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Phytoconstituents, Antioxidant and Bitterness Value of *Swertia chirayita* from Four Different Geographical Region of Nepal

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Abstract

The current study was focused on preliminary phytochemical screening in hexane, methanol and 50% ethanolic extract of plant *Swertia chirayita* (Roxb. ex Fleming) of different region. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) was quantified by using Folin-ciocalteau reagent (FCR) and aluminium chloride assay by spectrophotometric method before accessing antioxidant activity of the methanolic extracts by using 2, 2 -Diphenyl-1-picrylhydrazyl (DPPH) assay method. Bitter principal of plant material was determined by procedures as mentioned in Ayurveda Pharmacopeia of India and the percentage bitterness values of four different geographical regions were found to be Ilam (1.31) Rasuwa (1.50) Dolakha (1.43) and Bhojur (1.46).

Keywords*: Bitterness value, DPPH free radical, Scavenging activity, TFC, TPC*

Introduction

Swertia chirayita is a critically endangered Himalayan medicinal plant (Kumar, 2016; Khan, 2018). Dried powder plant of *Swertia chirayita* (Roxb. ex Fleming) Karsten (syn. S. chirata Buch.-Ham. ex. C.B. Clarke) Fam. Gentianaceae is used as medicine traditionally in small dose for various purposes (Kumar, 2016). A branched herb 60-125cm tall distributed in east to central of Nepal at an altitude 1200 to 3600 m commonly known as Chiraityo in Nepali (Hindi-Chirayata Eng.-Bitter stick) (DPR, 2007; Tabassum, 2012). *Swertia* species are known for bitter principles. Among *Swertia* species, *S. chirayita* is most valuable and is used in medicines on large scale particularly in India and Nepal for its bitter principles (Kumar, 2016). The active constituents contain swertianin, amarogentin, ameroswerin, mangiferin, gentiopicrin, sweroside, swerchirin, chiratanin, swertiamarin, bellidifolin (Negi, 2011; Latif, 2014). The unique structure of xanthones i.e. main secondary metabolites including catecholic moiety and completely conjugated system enables them to be promising antioxidants (Phoboo, 2010; Negi, 2011). The plant is hepatoprotective, anti-inflammatory, hypoglycemic, antihelminthic, antifungal, antimicrobial and excellent drug for intermittent fever, skin diseases, intestinal worms and bronchial asthma (Khan, 2017; Khan, 2018). The present investigation was undertaken for comparative study of polyphenol, bitter principle and antioxidant activity of four geographical regions of Nepal.

Materials and Methods

Plant Materials

Aerial parts of whole herb of *S. chirayita* were collected from different part of Nepal (Ilam, Rasuwa, Bhojpur, Dolakha) in the month of July-August. The plant was properly identified from the herbarium and literature available. Plants were authenticated by Pharmacognosy section of Natural Plant Research Laboratory, DPR and National Herbarium and Plant Laboratory, Godavari, Lalitpur. The plant sample was air dried, crushed and sieved to coarse powder mechanically and stored in air tight container for further use.

Percolation method

20 g each dried powdered plant sample were extracted with 250 ml of different solvents on Percolator for 72 hours on 2074/11/05. The residue was extracted successively with non-polar to polar solvents respectively. The extracts were filtered and solvents were evaporated in Rotatory Evaporator under reduced pressure.

Preliminary phytochemical Screening

The extracts were used for the preliminary phytochemical analysis. All the tests were performed in triplicate mode by standard operating procedures mention (Harborne, 1969; Sofowora, 1993) shown in table 2.

Volatile oils: Methanolic solution of extracts was put on filter paper by means of capillary tube & visualize. Transparent filter paper with no yellow color persist means presence of volatile oils.

Alkaloids: Test solution was tested with 2-3 drops of potassium mercuric iodide (Mayer's reagent) gives white yellowish or creamy colored precipitate.

Flavonoids: Test solution was tested with Mg metal and 5-6 drops of conc. HCl. Red color for flavonoid, orange for flavones, and violet for flavonones.

Steroids: 1 mL of extracts was dissolved in 10 mL of chloroform and equal volume of conc. H_2SO_4 was added by sides of the test tube. The upper layer shows green with yellow fluorescence.

Terpenoids: Crude alcoholic extracts was dissolved in 2 mL chloroform and 3 mL conc. H_2SO_4 and heated for 2 minutes. A grayish reddish brown coloration of the interface indicated the presence of terpenoid.

Tannins/Phenol: To 0.5 mL of alcoholic extract 1 mL water and 2-3 drops of 0.1% $FeCl_3$ was added. Bluish black or greenish black indicates the presence of tannins or phenols.

