

Phytochemical Analysis of *Ampelopteris Prolifera* (Retzius) Copeland

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Abstract

The fronds of *Ampelopteris prolifera* are used in stomach ache as well as vegetable. Dried and powdered fronds of *A. prolifera* was extracted successively with hexane, chloroform, ethyl acetate, methanol and 50% aqueous methanol. The results of phytochemical screening revealed that alkaloids were absent in all extracts. Hexane extract showed the absence of all the tested phytochemicals while chloroform extract showed the presence of only terpenoids and quinones. Ethylacetate extract showed the presence of all tested phytochemicals except quinones and saponins. Methanol and 50% aqueous methanol extracts showed the presence of phenolics, flavonoids, terpenoids, glycosides, tannins, quinones and saponins. The total content of phenolics, flavonoids, tannins and sugars as well as antioxidant activity were determined in ethyl acetate, methanol and 50% aqueous methanol extracts. Total phenolic content was measured spectrophotometrically by using Folin-Ciocalteu, total flavonoid content by using aluminium chloride, total hydrolyzable tannin content by using Folin-Ciocalteu, total condensed tannin content using vanillin-HCl and total sugar content by using anthrone reagent. Gallic acid was used as the standard for the calibration of phenolics, catechin for flavonoids and condensed tannins, tannic acid for hydrolyzable tannins and glucose for carbohydrates. The highest amount of phenolic was detected in 50% methanol extract (114.27±10.37 mg GAE/g). The highest amount of flavonoid was detected in ethylacetate extract (151.47±3.57 mg CE/g extract). The highest amount of hydrolyzable tannin was detected in 50% methanol extract (31.21±2.14 mg TAE/g extract). The highest amount of condensed tannin was detected in ethylacetate extract (337.50±5.00 mg CE/g extract), and the highest amount of sugar was detected in methanol extract (809.74±7.86 mg GE/g). The antioxidant activity was determined by using DPPH free radical and IC₅₀ value was determined. All the tested extracts showed only weak antioxidant activity. Among the tested extracts, 50% aqueous methanol extract showed better activity with an IC₅₀ value of 185.03µg/ml. The HPLC-ESI-MS analysis of methanol extract allowed the tentative identification of peganine, 1-methyltryptophan and rutin. The study thus demonstrates the potential value of *A. prolifera* in the medicinal application.

Keywords

Antioxidant, HPLC-ESI-MS, total flavonoid, total phenolic, total tannin sugar

Introduction

Plants and herbal preparations are generally used as foodstuffs and traditional medicine. However, their value can be explored only by phytochemical analysis. The earliest evidence of the use of plants by a human for healing can date back to the Neanderthal period (Winslow and Kroll 1998), and the information is readily available for

modern scientific research on drug discovery. The World Health Organization estimates that about 85% of traditional medicine involves the use of plant extracts that provide primary healthcare for approximately 3.5 to 4 billion people worldwide (Farnsworth *et al.*, 1985).

Nowadays, drugs have become a daily necessity for many people, especially the

elderly, with multiple health problems. Plants contain hundreds of active ingredients, and their combined action enhances the synergetic effect that may be potentially useful for the development of prophylactic as well as therapeutic agents for multiple targets. Drug discovery and development should not always be limited to the discovery of a single molecule and the current belief on one drug one disease approach. Preparation of standardized plant extracts or isolation and identification of phytochemicals from plant materials are therefore crucial for drug discovery as they provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Patwardan and Mashelkar 2009).

Although many types of drugs are available in the market, developing new drugs for the treatment of the world's major diseases such as malaria, trypanosomiasis, filariasis, leishmaniasis and tuberculosis are still in high demand. Chronic and difficult to treat diseases such as cancers, cardiovascular disease, diabetes, rheumatism all require new effective drugs. Some available synthetic drugs have adverse and unacceptable side effects as well as they developed resistance. Therefore, the search for the new classes of the drug is one of the most critical challenges. Medicinal plants used in traditional medicines could be an alternative source for the development of new drugs (Patwardan *et al.*, 2004). Nepalese medicinal plants could be the potentially valuable sources for the development of phytomedicines, nutraceuticals or food supplements and personal care products. However, for the value addition, it is essential to establish phytochemical and pharmacological information as well as the effectiveness and safety of the products. Therefore, the assessment of such properties remains an exciting and useful task (Pan *et al.*, 2013).

