depressa* (Lamichhane *et al.* 2014).

Table 2: Preliminary screening of major phytochemicals present in the methanol and aqueous extracts of selected species of *Gentiana*

Plant Extracts	Alkaloids	Resins	Phenols	Flavonoids	Glycosides	Diterpenes	Tanins	Phytosterols
GCA-Aq	+	-	-	-	+	-	-	+
GCA-Met	+	-	+	+	+	+	+	+
GDE-Aq	+	+	-	+	-	-	-	+
GDE-Met	+	+	+	+	+	+	-	-
GOR-Aq	+	-	+	-	+	+	+	-
GOR-Met	+	+	+	+	+	+	+	+
GUR-Aq	+	-	+	-	+	-	-	+
GUR-Met	+	+	+	+	+	+	+	+

Legend: GCA - *G. capitata*, GDE - *G. depressa*, GOR - *G. ornata*, GUR - *G. urnula* ; Aq- Aqueous extract; Met-methanol extract '+'= presence, '-'= absence

3.3 Total Flavonoid Content

The methanolic extracts possessed a significantly higher ($p \leq 0.05$) amount of total flavonoid content (TFC) than aqueous extracts. In the case of methanolic extracts highest (19.09±0.97 mg QE/g) and lowest (11.31 ± 0.49 mg QE/g) amount of TFC was observed in *G. capitata* and *G. ornata*, respectively (Figure1A). However, in aqueous extracts, the highest (7.85 ± 0.37 mg QE/g) and lowest (4.22±0.66 mg QE/g) values of TFC were observed in *G. ornata* and *G. urnula*, respectively. Flavonoids consist of flavones, isoflavones, flavonols, catechins, anthocyanidins, and chalcones and show a broad range of biological effects such as anti-inflammatory, antiviral, antioxidant, and anticancer effects (Havsteen 2002). Among the species of *Gentiana* from the Himalayas, *G. kurroo* is the most extensively used medicinal plant. Baba and Malik (2014) reported TFC content of 42 ± 2.2 and 20 ± 1.5 mg rutin/g in methanolic extracts of roots and leaves, respectively, in *G. kurroo*, Similarly, Khanal *et al.* (2015) reported higher TFC content in the range of 18.41 ± 0.19 to 26.16 ± 0.25 mg QE/g in methanolic extracts of different species of *Swertia* from Nepal. This variation in TFC might be due to species diversity, geographical locations, and extraction methods.

3.4 Total Phenolic Content

The total phenolic content (TPC) present in the methanol and aqueous extracts of different species of *Gentiana* is presented in Figure 1*B*. For the methanol extracts, the highest TPC (79.2±6.39 mg GAE/) was found in *G. depressa* and the lowest TPC (44.6±3.97 mg GAE/g) in *G. capitata*. For the aqueous extracts, *G. depressa* had the highest (59.64±3.64 mg GAE/g), and *G. urnula* had the lowest (37.11±2.18 mgGAE/g) TPC. All species, except *G. capitata*, showed significantly higher ($P<0.05$) TPC in the methanol extracts than in aqueous extracts. TPC in *G. depressa* and *G. ornata* were significantly higher ($P<0.05$) than that of the remaining two *Gentiana* species. For the aqueous extracts, TPC in *G. depressa* was significantly higher than in all other species ($P<0.05$), however, no statistically significant difference in the TPC was observed among aqueous extracts of *G. urnula*, *G. ornata*, and *G. capitata*. Phenolic components are the primary antioxidants that donate hydrogen to free radicals and scavenge radicals (Dembinsca-Kiec *et al.* 2008). It has been recognized that the phenolic-linked antioxidant property in medicinal plants are good antioxidants since the generation of reactive oxygen species has been linked to most systemic diseases (Chen *et al.* 2011).

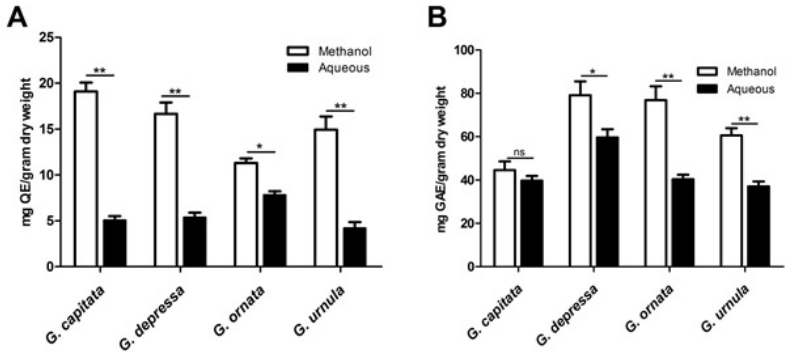

Fig. 1: Total flavonoids (A) and total polyphenol content (B) of the methanol and aqueous extracts of selected species of *Gentiana*.Mean±SD values are reported (n=3). Within each species, * represents a statistically significant difference (P<0.05), and ** represents a statistically significant difference between methanol and aqueous extracts (P < 0.01).

