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Screening of phytochemicals, estimation of total phenolic and total flavonoid content and Antioxidant activity in *Achyranthes aspera* L

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Abstract
In recent times, plants sources have attracted a wide range of interest across the world. This is due to growing concern for safe and alternative sources of antioxidants. The leaves and stem of *Achyranthes aspera* L. (Amaranthaceae) were screened for the presence of its phytochemical composition, total phenolic content (TPC), and total flavonoid content (TFC) and antioxidant activities. Estimation of TPC and TFC was performed by Folin-Ciocalteau reagent and aluminum chloride assay method using spectrophotometrically. Antioxidant activity of methanol, 50 % ethanol and aqueous extract was screened by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Total phenol content was expressed as mg gallic acid g^{-1} phenol and total flavonoid content as mg quercetin g-1 flavonoid using the standard curves; Standard curve equation y = 0.0106x+0.0151, R^2= 0.9879 for gallic acid and standard curve equation y = 0.004x+0.0073, R^2= 0.9922 for Quercetin. The maximum total phenolic content in methanolic extracts stem was 374.95 mg GAE/g and minimum aqueous extracts in leaf was 200.00 mg GAE/g. Similarly, the maximum total flavonoid content in methanolic extracts stem was 190.875 mg QE/g and minimum aqueous extracts in leaf was 53.375 mg QE/g. IC_{50} value of methanolic extract of stem was 23.97 and that in leaf was 34.28. The higher the IC_{50}values lower will be its total phenolic and flavonoid content.

Keywords: Folin-Ciocalteau reagent, Gallic acid, methanolic extracts

Introduction
Achyranthes aspera L. known as prickly chaff flower plant (Eng.) [1, 3], Datiwan in Nepali [2, 3] and Apamarga in Sanskrit [1, 3] is a 30-90 cm tall herb with quadrangular branches thickened just above the node [1, 2, 4] leaves simple, short petiole, opposite, velvety tomentose, 10-12 cm long and 7.5 cm wide, rounded at the apex, elliptic, ovate or orbicular [1, 5]. Flowers aresmall, greenish white [6]. Fruits are easily disarticulating[7]. The plant flowering season is June-July [1, 8] and whole part of the plant is used in various purposes [9, 10]. The uses of the plant is in purgative, diuretic, and also used in leprosy, piles, boils, skin eruption, colic, and snake bite. Infusion of root is astringent [10, 11, 12]. In Nepal it is distributed in 600-1800 m, east to west [11]. In the recent time, *A. aspera* is reported to have array of medicinal compounds and medicinal properties. Plant yield alkaloids. The root contains ecdysterone and oleanolic acid [12, 13]. Seeds yields sapiens, oleanolic acids and its ester [13, 14].
The dried leaves of *A.aspera* are reported to cure asthma [14, 15]. *A. aspera* is used for the treatment of fever, dysentery, asthma, hypertension and diabetics [15, 16, 17]. *A. asper*sed were reported to contain emetic and hydrophobic properties. *A. asper a*re ported to possess wound healing activity, immune-stimulatory properties [15, 16, 17], larvicidal activity, antibacterial activity and antifungal activity [17, 18]. Roots of *A. aspera* reported to possess antioxidant activity and anti-inflammatory properties [18, 19].

Antioxidants are those substances which possess free radical chain reaction breaking properties [19]. Oxidative stress contributes to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease, the pathologies caused by Diabetes, a Rheumatoid Arthritis [20], Atherosclerosis, Ischemic heart disease, Ageing, Immune suppression, Neurodegenerative diseases, Cancer and others [21, 22, 23]. The most effective path to eliminate and diminish the action of free radicals which cause the oxidative stress is "ant oxidative defense" mechanisms [22]. A great number of aromatic, medicinal, spice and other plants contain chemical compounds exhibiting antioxidant properties. The synthetic antioxidant showed the toxic effects like liver damage mutagenesis [23]. Hence, nowadays search for natural antioxidant source is gaining much importance.
With this background and abundant source of unique active components present in this plant, the present study was taken up on medicinal plant namely *A. aspera* which is a common weed as well as indigenous medicinal plant of Asia, South America and Africa [24], belonging to the family Amaranthaceae [25, 26]. Itis one of the major ingredients in Ayurveda reputed to be a laxative, stomachic, depurative, pectoral and astringent*;* its juice is administered in diarrhea, dysentery, monorrhagia, piles, rheumatism, inflammation of internal organs, coughs, enlarged cervical glands, eruptions, boils, etc. Plant possesses abortifacient activity [26, 27, 28], hypoglycemic, hypolipidemic activity, anti-inflammatory activity [27], antifungal [28], anti – feedant activity [29] and antibacterial properties [30], gynecological disorders, estrogenic and pregnancy interceptor effects, diabetes mellitus [30, 31, 32] In the present study, the antioxidant potential of the methanolic, 50% ethanolic and aqueous extracts was determined on the basis of phytochemical screening, and