Pteridophytes are not well investigated phytochemically in comparison to angiosperms. It prompted us to investigate the pteridophyte, *Ampelopteris prolifera*. Locally, it is known as 'Neuro' and found on sandy flooded plains by streams in sunny areas from 100 m to 1000 m of altitude in tropical and subtropical regions. The young fronds are eaten as vegetable in terai region and also used in a stomach ache. A survey of the recent literature showed minimal data on

A. prolifera. Only phytochemical screening of rhizome and frond of *A. prolifera* collected from India was available (Bharati 2018).

Therefore, the present study was carried out to estimate the total content of phenolic, flavonoid, tannin and sugar in different extracts and their antioxidant activity by DPPH method and the determination of chemical constituents of methanol extract by LC-MS technique.

Methodology

Plant materials

The plants were collected from Rupandehi district of western Nepal in April 2017 and authenticated by comparing with the voucher specimens deposited at Central Department of Botany Tribhuvan University, Kathmandu, Nepal. The voucher specimen (#MP 17) was deposited at Research Centre for Applied Science and Technology, RECAST, TU. The fronds were shade dried, powdered and kept in an airtight container for further use.

Chemicals and equipment

DPPH and authentic (\pm)- catechin were purchased from Sigma Chemical Company, USA. Gallic acid was purchased from Merck, Darmstadt, Germany. Aluminium chloride (SD fine-chemicals), Folin-Ciocalteu (SD fine-chemicals) and anthrone (Thomas baker) were purchased from a local vendor. All other chemicals and solvents were of analytical grade and purchased from local vendors. Absorbance was measured using Chemito UV-VIS Spectrophotometer. LC-MS was carried out in Agilent 1200 HPLC system connected to Binary pump G4220A, Column compartment G1316C, Diode-array detector 4212 A and Agilent MS Q-TOF, G6540A mass spectrometer.

Extraction

The dried and powdered plant material (100 g) was extracted successively with hexane (400 mL), chloroform (300 mL), ethyl acetate (300 mL) and methanol (300 mL) by Soxhlet extraction method. The remaining residue after extraction with methanol was refluxed with 50% aqueous methanol (200 mL) for 2 hours then allowed to cool and filtered. The extracts were concentrated using rota vapour and the solid or semi-solid mass

obtained was kept in a freezer for further analysis.

Phytochemical Screening

The presence of main classes of natural constituents in different extracts was analyzed by using specific reagent according to the standard protocol (Culi 1982).

Estimation of total phenolic content in different extracts

The total phenolic content in plant extracts was estimated by using the Folin-Ciocalteu colorimetric method (Waterhouse 2002). Gallic acid was used as the standard for the construction of the calibration curve. Various concentrations of gallic acid solutions in methanol (10, 25, 50, 75 and 100 $\mu\text{g}/\text{mL}$) were prepared. In a 20 mL test tube, 1 mL gallic acid solution of each concentration was added and to that 5 mL Folin-Ciocalteu reagent (10%) and 4 mL 7% sodium carbonate were added to get a total volume of 10 mL. The blue coloured mixture was shaken well and incubated for 30 minutes at 40 $^{\circ}\text{C}$ in a water bath. Then, the absorbance was measured at 760 nm against a blank. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve. Various concentrations of the extracts (200, 100, 50 and 25 $\mu\text{g}/\text{mL}$) were prepared. Following the procedure described for standard, absorbance for each concentration of the extract was recorded. Total phenolic content of the extracts was expressed as mg gallic acid equivalents (GAE) per gram dry extract (mg/g).

Estimation of total flavonoid content in different extracts

The total flavonoid content in plant extracts was estimated by using the aluminium chloride colorimetric method (Zhishen *et. al.*, 1999). Catechin was used as the standard for the construction of the calibration curve. Various concentrations of catechin (10, 25, 50, 75 and 100 $\mu\text{g}/\text{mL}$) were prepared. An aliquot of 1 mL catechin of each concentration in methanol was added to a 10 mL test tube containing 4 mL double distilled water. At the zero time, 0.3 mL 5% sodium nitrite was added, after 5 min, 0.3 mL of 10% AlCl_3 was added, and after 1 min, 2 mL of 1 M sodium

hydroxide 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. The absorbance of the pink coloured mixture was determined at 510 nm against a blank containing all reagents except catechin. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of catechin were used to plot the calibration curve.