Wijekoon *et al.* (2011) depicted water as the least effective solvent for the extraction of phenolic compounds. The phenols dissolve better in alcohol than in water, which is also reflected in the present observation. They reported higher phenolic content in 50% methanol extracts compared to the water extract of *G. lutea*. However, Nastasijević *et al.* (2012) reported slightly higher total polyphenol content in water than different concentrations of aqueous ethanol and methanol extracts in *G. lutea*. Baba and Malik (2014) reported TPC in the methanolic root and leaf extract of *G. kurroo* to be 68±2.4 GAE/g DW and 34±1.8 GAE/g DW, respectively. Similarly, Nastasijević *et al.* (2012) reported the phenolic content in the 80% methanol extract of *G. lutea* to range between 20.74 mgGAE/g in rhizomes 37.02 mgGAE/g in generative parts. In the present investigation, TPC in methanol extracts of the whole plant of *G. depressa*, *G. ornata* and *G. urnula* were found in the range between those reported for *G. lutea* and *G. kurroo*.

3.5 DPPH Free Radical Scavenging Assay

The percentage radical scavenging activity of ascorbic acid and all the methanol extracts showed a concentration-dependent inhibition. A plant with higher radical scavenging activity depicted a lower IC_{50} value. The plant extract with the lowest IC_{50} value contains better antioxidant properties. Methanol and aqueous antioxidant activity methanol, and aqueous was evaluated by DPPH Free Radical Scavenging Assay using ascorbic acid as standard (Fig. 2A and 2B). Antioxidants are important compounds that protect the body from damages caused by free radical-induced oxidative stress by acting as modulators of cellular signalling processes (Rammal *et al.*, 2012).

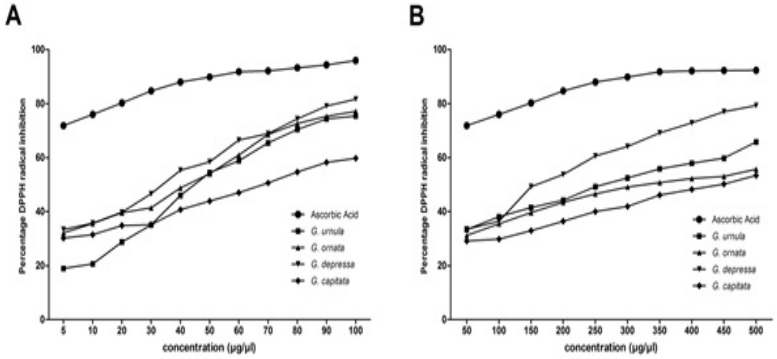

Fig. 2. Radical Scavenging activity (%) of the methanol (A) and aqueous (B) extracts of selected species of *Gentiana*

The methanol extracts showed better scavenging properties than their aqueous extracts in all *Gentiana* species, with significant differences ($P<0.05$) in their IC_{50} values. This variation can be attributed to the significantly higher TPC and TFC in methanol extracts. The IC_{50} value for ascorbic acid (standard) was 29.55±2.13µg/mL. The lowest and highest IC_{50} values were observed in methanol extracts of *G. depressa* (39.57±0.95 µg/mL) and *G. capitata* (80.12±3.69 µg/mL) (Fig. 3). The species with lower IC_{50} are considered better antioxidants (Roy *et al.* 2010). Therefore, *G. depressa* has the best antioxidant activity among the studied *Gentiana* species. The IC_{50} value of ascorbic acid was significantly ($P<0.05$) lower than that of all four *Gentiana* species. Among the species, the IC_{50} value of the methanol extract of *G. depressa* was significantly ($P<0.05$) lower than other species. Likewise, the IC_{50} value of *G. ornata* and *G. urnula* were significantly lower than that of *G. capitata*. Nastasijevic *et al.* (2012) reported the IC_{50} values of 80% methanol extracts of *G. lutea* to be higher than 200µg/mL. The IC_{50} values of the *Gentiana* species investigated here were lower than that of *G. lutea*.