quantification of total phenolic content and total flavonoid content.

Materials and Method

Plant Material Collection and Authentication:

Whole plant of *A. aspera* was collected from the local market vendor of Asan Bazaar Kathmandu district in Bhadra 2073(August 2016). The plants were washed, separated, cut into small pieces and air dried and store in a plastic air tight container for phytochemical analysis, total phenolic content, total flavonoid content and antioxidant activity of the stem and leaves. The plants research was carried in Natural Product Research Laboratory, Tha pathali, and Kathmandu. Plant was identified and authenticated by Asso. Prof. Dr. Ila Shrestha, Department of Botany, Patan Multiple Campus, Latipur.

Chemicals and reagents

Chemicals and Reagents were of analytical grade. Folin–Ciocalteu's phenol reagent, gallic acid, DPPH, was purchased from sigma chemical company, USA. All the solutions are prepared in distilled water. The plant extracts were prepared by using laboratory grade solvent methanol, ethanol and aqueous extract.

Processing of plant for the preparation of extracts

The Plant of *A. aspera* was collected and washed thoroughly in distilled water and cut in to small pieces. The stem was dried in shade at room temperature. Dried stem pieces were uniformly grinded using mechanical grinder to make fine powder. The powder was stored in an airtight container and used for solvent extraction using a Soxhlet apparatus. These extracts were concentrated under reduced pressure with a Rotary evaporator and dried using lyophilizer. Dried extract was collect in air tight container and stored at 4°C for further use.

Qualitative phytochemical analysis

A. aspera extracts were subjected to the analysis of macromolecules and secondary metabolites such as alkaloids, flavonoids, sterols, lignin, gum, starch, terpenes, saponins, tannins, proteins, and phenols by using standard methods [32].Phytochemical screening of the stem and leaf extracts of *A. aspera* was carried out by using the standard protocols as described by JB Harborne[33].

Quantitative phytochemical analysis

Aluminum chloride colorimetric method [34] was used for flavonoids determination. Total phenols were determined by using Folin Ciocalteu's reagent [35] and Antioxidant activity was determined by DPPH method [36, 37].

Estimation of total phenolic content

Total phenolic content of the aqueous, 50% ethanolic and methanol extract of *A. aspera* stem was determined using the Folin-Ciocalteu reagent. The crude aqueous and methanol extracts were dilutedin methanol to obtain different concentrations. 50 µl of eachextract was mixed with 2.5 ml of Folin- Ciocalteu's reagent (1/10 dilution in purified water) and 2 ml of 7.5% Na2CO3 (w/v in purified water). The mixture was incubated at 45˚C for 15min. The absorbance was measured at 765 nm. Na2CO3 solution (2 ml of 7.5% Na2CO3 in2.55 ml of distilled water) was used as blank. The results were expressed as Gallic acid equivalence.

Estimation of total flavonoid content

Total Flavonoid content was determined by $AlCl_3$ colorimetric assay. Concentration of standard quercetin was prepared by serial dilution of stock solution. 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.

DPPH radical scavenging activity

The DPPH radical scavenging activity was performed according to the method of Gunjan *et al*. with few modifications. The plant extracts were diluted in distilled water to make 5, 10, and 15 µg/ml dilutions. Two milliliters of each dilution was mixed with 1 ml of DPPH solution (0.2 mM/ml in methanol) and mixed thoroughly. The mixture was incubated in dark at 20˚C for 40 min. Absorbance was measured at 517 nm using UV–Vis spectrophotometer with methanol as blank. Gallic acid was used as positive control. The percentage scavenging of DPPH by the extracts was calculated according to the following formula:

% DPPH Radical scavenging= [(Ac - At) / Ac] × 100

Here Ac is the absorbance of the control (DPPH). At is the absorbance of test sample.