Various concentrations of the extracts (200, 100, 50 and 25 $\mu\text{g}/\text{mL}$) were prepared. Following the procedure described for standard, absorbance for each concentration of the extract was recorded. Total flavonoid content of the extracts was expressed as mg catechin equivalents (CE) per gram of dry extract (mg/g).

Estimation of total tannin content in different extracts

The total tannin content in plant extracts was estimated by using two different methods (Katoch 2011). Total hydrolysable tannin content was estimated by using Folin-Ciocalteu colourimetric method. Tannic acid was used as the standard for the construction of a calibration curve. Various concentrations of tannic acid such as 10, 25, 50, 75 and 100 $\mu\text{g}/\text{mL}$ were prepared. An aliquot of 1 mL tannic acid of each concentration in distilled water was poured to 10 mL test tube containing 8.4 mL of distilled water to which 0.5 mL of Folin-Ciocalteu reagent and 0.1 mL of 7% Na_2CO_3 solution were added. The solution was then shaken well and allowed to stand for 30 minutes and then absorbance was taken at 700 nm against a blank. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of tannic acid were used to plot the calibration curve. Various concentrations of the extracts (1000, 500, 250 and 125 $\mu\text{g}/\text{mL}$) were prepared. Following the procedure described for standard, absorbance for each concentration of the extract was recorded. The total tannin content of the extracts was expressed as mg tannic acid equivalents (TAE) per gram of dry extract (mg/g).

Total condensed tannin content was estimated by using vanilin-HCl colorimetric method. Catechin was used as the standard for

the construction of a calibration curve. Various concentrations of catechin such as 10, 25, 50, 75 and 100 µg/ml were prepared. Vanillin hydrochloride reagent was prepared by mixing equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol just before use. An aliquot of 1 mL catechin of each concentration in methanol was poured to 10 mL test tube and added 5 ml of vanillin hydrochloride reagent and allowed to stand for 20 minutes. Then the absorbance was taken at 500 nm against a blank containing 5 ml vanillin hydrochloride reagent and 1 mL methanol. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of catechin were used to plot the calibration curve. Various concentrations of the extracts (1000, 500, 250 and 125 µg/mL) were prepared. Following the procedure described for standard, absorbance for each concentration of the extract was recorded. The total condensed tannin content of the extracts was expressed as mg catechin equivalent (CE) per gram of dry extract (mg/g).

Estimation of total sugar content in different extracts

The total sugar content in plant extracts was estimated by using anthrone reagents (Hedge & Hofreiter 1962). Glucose was used as the standard for the construction of a calibration curve. Various concentrations of D-glucose (10, 25, 50 and 75 µg/mL) were prepared. An aliquot of 2 mL glucose of each concentration and 8 mL of freshly prepared anthrone reagent (200 mg of anthrone in 100 mL of ice-cold 95% H₂SO₄) was mixed in 15 mL test tube. The mixture was shaken well and heated for 8 minutes at boiling water bath. Then cooled rapidly and the absorbance of green coloured solution was measured at 630 nm against a blank containing all reagents except sugar. All the experiments were carried out in triplicate. The absorbance values obtained were used separately to plot calibration curve.

An amount of 100 mg each extract was dissolved in methanol and added 5 mL of 2.5 N HCl and subjected to hydrolysis by keeping it in a boiling water bath for 3 hrs then cooled and neutralized with solid sodium carbonate and make up the volume to 100 mL (1 mg/mL). Serial

dilutions were carried out to get the concentration of 100, 200, 400 and 600 µg/mL. To these diluted solutions, anthrone reagent was added and heated for 8 minutes as in the case of glucose and absorbance was measured at 630 nm against a blank.

Calculation

The total phenolic, flavonoid, and tannin sugar content were calculated in all the extracts separately using the formula,

Where,

C = Total content of flavonoid/phenol/tannin sugar compounds in mg/g,

c = Concentration of gallic acid/ catechin/ tannic acid/ D-glucose established from the calibration curve in mg/ml, V = Volume of extract in ml, m = Weight of plant extract.