In contrast to the methanol extracts, aqueous extracts reported significantly reduced antioxidative capacity. The IC_{50} values of each aqueous extract were greater than 150µg/mL, revealing that aqueous extracts had less antioxidant capacity while using the crude extracts in ethnomedicine. Among the aqueous extracts, *G. depressa* showed the lowest IC_{50} of 183.193±28.75 µg/mL (Figure3). The plant with the highest IC_{50} or least radical scavenging activity was *G. capitata* at 433.30±42.83 µg/mL. The IC_{50} value of the aqueous extract of *G. depressa* was significantly ($P<0.05$) lower than the other *Gentiana* species. Most antioxidant activities from plant sources are correlated with phenolic-type compounds (Cowan 1999). *Gentiana* species extracts possess many phenolic compounds with a wide range of biological and pharmacological properties, including antioxidant, analgesic and hepatoprotective activities (Mirzaee *et al.* 2017; Singh 2008). This justifies the preferential selection of species of *Gentiana* except for *G. capitata* for various ethnopharmacological uses by local communities.

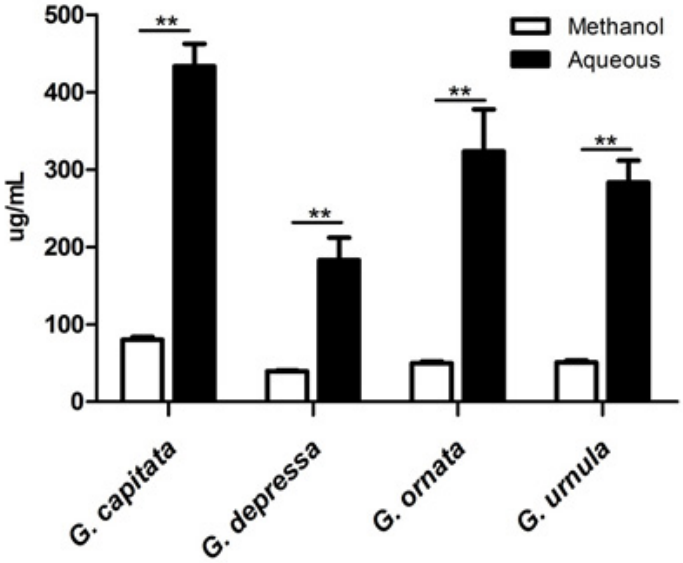

Fig. 3. Radical Scavenging activity of extracts of selected species of *Gentiana* expressed as IC_{50}:Mean±SD (n=3). Within each species, * represents a statistically significant difference ($P<0.05$) and ** represents a statistically significant difference between methanol and aqueous extracts ($P<0.01$)

3.6 Antibacterial Activity

With the increase in antibiotic resistance, researchers are turning to ethnopharmacology as medicinal plants serve as the primary tool for developing alternative drugs. Since methanol extracts of various species of *Gentiana* contained more bioactive and pharmacologically important compounds like phenolics and flavonoids than aqueous extracts, methanol extracts showed higher antibacterial activity as expected. However, very weak antibacterial activity was observed in the extracts of *Gentiana* in the present investigation. Growth inhibition for Salmonella typhii was observed at 100mg/mL, 50 mg/mL and 25mg/mL for all samples, while that for *E. coli* was observed at concentrations of 50mg/ml and above in both methanol and aqueous extracts. In the case of other bacterial strains, inhibition was observed only at 100 mg/mL in all the extracts (Table 3). The methanol extracts of *G. depressa* and *G. ornata* inhibited E. coli at all concentrations, while that of the remaining two species showed inhibition only at higher concentrations (50 and 100 mg/mL). Methanolic extracts of all the species tested showed antibacterial activity against *Klebsiella pneumoniae* at concentrations. Among the species tested, the extracts of *G. depressa* and *G. capitata* showed the highest zone of inhibition, possibly due to high TPC and TFC in their extracts.