Statistical Analysis

All tests were conducted in triplicate. Data are reported as means value. Results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA). 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.

Results and Discussion

The research work was carried out on the medicinal plant which shows the potent phytochemical constituent's summarized in Table 1.

Table 1: Qualitative Preliminary phytochemical screening of *A. aspera* Leaf and Stem. Note: Result + means presence – means absence of phytochemicals

S. No.	Chemical Compounds	Test	Colour	Solvent	Leaf	Stem
1	Volatile oils	Spot/Residue test	Transparent filter paper with no yellow colour persist	MeOH	+	+
				EtOH	-	-
				H_2O	-	-
2	Alkaloids	Mayer's Regent Test	White yellowish ppt.	MeOH	+	+
				EtOH	-	+
				H_2O	-	+
3	Flavonoid	Shinoda test	Pink Scarlet	MeOH	-	+
				EtOH	-	+
				H_2O	-	-
4	Steroids	Steroid test	Yellow with green fluorescence	MeOH	-	-
				EtOH	-	-
				H_2O	-	-
5	Terpenoids	Terpenoids test	A grayish colour	MeOH	+	+
				EtOH	+	+
				H_2O	+	+
6	Tannins	0.1% $FeCl_3$ Test	Bluish black/greenish black	MeOH	+	+
				EtOH	+	+
				H_2O	+	+
7	Reducing sugar	Fehling's Test	Reddish brick ppt.	MeOH	+	+
				EtOH	+	+
				H_2O	-	-
8	Glycosides	Salkowski's test	Reddish brown	MeOH	+	+
				EtOH	+	+
				H_2O	-	-
9	Phenols	Phenolic test	Blue green	MeOH	+	+
				EtOH	+	+
				H_2O	+	+
10	Saponins	Froth/ Foam Test	Foam	MeOH	+	+
				EtOH	+	+
				H_2O	+	+
11	Protein	Ninhydrin Test	Violet	MeOH	-	+
				EtOH	-	+
				H_2O	-	-
12	Carbohydrate	Molish Test	Violet ring at junction	MeOH	+	+
				EtOH	+	+
				H_2O	-	-

Calibration curve for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The total phenolic content in plant extract was determined by using Folin-Ciocalteu colourimetric method. The absorbance values obtained at different concentrations of gallic acid was used for the construction of calibration curve. Absorbance values for gallic acid measured at 765 nm using Folin-Ciocalteu reagent. Similarly the total flavonoid contents were determined by a colorimetric assay using aluminum chloride. Absorbance values for quercetin measured at 510 nm using aluminum chloride colourmetric assay were shown in Table 2 and calibration curve in Figure 1 and 2.

Table 2: Absorbance recorded for standard gallic acid and quercetin for calibration at 760nm and 430nm

Standard for calibration of phenols (Gallic acid)		Standard for calibration of flavonoid (Quercetin)	
Concentration (µg/ml)	Absorbance for Gallic acid measured	Concentration (µg/ml)	Absorbance values for Quercetin measured
10	0.14	10	0.03
25	0.28	25	0.12
50	0.54	50	0.22
75	0.74	75	0.31
100	1.12	100	0.4

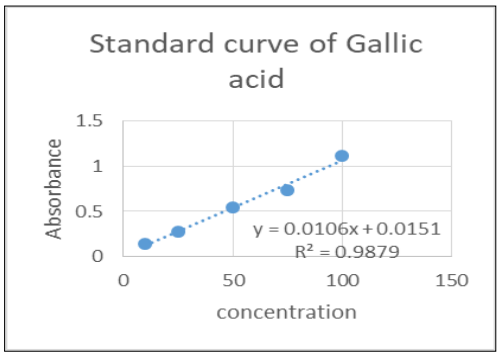

Fig 1: Calibration curve for authentic gallic acid

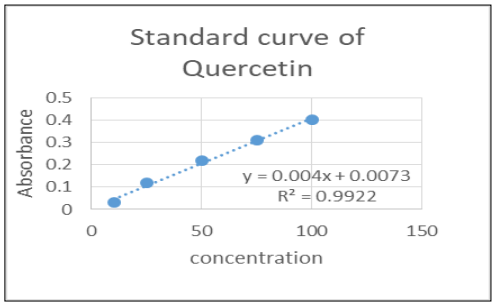

Fig 2: Calibration curve for authentic quercetin

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 was calculated using the formula C=cV/m and expressed as mg gallic acid equivalents (GAE) per g of extract in (mg/g). TFC of the extracts were calculated using the formula, C=cV/m and expressed as mg quercetin equivalents (QE) per gram extract in (mg/g). The TPC & TFC was calculated given in Table 3.