Reducing sugar: 0.5 mL extract solution was added with 1 mL water acidified with dil. HCl, neutralized with alkali and heated with 0.5 mL Fehling solution A + B gently. A reddish brick precipitate indicates the presence of reducing compounds.

Glycosides: The extract was mixed with 2 mL chloroform. H_2SO_4 was added carefully and shaken gently. A brown ring at the interface indicates the presence of cardiac glycosides.

Saponins: Extracts were diluted with distilled water to 20 mL and this was shaken in a graduated cylinder for 15 minutes. If foam produced persists for ten minutes it indicates the presences of saponins.

Protein: Crude extract boiled with 2 mL of 0.25% w/v solution of Ninhydrin, violet blue color appeared suggesting the presence of the protein.

Total Phenolic Content and Total Flavonoid Content

Preparation of standard for phenolic content and flavonoid content: The TPC of extract was estimated by Folin-Ciocalteau reagent described by Singleton and Rossi (Singleton & Rossi, 1965). Gallic acid stock solution was prepared by dissolving 1 mg gallic acid in 1 mL of methanol (1 mg/ mL). Various concentrations of gallic acid such as were prepared by serial dilution of stock solution. An aliquot of 1 mL gallic acid of each concentration in methanol was added to 20 mL test tube. To that 5 mL of Folin-Ciocalteu reagent (10%) and 4 mL of 7% Na_2CO_3 were added to get a total of 10 mL. The blue colored mixture was shaken well and incubated for 30 minutes at 40ºC in a water bath. Then the absorbance was measured at 760 nm against blank. Similarly, TFC was determined by $AlCl_3$ colorimetric assay (Acharya, 2013). Concentration of standard quercetin was prepared by serial dilution of stock solution of concentration of 4 mg/ mL. An aliquot of 1 ml quercetin of each concentration in MeOH was added to 10 mL v.f. containing 4 mL of double distilled water. At the zero time, 0.3 ml, 5% sodium nitrite was added to the flask. After 5 min, 0.3 mL of 10% $AlCl_3$ was added to the flask. At 6 min, 2 mL of 1 M NaOH was added to the mixture. Immediately, the total volume of the mixture was made up to 10 mL by the addition of 2.4 mL double distilled water and mixed thoroughly. Absorbance of the pink colored mixture was determined at 510 nm versus a blank containing all reagents except quercetin. Absorbance values obtained at different

concentrations of quercetin were used to plot the calibration curve.

Preparation of samples for Phenolic content and flavonoid content: Stock solutions of all extracts were prepared by dissolving 1 mg in 1 mL of MeOH. Serial dilutions were carried out to get the concentration of different µg/ mL. To these diluted solution FCR and Na_2CO_3 were added and incubated for 30 minutes as in the case of standard gallic acid preparation and absorbance was measured at 760 nm. Similarly, various concentrations of the extracts viz, 2 mg/ mL, 1 mg/ mL, 0.5 mg/ mL and 0.25 mg/ mL were prepared. Following the procedure described above in flavonoid, absorbance for each concentration of extract was recorded. TFC of the extracts was expressed as mg quercetin equivalents (QE) per gram of extract in dry weight (mg/g).

Calculation for TPC and TFC and Statistical Analysis: The total phenolic content and flavonoid content was calculated using the formula: $C = \frac{cV}{m}$ …(1) where C= total contents of compounds in mg/g, in mg GAE/ g or total flavonoid content mg QE/ g dry extract, c= concentration of gallic acid established from the calibration curve in mg/ mL or concentration of quercetin obtain from calibration curve, mg/ mL, V= the volume of extract in mL, m= the weight of plant extract in g. Calculation of linear correlation coefficient R^2 and correlation analysis were carried out using Microsoft Office Excel 2007. The linear regression equation is given as, y = mx + C…(2), where y = absorbance of extract, m= slope of the calibration curve, x= concentration of the extract, C=intercept.

Determination of antioxidant activity using DPPH free radical method

DPPH radical scavenging activity of extracts was carried out according to Brands et al Method (Brand-Williams, 2012). DPPH solution (0.1 mM) in MeOH was prepared by dissolving 3.9 mg of DPPH in 100 ml methanol and stirred overnight at 4ºC. Thus prepared purple colored DPPH free radical solution was stored at -20°C for further use.