Table 3. Antibacterial activities of extracts of selected species of *Gentiana*

Species	Bacterial Strain	Zone of Inhibition (mm) at different concentrations of Extracts								+ve control
		100 mg/mL		50 mg/mL		25 mg/mL		12.5 mg/mL		
		MET	AQ	MET	AQ	MET	AQ	MET	AQ	
	ECO	7±1.4	8 ± 1.78	6 ± 1.64	7.5 ±1.55	-	-	-	-	25 ± 3.67
	EFA	7 ± 2.6	9.5 ± 2.21	6 ± 2.04	-	-	-	-	-	28 ± 4.27
GCA	KPN	11 ± 1.83	9 ± 1.61	9 ± 2.56	7 ± 1.82	7 ± 1.98	6.5 ± 1.5	-	-	37 ± 6.47
	SAU	-	8 ± 1.66	-	-	-	-	-	-	29 ± 3.86
	STY	10 ± 2.8	9.5 ± 1.84	7 ± 1.84	9 ± 2.14	6 ± 0.8	7 ± 0.84	-	-	30 ± 4.94
	ECO	12 ± 2.23	8 ± 1.41	9 ± 1.27	8 ± 1.25	8 ± 1.18	-	8 ± 1.15	-	25 ± 5.63
	EFA	9 ± 2.07	7.5 ± 2.15	8 ± 2.3	-	6 ± 1.75	-	-	-	28 ± 4.84
GDE	KPN	14 ± 2.3	8.5 ± 2.1	12 ± 2.1	7.5 ±1.58	10 ± 1.47	-	7 ± 2.2	-	36 ± 4.82
	SAU	12 ± 1.83	7 ± 1.52	9 ± 1.41	7 ± 1.5	6 ± 1.12	-	-	-	29 ± 3.45
	STY	10 ± 1.85	9 ± 0.95	8 ± 1.56	8 ± 1.68	7 ± 1.06	8 ± 0.88	-	-	30 ± 4.66
	ECO	14 ± 2.29	8 ± 1.54	12 ± 2.04	8 ± 1.36	8 ± 1.62	-	6 ± 1.08	-	25 ± 3.88
	EFA	14 ± 3.17	7 ± 1.55	12 ± 2.74	-	8 ± 2.08	7 ± 1.84	6 ± 1.88	-	28 ± 5.64
GOR	KPN	18 ± 2.94	9.5 ± 1.97	16 ± 2.63	8 ± 1.5	16 ± 1.55	8 ± 1.27	12 ± 1.26	-	41 ± 5.08
	SAU	10 ± 3.8	8.94 ± 1.68	8 ± 2.6	-	6 ± 1.07	-	-	-	29 ± 3.46
	STY	9 ± 2.6	10 ± 2.23	7 ± 2.4	9 ± 2.14	6 ± 1.34	8 ± 1.17	-	-	30 ± 3.34
	ECO	10 ± 2.14	9 ± 2.24	8 ± 2.56	7 ± 1.84	7 ± 1.5	-	-	-	25 ± 4.63
	EFA	12 ± 3.89	6.5 ± 2.08	8 ± 2.24	-	7 ± 1.81	6.5 ±1.34	6 ± 1.64	-	28 ± 4.58
GUR	KPN	11 ± 2.12	6.5 ± 1.63	8 ± 1.83	-	6 ± 1.22	6.5 ± 2.89	-	-	38 ± 6.14
	SAU	9 ± 1.98	6.5 ± 2.96	18 ± 2.41	5.5 ±1.41	6 ± 1.06	-	-	-	29 ± 3.85
	STY	7 ± 2.08	10 ± 2.08	6 ± 0.89	-	-	-	-	-	30 ± 3.58

Legend: GCA - *G. capitata*, GDE - *G. depressa*, GOR - *G. ornata*, GUR - *G. urnula*; Aq- Aqueous extract; Met-methanol extract; ECO- *Escherichia coli*, EFA- *Enterococcus faecalis*, KPN- *Klebsiella pneumoniae*, SAU- *Staphylococcus aureus*, STY- *Salmonella typhiimurium.*

Previous studies like that of Bhattarai *et al.* (2009) also reported the antimicrobial activity of *G. robusta* extracts on *S. aureus, B. subtilis, P. aeruginosa* and *E. coli.* Similarly, Lamichhane *et al.* (2014) proved the medicinal importance of *G. depressa* by showing the antimicrobial activity of its methanol extract against *S. typhii, Cytobacter freundii, P. aeruginosa, E. coli* and *Shigella dysenteriae*. Inhibition by the methanol extract is due to the presence of tannins, flavonoids, alkaloids and glycosides known to possess antimicrobial potential against bacteria and fungi (Scalbert, 1991). These compounds confer antimicrobial properties by disrupting the the cytoplasmic membrane, disrupting the proton motive force and coagulation of cell contents (Kotzekidou *et al.* 2008).