Statistical analysis

Data were recorded as a mean ± standard deviation of three determinations of absorbance for each concentration, from which the linear correlation coefficient (R²) value was calculated using MS Office Excel 2007. The linear regression equation for a straight line is, $y = mx + c$ where, y = absorbance of extract, m = slope of the calibration curve, x = concentration of extract, c = intercept. Using this regression equation, concentrations of extracts were calculated. From the calculated values of concentration of each extract, the total phenolics, flavonoid and sugar content were calculated.

Determination of antioxidant activity using 2, 2-Diphenyl – 1-picrylhydrazyl radical scavenging assay

Antioxidant activity of the selected extracts was assessed using DPPH free radical (Brand-William *et. al.*, 1995). DPPH solution (0.1 mM) was prepared by dissolving 3.9 mg of DPPH in 100 mL methanol and stirred overnight at 4C. Thus, prepared purple-coloured DPPH free radical solution was stored at -20C for further use.

Different concentrations (5, 10, 15 and 20 µg/mL) of methanolic solutions of each extract were prepared by the serial dilution of the stock solution. 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 methanol and 2.5 ml 0.1 mM methanolic DPPH solution. These samples were shaken well and kept in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against the blank solution consisting of methanol. The radical scavenging activity was expressed as the radical scavenging percentage.

The IC₅₀ 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 the concentration of extracts.

High-performance liquid chromatography-mass spectrometry analysis of methanol extract of *A. prolifera*

Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) measurements were carried out on Agilent 1200 HPLC system interfaced with Agilent G6540A hybrid quadrupole time of flight mass spectrometer (Agilent technologies, USA). The HPLC system was equipped with a binary pump (G4220A), column compartment (G1316C) and diode-array detector (4212A).

Chromatographic condition

Chromatographic separations were performed using a Phenomenex Luna Omega C18, column (50 mm×2.1 mm, 1.6 μm) operated at 25 °C employing a gradient elution using 0.1% formic acid in water (A) and 0.1% formic acid in

acetonitrile (B) as mobile phase at a flow rate of 0.6 mL/min and 1000 bar pressure. The elution consisted of a gradient of 2%–98%, 0–15 min, 95%–5%, 16–17 min and initial condition was maintained for 5 min. The sample injection volume was 1 mL. The diode array detector was monitored at 220 and 254 nm while UV spectra from 190 to 640 nm were recorded for peak characterization.

Mass spectrometric condition

The mass spectrometer was operated in positive and negative electrospray ionization mode, and spectra were recorded by scanning the mass range from *m/z* 80 to 1400 in MS mode. Nitrogen was used as drying, nebulizer and collision gas. Drying gas flow rate was 8 L/min. The heated capillary temperature was set at 300 °C and nebulizer pressure at 50 psi. The source parameters such as capillary voltage were set at 3500 V, fragment, skimmer and octopole voltages were set at 120 V, 45 V and 750 V. The accurate mass data of the molecular ions were processed through the Agilent Mass Hunter Workstation (version B 04.00) software.

Results and Discussion

Extractive values of different extracts

The plant material (100 g) was successively extracted with hexane, chloroform, ethylacetate and methanol in a Soxhlet extractor. The remaining residue was refluxed with 50% aqueous methanol.

Table 1. The yield of extracts in gram and results of phytochemical screening of *A. prolifera* extracts

Yield and classes of phytochemicals	Hexane	CHCl ₃	EtOAc	MeOH	50% MeOH
The yield of extracts from 100 g material	3.05	1.78	4.26	5.46	7.34
Phenolics	-	-	+	+	+
Flavonoids	-	-	+	+	+
Alkaloids	-	-	-	-	-
Terpenoids	-	+	+	+	+
Glycosides	-	-	+	+	+
Saponins	-	-	-	+	+
Quinones	-	+	-	+	+
Reducing Sugar	-	-	+	+	+
Tannins	-	-	+	+	+

(+) indicates present and (-) indicates absent

Altogether five different extracts were prepared. The highest amount of extract was obtained with 50% aqueous methanol, and the lowest amount was obtained with chloroform. The results of the yield of the different extracts are shown in Table 1.