Table 3: Total Phenolic content (TPC) and Total Flavonoid Content (TFC) in leaf and stem extract of *A. aspera* L.

TPC and TFC content (Mean)		MeOH extract	50% EtOH extract	H_2O extract
mg GAE/ g (Mean TPC)	Leaf	271.17	219.29	200.00
	Stem	374.95	299.45	233.33
QE mg/ g (Mean TFC)	Leaf	115.875	90.875	53.375
	Stem	190.875	140.875	90.875

DPPH assay for antioxidant activities

Antioxidant potential of the methanol, 50% ethanol and aqueous extracts were measured by DPPH radical scavenging activity was carried out and absorbance values measured at wavelength 517 nm for different concentrations and the control of stem extract and leaf extracts are shown in Table 4 and Table 5.

The calculated percentage of inhibition showed that extract antioxidant activity potential. Methanol extract showed high antioxidant activity than that of aqueous extract. The DPPH radical scavenging activity was found to be increasing as dose increases. Earlier, *A. aspera* roots and leaves have been reported to possess DPPH radical scavenging activity with IC_{50} values. Presented study reports the high DPPH radical scavenging activity of *A. aspera* stem than that of leaves.

Folin-Ciocalteu (FC) method was applied for the determination of total phenolic using gallic acid as a standard based on the transfer of electrons in alkaline medium from the phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue colored complexes, $(PMoW_{11}O_{40})^{4-}$ that are determined spectrophotometric ally at 765 nm.

Table 4: Absorbance and a control at wavelength 517 nm in the DPPH assay, % inhibition and IC_{50} of stem

Plant extract	Conc. µg/ml	Absorbance	% Inhibition	IC_{50}	TPC(mg GAE/g)	TFC (mg QE/g)
Methanolic	5	0.71	23.65	23.97	374.95	190.875
	10	0.65	30.10			
	15	0.58	37.63			
50 % Ethanolic	5	0.76	18.27	33.41	299.45	140.875
	10	0.75	23.35			
	15	0.73	29.50			
Aqueous Extract	5	0.91	2.15	49.48	233.33	90.875
	10	0.85	8.60			
	15	0.82	11.82			
Control		0.93				

Table 5: Absorbance and a control at wavelength 517 nm in the DPPH assay, % inhibition and IC_{50} of Leaf

Plant extract	Conc. µg/ml	Absorbance	% inhibition	IC_{50}	TPC(mg GAE/g)	TFC (mg QE/g)
Methanolic	5	0.72	22.58	34.28	271.17	115.875
	10	0.70	24.73			
	15	0.63	32.25			
50 % Ethanolic	5	0.81	12.90	54.98	219.29	90.875
	10	0.79	15.05			
	15	0.74	20.43			
Aqueous Extract	5	0.92	1.07	63.53	200.00	53.375
	10	0.87	6.45			
	15	0.85	8.60			

The DPPH assay is based on the capability of an antioxidant to donate hydrogen radical which is stable free radical with deep violet color. When an odd electron become paired in the presence of free radical scavenger of antioxidant agent, DPPH radicals get reduced to corresponding hydrazine, DPPH-H form and the solution gets decolorized from its initial deep violet to light yellow color.

Conclusions

The qualitative phytochemical screening for methanol, 50 %ethanol and aqueous extract of *A. aspera* stem and leaf was performed and then inferred the total phenolic content and total flavonoid content in the plant to know the polyphenol present. The results of phytochemical, polyphenols and antioxidant reveals that the leaf and stem of this plant extract were vital and enough to show a protective mechanism in vivo. The maximum polyphenol and antioxidant activity is present in methanolic extracts and furthermore, some pharmacological activities have to be performed to establish the importance of certain phytoconstituents having protective action. This research will certainly help to quantify the total phenolic and flavonoid content and antioxidant effect of Nepalese *A. aspera plant.* The Polyphenols from leaves can be used as potent biochemical and showed antioxidant property so that they could be rich source of natural antioxidants.

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