3.7 Identification and Estimation of Amarogentin, Swertiamarin and Mangiferin

All four *Gentiana* species were tested for their methanolic extracts' phytochemicals like Amarogentin, Mangiferin, and Swertiamarin using Thin Layer Chromatography (TLC). The highest content of amarogentin (0.075±0.005 mg/g) was observed in *G. capitata* and the lowest (0.026±0.006 mg/g) in *G. depressa* extracts. The content of amarogentin in other species was in between these two extremes (Fig. 4). The amarogentin content of *G. capitata* was significantly ($P<0.05$) higher than that of the rest of the species. Amarogentin is the bitterest compound that is responsible for Gentiana's bitter taste (Quercia *et al.* 1980). Singh (2008) mentions that amarogentin can be obtained from *G. lutea* and *G. macrophylla,* along with *Swertia chirayita* and a few other plants from Gentianaceae. Amarogentin content was present in 0.05 to 0.025% of plant dry weight in methanol extracts of Gentian roots by HPLC (Quercia *et al.* 1980). However, Azman *et al.* (2014) did not observe amarogentin content in detectable amounts in 50% methanol extract and aqueous extract. The amount of amarogentin found in this study (0.026 to 0.075 mg/g) is lower than the value reported for *G. lutea* (Aberham *et al.* 2007).

The methanol extract of *G. ornata* showed the highest (0.109±0.013 mg/g), while that of *G. depressa* showed the lowest (0.038±0.002 mg/g) swertiamarin content. The swertiamarin content of the methanol extract of *G. ornata* was significantly greater than that of the rest of the species tested (Fig. 4). Besides, the swertiamarin content in *G. urnula* was also significantly ($P<0.05$) greater than that of *G. capitata* and *G. depressa*. Olennikov *et al.* (2015) quantified swertiamarin in decoctions of *G. algida, G. decumbens, G. macrophylla* and *G. triflora* by microcolumn-RP-HPLC-UV procedure. They reported swertiamarin content ranging from trace amount in *G. triflora* and *G. decumbens* to as high as 26.38±0.47µg/mL in *G. algida.* Swertiamarin was also quantified in three species of *Gentiana* (*G. davidiivar. formosana, G. arisanensis* and *G. scabrida var. punctulata*) from Taiwan and compared with that in *G. scabra.* The swertiamarin content in *G. davidii* and *G. scabra* was reported to be substantially higher, 2.66 and 2.52mg/g, respectively (Huang *et al.* 2013). Aberham *et al.* (2007) reported the swertiamarin content in 12 commercial samples of *G. lutea* root to be between 0.21% and 0.45%. These findings in different *Gentiana* species are notably higher than the values obtained in this study. This difference in swertiamarin content may be due to species diversity, habitat, extraction methods used, the efficiency of the method used for quantification or other criteria influencing the handling of the samples.

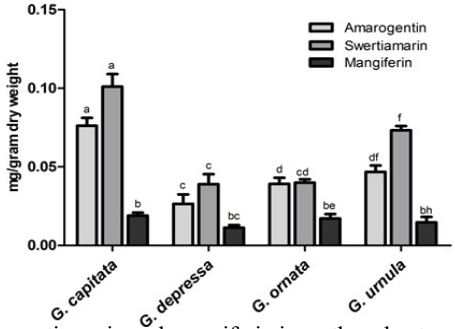

Fig. 4. Estimation of amarogentin, swertiamarin and mangiferin in methanol extracts: Semi-quantitative estimation of three major compounds from methanol extracts of different *Gentiana* species. Tukey's Honest Significant Difference Test employed on Mean±SD values of each compound content. Mean values with different letters indicate significant differences ($P<0.05$) among different species for each compound.

The highest content of mangiferin (0.018±0.001 mg/g) was reported in *G. ornata* and the lowest (0.012±0.002 mg/g) in *G. Depressa* (Fig. 4). The semi-quantitative estimation of mangiferin showed no significant differences in mangiferin content. The semi-quantitative estimation of mangiferin reported no significant differences in mangiferin content amongamong *Gentiana* species in the present investigation. Mangiferin was detected in lower amounts, with the highest content occurring in *G. ornata* compared to methanol extracts of other *Gentiana* species. Olennikov *et al.* (2015) reported mangiferin for the first time in *Gentiana triflora* at 19.86 µg/mL concentration. It was, however, not detected in *G. macrophylla, G. algida* and *G. decumbens*. The authors also concluded that this compound occurs sporadically in the members of *Gentiana*ceae. A detectable amount of Mangiferin was also reported in chloroform fraction, ethyl acetate fraction and n-butanol fraction of *G. asclepiadea* (Mihailovic *et al.*, 2011). However, Phoboo *et al.* (2010) reported a higher amount of 0.46 and 0.4 mg/g of mangiferin, respectively in aqueous and ethanolic root extracts of *S. chirayita* from Nepal. All four of the *Gentiana* species used in this study showed the presence of swertiamarin, amarogentin and mangiferrin, albeit in low concentrations. Detection of these bioactive compounds and the demonstration of a certain antimicrobial activity to various pathogenic bacteria support their frequent use in traditional medicine in various parts of Nepal.