Three different concentrations (5, 10 and 15 µg/ mL) of methanolic solutions of each extracts were prepared by the serial dilution of the stock solution of the respective extract. To each 0.5 mL extract solution, 2.5 mL, 0.1 mM methanolic DPPH solution was added. A control was prepared by mixing 0.5 mL distilled water and 2.5 mL 0.1 mM methanolic DPPH solution. These samples were well shaken and kept in dark for 30 min at a room temperature. The absorbance of the mixture was measured spectrophotometrically at 517 nm against the blank solution consisting 2.5 mL MeOH and 0.5 mL ml distilled water. The radical scavenging activity was expressed as the radical scavenging percentage using the following equation: DPPH % scavenging activity $= \left[\frac{(Ac - As)}{Ac}\right] \times 100$ Where, A_C=absorbance of control and methanol, A_S= absorbance of sample solution and DPPH radical. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the plotted graph of radical scavenging activity against concentration of extracts. The antioxidant activity was determined by DPPH assay and the free radical scavenging activity (IC_{50}) value was calculated.

Table 1: Absorbance value for gallic acid and quercetin measured for calibration curve

Gallic acid used as standard for calibration of phenols		Quercetin is used as standard for calibration of flavonoid	
Concentration (µg/mL)	**Absorbance for gallic acid measured**	**Concentration (µg/ mL)**	**Absorbance values for quercetin measured**
25	0.156	25	0.098
50	0.381	50	0.167
75	0.469	75	0.246
100	0.669	100	0.316
125	0.798	125	0.428

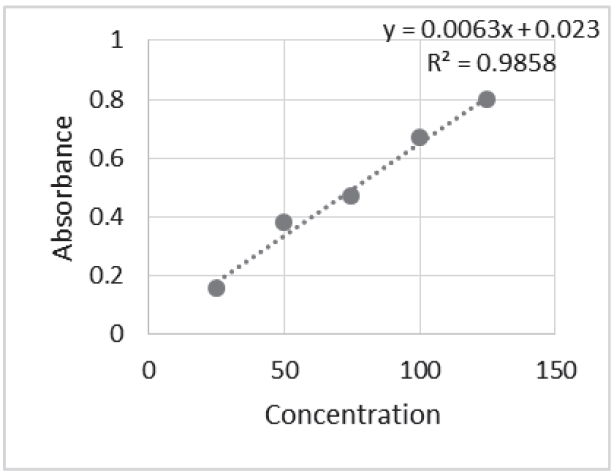

Figure 1: Calibration curve for authentic gallic acid

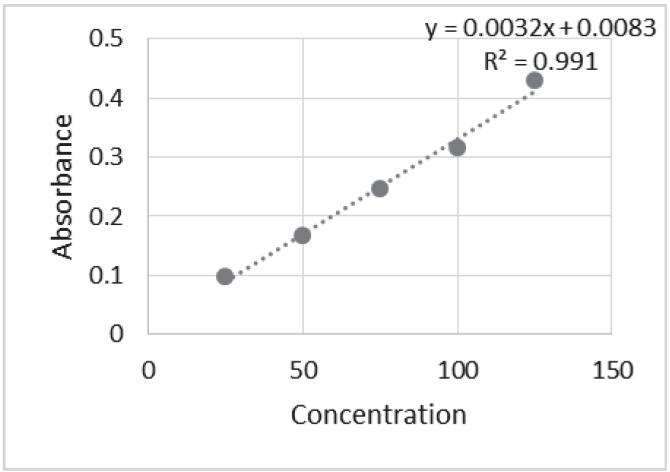

Figure 2: Calibration curve for authentic quercetin

Calculation of total phenolic and total flavonoid contents in extracts

The concentration of phenolic and flavonoid in extract was calculated from the calibration curve by regression equation. The TPC and TFC was calculated using the formula C=cV/m and expressed as mg gallic acid equivalents (GAE) per g of extract in (mg/g) and mg quercetin equivalents (QE) per gram extract in (mg/g). The TPC & TFC was calculated given in Table 3.

Determination of Bitterness principle

The bitterness principle is determine for all the four samples of four different district separately with repeatability test following the procedures as mentioned in Ayurvedic Pharmacopeia of India, volume 1 part 1;1986.The details are as follows:

2 g powder (No. 60 sieve) of *Swertia chirayita* was mixed with boiling water containing 0.5 g of calcium carbonate and extracting with boiling water till the last portion of the extract is devoid of bitterness, concentrate in vacuum (Life lysed) and dissolve the residue in hot alcohol. Filtering while hot and wash the residue thrice on the filter with 10 mL portions of hot alcohol and remove the alcohol from the filtrate and take up the residue repeatedly with 25,15,15, 15, and 15 mL of hot water. Shaking the aqueous extract repeatedly with 25, 20, 15, 15 and 10 mL of ethyl acetate, collect the ethyl acetate extracts, evaporate, dry and Weigh. The powder of four samples from the specific region were taken repeatedly and replicated thrice for the consistency in the result.