4. CONCLUSION

Different species of *Gentiana* showed the presence of most of the phytochemicals tested in their methanol extracts. The quantitative study showed the presence of phenolics and flavonoids in substantial quantities. All the four-species showed a considerable level of antioxidant activity, which was much higher in methanolic extracts than in aqueous extracts. The extracts of all the species showed weak antibacterial activity. This study affirms the presence of pharmacologically important phytochemicals, mainly amarogentin and swertiamarin, in four species of *Gentiana* tested. Mangiferin was also found in all the species but quantities much less than amarogentin and swertiamarin. These properties of extracts in the tested species of *Gentiana* may be the reason behind their use in ethnomedicine. Though *Gentiana* species are commonly studied in Europe and China, the species from Nepal Himalaya have not been studied properly. This paper presents the first report of preliminary estimation of Gentian phytochemicals, and their antioxidant and antimicrobial properties from Nepal. It also paves the way and necessitates indulging in further research into different species of *Gentiana* from Nepal.

ACKNOWLEDGEMENT

The authors would like to thank the Department of National Parks and Wildlife Conservation (DNPWC) and Langtang National Park, Government of Nepal, for permission to collect the plant samples. Similarly, the authors would also like to thank the National Herbarium and Plant Laboratories, Godawari (KATH), for the identification and deposition of collected specimens. The authors are also indebted to the Central Department of Biotechnology, Tribhuvan University, for availing facilities to conduct the research work.

REFERENCES

Aberham, A., S. Schwaiger, H. Stuppner and M Ganzera, 2007. Quantitative analysis of iridoids, secoiridoids, xanthones and xanthone glycosides in *Gentiana lutea* L. roots by RP-HPLC and LC–MS. Journal of Pharmaceutical and Biomedical Analysis 45(3): 437-442. https://doi.org/10.1016/j.jpba.2007.07.001.

Anonymous, 2016. Medicinal Plants of Nepal. Department of Plant Resources, Thapathali, Kathmandu.

Anonymous, 2018. European union monograph on *Gentiana lutea* radix. European Medicines Agency. https://www.fitoterapia.net/archivos/201905/european-herbal-monograph-*gentiana*-lutea-l-radix-revision-1_en.pdf?1.

Azman N. A. M., F. Segovia, X. Martínez-Farré, E. Gil and M. P. Almajano, 2014. Screening of antioxidant activity of *Gentiana lutea* root and its application in oil-in-water emulsions. Antioxidants3(2):455-71. https://dx.doi.org/10.3390%2Fantiox3020455.

Baba S. A. and S. A. Malik, 2014. Evaluation of antioxidant and antibacterial activity of methanolic extracts of *Gentiana kurroo* Royle. Saudi Journal of Biological Sciences 21(5):493-8. https://doi.org/10.1016/j.sjbs.2014.06.004.

Bhattarai, S., R. P. Chaudhary, R. S. Taylor and S. K. Ghimire, 2009. Biological Activities of some Nepalese Medicinal Plants used in treating bacterial infections in Human

beings. Nepal Journal of Science and Technology 10: 83-90.

Chakraborty, T., S. Saha, N. S. Bisht, 2017.First report on the ethnopharmacological uses of medicinal plants by Monpa tribe from the Zemithang region of Arunachal Pradesh, eastern himalayas, India. Plants 6(1): 13. https://doi.org/10.3390/plants6010013.

Chang, C., M. Yang, H. Wen and J. Chem, 2002. Estimation of total flavonoid content in Propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis 10:178-182. https://doi.org/10.38212/2224-6614.2748.