Results and Discussion

Phytochemical analysis

Table 2: Phytochemical screening of aerial parts of *Swertia chirayita* in different solvents-extracts

S.N.	Experiment	Region	Hexane extract	50% EtoH Extract	MeOH Extract
1.	Volatile oils spot test	I	-	-	-
		R	-	-	-
		B	-	-	-
		D		-	-
2.	Alkaloids Mayers teest	I	-	+	++
		R	-	+++	+++
		B	-	+	++
		D	-	++	++
3.	Flavonoid Shinoda test	I	-	++	+++
		R	-	++	++
		B	-	+++	++
		D	-	+++	++
4.	Steroids	I	+	++	+
		R	++	++	+
		B	+	++	++
		D	++	++	+
5.	Terpenoids	I	-	++	++
		R	-	+	++
		B	-	+	++
		D	-	++	++
6.	Tannins	I	-	+++	+++
		R	-	++	+++
		B	-	++	++
		D	-	+++	+++
7.	Reducing sugar	I	+	++	+
		R	+	++	+
		B	+	++	+
		D	++	++	+
8.	Glycosides	I	-	+	+
		R	-	+	+
		B	-	+	+
		D	-	+	+
9.	Saponins	I	-	+	+
		R	-	+	+
		B	-	++	++
		D	-	+	+
10.	Protein	I	-	-	++
		R	-	+	++
		B	-	-	+
		D	-	-	+

Indications: Result + trace amount, ++ moderate amount, +++ high amount – means absence of phytochemicals and I= Ilam, R= Rasuwa, D=Dolakha B= Bhojpur

Calculation of total phenolic and total flavonoid contents in extracts and DPPH assay for antioxidant activities

The DPPH assay was carried out and absorbance values measured at wavelength 517 nm for different concentrations and the control. The calculated percentage of inhibition showed that extract antioxidant activity at 5, 10, and 15µg/mL. The TPC, TFC, % inhibition and IC_{50} value was calculated and shown in Table 3.

Table 3: TPC, TFC, % inhibition, and IC_{50} in different methanolic extract of *Swertia chirayita*.

Region of *S. chirayita*	mg GAE/ g (Mean TPC± S.D)	QE mg/ g (Mean TFC±S.D)	% inhibition	IC_{50} value
Ilam	87.44 ± 0.30	25.09 ± 0.31	15.23	49.39
			18.40	
			23.12	
Rasuwa	97.33 ± 0.88	31.92 ± 0.61	24.24	24.18
			29.56	
			37.93	
Dolakha	91.22 ± 0.66	29.10 ± 0.39	21.13	27.71
			27.41	
			33.85	
Bhojpur	79.80 ± 0.25	24.15 ± 0.59	6.14	50.64
			10.54	
			15.78	

Bitterness Value

Table 4: Bitterness value of four samples of *Swertia chirayita*

S.N.	Region	Weight extract (g)	% Bitterness	Average
1	Ilam	0.0258	1.29	1.31
		0.0266	1.33	
		0.0262	1.31	
2	Rasuwa	0.0306	1.53	1.50
		0.0294	1.47	
		0.030	1.50	
3	Dolakha	0.0292	1.46	1.43
		0.0286	1.43	
		0.0284	1.42	
4	Bhojpur	0.0288	1.44	1.46
		0.0296	1.48	
		0.0292	1.46	

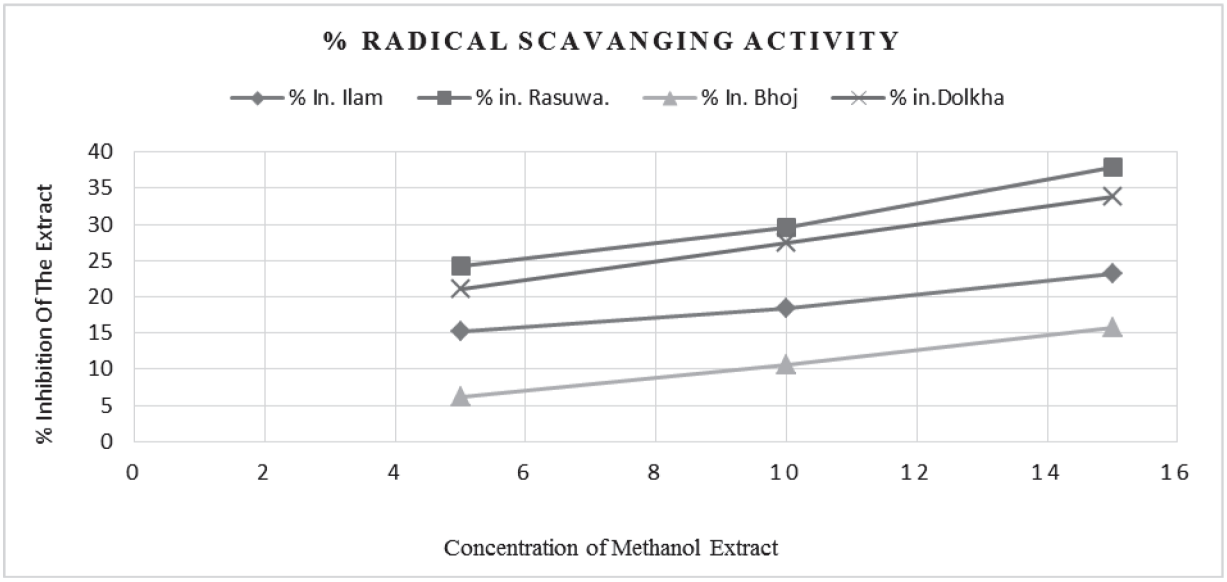

Figure 3: Percentage inhibition of the methanolic extract shows antioxidant activity of four samples

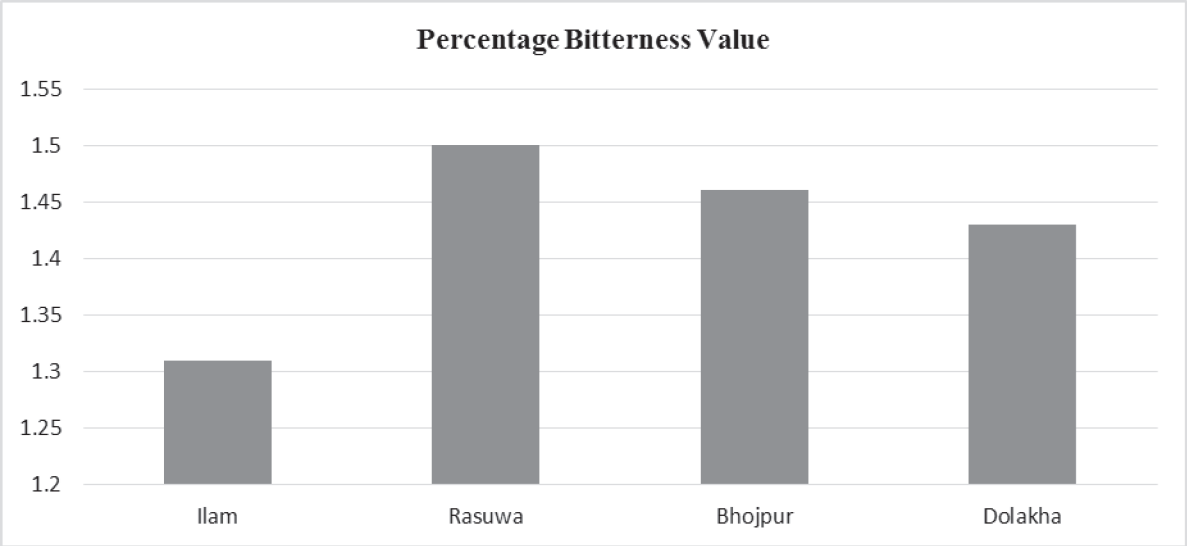

Figure 4: Bitterness value of *S. chirayita* in the different region

Conclusions

The phytochemical analysis revealed that the maximum bioactive compounds are present in methanolic and 50% ethanolic extract. The plant is mostly found in high altitude from were sample is collected shows potent bitterness value and antioxidant activity. The result showed that this plant is potent antioxidant property and maximum phenolic content in methanolic extracts so they could be the rich source of natural antioxidants in herbal medicine for various ailments. The highest the phenolic content, the lowest the IC_{50} Value. The annual demand of this plant is very high in the national as well as international market but plant is in threatened due to its over exploitation so expansion of cultivation and variety development of this plant is recommended. Cultivation practices need to be standard. This research will certainly help to analysis and compare the result of phytochemicals; quantify the total phenolic and flavonoid content, antioxidant effect, and bitterness principle of Nepalese *S. chirayita* in four different regions.

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