Chen, Y., B. Huang, J. He, L. Han, Y. Zhan and Y, Wang, 2011. In vitro and in vivo antioxidant effects of the ethanolic extract of Swertia chirayita. Journal of Ethnopharmacology 136(2): 309-15. https://doi.org/10.1016/j.jep.2011.04.058.

Cowan, M. M., 1999. Plant products as antimicrobial agents. Clinical microbiology reviews 1999;12(4):564-82. https://doi.org/10.1128/CMR.12.4.564.

Cragg, G. M., D. J. Newman, and K. M. Snader, 1997. Natural products in drug discovery and development. Journal of Natural Products 60(1):52-60.https://doi.org/10.1021/np9604893.

Dembinsca-Kiec, A., O. Mykkanen, *B.* Kiec-Wilk and H. Mykkanen, 2008.Antioxidant phytochemicals against type 2 diabetes. British Journal of Nutrition 99 E Supplment 1: ES109-117. https://doi.org/10.1017/s000711450896579x.

GelQuant.NET version 1.7.8, 2011.http://biochemlabsolutions.com/GelQuantNET.html. (17 December 2017, date last accessed).

Gyatso, T. and C. Hakim, 2010. Essentials of Traditional Tibetan medicine.North Atlantic Books, California, p162.

Harborne, A., 1998. Phytochemical methods a guide to modern techniques of plant analysis. Springer Science & Business Media.

Havsteen, B. H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacology & Therapeutics 96(2): 67-202. https://doi.org/10.1016/S0163-7258(02)00298-X.

Hostettmann-Kaldas, M., K. Hostettmann and O. Sticher, 1981 . Xanthones, flavones and secoiridoids of American *Gentiana* species. Phytochemistry 20(3):443-6. https://doi.org/10.1016/S0031-9422(00)84162-X.

Huang, S. H., C. T. Wu, C. L. Kuo, and H. S. Tsay, 2013. Comparative analysis among three Taiwan-specific *Gentiana* species and Chinese medicinal plant *Gentiana scabra.* Botanical Studies 54(1):54. https://dx.doi.org/10.1186%2F1999-3110-54-54.

Jensen, S. R. and J. Schripsema, 2002. Chemotaxonomy and pharmacology of Gentianaceae. In: Struwe, L. and V. A. Albert, editors. Gentianaceae: *systematics and natural history*. Cambridge University Press, Cambridge, pp:573-631.

Joshi, A. R., D. P. Joshi and K. Joshi, 2000. Status of some endemic plants in Nepal. Tiger Paper 27(3): 15-20.

Khanal, S., N. Shakya, K. Thapa and D. R. Pant, 2015. Phytochemical investigation of crude methanol extracts of different species of *Swertia* from Nepal. BMC Research Notes 8: 821. https://doi.org/10.1186/s13104-015-1753-0.

Kotzekidou, P., P. Giannakidis and A. Boulamatsis, 2008. Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens in vitro and on the fate of inoculated pathogens in chocolate. LWT-Food Science and Technology 41(1):119-127. https://doi.org/10.1016/j.lwt.2007.01.016.

Lamichhane, J., S. B. Chhetri, M. Bhandari, S. Pokhrel, A. Pokharel and J. K. Sohng, 2014. Ethnopharmacological survey, Phytochemical screening and Antibacterial activity measurements of high altitude medicinal plants of Nepal: A bioprospecting approach. *Indian Journal of Traditional Knowledge* 13(3): 496-507. http://nopr.niscair.res.in/handle/123456789/29120.

Mihailović, V., N. Vuković, N. Nićiforović, S. Solujić, M. Mladenović, P. Mascaron and M. S. Stankovic, 2011. Studies on the antimicrobial activity and chemical composition of the essential oils and alcoholic extracts of *Gentiana asclepiadea* L. *Journal of Medicinal Plants Research* 5(7): 1164-1174.

Mirzaee, F., A. Hosseini, H. B. Jouybari, A. Davoodi, and M. Azadbakht, 2017. Medicinal, biological and phytochemical properties of

Gentiana species. Journal of Traditional and Complementary Medicine 7(4): 400-408. https://dx.doi.org/10.1016%2Fj.jtcme.2016.12.013.

Nastasijevic, *B.*, T. Lazarevic-Pasti, S. Dimitrijevic-Brankovic, I. Pasti, A. Vujacic, G. Joksic and V. Vasic, 2012. Inhibition of myeloperoxidase and antioxidative activity of *Gentiana lutea* extracts. Journal of Pharmaceutical and Biomedical Analysis 66:191-196. https://doi.org/10.1016/j.jpba.2012.03.052.

Olennikov, D. N., N. I. Kashchenko, N. K. Chirikova and L. M. Tankhaeva, 2015. Iridoids and flavonoids of four Siberian gentians: Chemical profile and gastric stimulatory effect. Molecules 20(10):19172-19188. https://dx.doi.org/10.3390%2Fmolecules201019172.

Pandey, M. R., 2006. Use of Medicinal Plants in Traditional Tibetan Therapy System in Upper Mustang, Nepal. Our Nature4: 69-82.

Phoboo, S., M. D. S. Pinto, P. C. Bhowmik, P. K. Jha and K. Shetty, 2010. Quantification of major phytochemicals of *Swertia chirayita*, a medicinal plant from *Nepal. Ecoprint: An International Journal of Ecology*.17:59-68.

Quercia, V., G. Battaglino, N. Pierini and L. Turchetto, 1980. Determination of the bitter constituents of the *gentiana* root by high-performance liquid chromatography. *Journal of Chromatography* A 193(1): 163-169. https://doi.org/10.1016/S0021-9673(00)81460-0.

Rammal, H., J. Bouayed, A. Hijazi, M. Ezzedine and R. Soulimani, 2012. Scavenger capacity of Momordica charantia for reactive oxygen species. *Journal of Natural Products* 5:54-9.

Roy, M. K., M. Koide, T. P. Rao, T. Okubo, Y. Ogasawara and L. R. Juneja, 2010. ORAC and DPPH assay comparison to assess the infusions' antioxidant capacity: Relationship between total polyphenol and individual catechin content. *International Journal of Food Sciences and Nutrition* 61(2): 109-24. https://doi.org/10.3109/09637480903292601.

Scalbert, A., 1999. Antimicrobial properties of tannins. Phytochemistry 30(12): 3875-83. https://doi.org/10.1016/0031-9422(91)83426-L.

Singh, A., 2008. Phytochemicals of Gentianaceae: a review of pharmacological properties. *International Journal of Pharmaceutical Sciences and Nanotechnology* 1(1):33-6. https://doi.org/10.37285/10.37285/ijpsn.1.1.4.

Singleton, V. L., R. Orthofer and R. M. Lamuela-Raventós, 1999. Analysis of total phenols and other oxidation substrates and antioxidants usinga folin-ciocalteu reagent. Methods in Enzymology. 1999;299:152-78. https://doi.org/10.1016/S0076-6879(99)99017-1.

Struwe, L., J. W. Kadereit, J. Klackenberg, S. Nisson, M. Thiv, K.*B.* Von Hagen and V. A. Albert, 2002. Systematics, character evolution, and biogeography of Gentianaceae, including a new tribal and subtribal classification. In: Struwe, L. and V. A. Albert, editors. Gentianaceae: *Systematics and Natural History*. Cambridge University Press, pp 21-309.

Timmermans, K., 2003. Intellectual property rights and traditional medicine: policy dilemmas at the interface. Social Science & Medicine 57(4): 745-756. https://doi.org/10.1016/S0277-9536(02)00425-2.

Wagner, H., S. Bladt and E. M. Zgainski, 1984. TLC Screening of an unknown commercial drug. In: Wagner, H. and S. Bladt, editors. Plant Drug Analysis. Springer. https://doi.org/10.1007/978-3-662-02398-3_17.

Wani, B. A., D. Ramamoorthy and B. A. Ganai, 2011. Preliminary phytochemical screening and evaluation of the analgesic activity of methanolic extract of roots of *Gentiana kurroo* Royle in experimental animal models. International Journal of Pharmacy and Pharmaceutical Sciences. 3(4):164-6..

Wijekoon, M. J. O., R. Bhat and A. A. Karim, 2011. Effect of extraction solvents on the phenolic compounds and antioxidant activities of bunga kantan (*Etlingera elatior* Jack.) inflorescence. Journal of Food Composition and Analysis 24(4): 615-619. https://doi.org/10.1016/j.jfca.2010.09.018.

Yeshi, K., Y. Gyal, K. Sabernig, J. Phuntso, T. Tidwell, T. Jamtsho, R. Dhondup, E. Tokar, and P. Wangchuk, 2019. An integrated medicine of Bhutan: Sowa Rigpa concepts, botanical identification, and the recorded phytochemical and pharmacological properties of the eastern Himalayan medicinal plants European journal of Integrative medicine 29: 100927. https://doi.org/10.1016/j.eujim.2019.100927.