Phytochemical Screening

The phytochemical screening of hexane, chloroform, ethyl acetate, methanol and 50% aqueous methanol extracts of *A. prolifera* indicated that alkaloids were absent in all extracts. None of the tested phytochemicals was detected in hexane extract. Hexane extract generally contains fatty acids, phytosterols and hydrocarbons. Only terpenoids and quinones were detected in chloroform extract. In ethyl acetate extract, all the phytochemicals were present except saponins and quinones. Methanol and 50% aqueous methanol extracts showed the presence of all tested phytochemicals. The results indicated that *A. prolifera* could be a rich source of bioactive phytochemicals. The results of the phytochemical screening are shown in Table 1.

Determination of total phenolic content in different extracts

The absorbance values measured at 760 nm using Folin-Ciocalteu reagent for different concentrations of gallic acid was used for the construction of the calibration curve. The absorbance values for different concentrations (125, 250, 500 and 1000 $\mu\text{g/mL}$) of each extract were also measured at 760 nm. The total phenolic content in different extracts was calculated from the calibration curve using regression equation $Y=0.0145x+0.0569$, $R^2= 0.9964$ followed by the formula $C= cV/m$ and expressed as mg gallic acid equivalents (GAE) per g of extract in dry weight (mg/g). The results indicated that the highest amount of phenolic was detected in the 50% methanol extract (114.27 ± 10.37 mg GAE/g extract) while the lowest amount was found in the methanol extract (53.28 ± 5.57 mg GAE/g extract). The results are shown in Table 2.

Quantification of phenolics in plant extract is influenced by the chemical nature of the extracts as well as assay method, selection of standards and presence of interfering substances (Naczka and Shahidi 2006). As the plant extracts contain different types of phenolics and other readily

oxidizable substances, the methods used for the determination of total phenolics are not perfect (Singleton *et. al.*, 1999). The Folin Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes $(\text{PMoW}_{11}\text{O}_{40})^{4-}$ that are determined spectroscopically at approximately 760 nm (Singleton & Rossi 1965). It is indeed a measure of total phenolics and other oxidation substrates. The other oxidation substrate present in a given extract sample can interfere with the total phenolics measurement in an inhibitory or additive manner. The inhibitory effects could be due to the oxidants competing with Folin reagent, and the additive effects occur from unanticipated phenols, aromatic amines, high sugar levels or ascorbic acid in the samples. The Folin-Ciocalteu assay is also considered as antioxidant capacity assay because its underlying mechanism is as oxidation/reduction reaction, although it has been used as a measurement of total phenolics content. Phenolics are the largest category of phytochemicals and display wide ranges of biological activities. Recently, phenolics have been considered powerful antioxidants and free radical scavengers *in vitro* and proved to be more potent antioxidants than Vitamin C and E and carotenoids (Rice-Evans *et. al.*, 1995, Rice-Evans *et. al.*, 1996, Sugihara *et. al.*, 1999). The high intake of fruit and vegetable and the low risk of oxidative stress associated diseases such as cardiovascular diseases, cancer or osteoporosis has been partly ascribed to phenolics (Hollman 1999, Scalbert *et. al.*, 2005). Phenolic compounds are also known to play an essential role in stabilizing lipids against peroxidation and inhibiting various types of oxidizing enzymes (Laughton *et. al.*, 1991; Cos and Calomme 1998).

Determination of total flavonoid content in different extracts

The absorbance values measured at 510 nm using aluminium chloride reagent for different concentrations of catechin was used for the construction of calibration curve. The absorbance values for different concentrations (125, 250, 500 and 1000 $\mu\text{g/mL}$) of each extract were also measured at 510 nm. The total flavonoid content in different extracts was calculated from the

calibration curve using regression equation $Y = 0.0028x + 0.0353$, $R^2 = 0.9625$ followed by the formula $C = cV/m$ and expressed as mg catechin equivalents (CE) per g of extract in dry weight (mg/g). The results of this investigation indicated that the highest amount of flavonoid was detected in the ethyl acetate extract (151.47 ± 3.57 CE/g) while the lowest amount was detected in the methanol extract (48.17 ± 6.44 CE/g extract). The results are shown in Table 2.

Flavonoids are the largest group of plant polyphenols, and they have been reported to possess many useful properties, including anti-inflammatory, oestrogenic, enzyme inhibition, antimicrobial, antiallergic, cytotoxic and antitumour activity (Cushnie and Lamb 2005). The anticancer activity of some flavonoids is due to their ability to scavenge free radicals, thus preventing the early stages of cancer promotion (Aron and Kennedy 2008).

Determination of total tannin content **Determination of total hydrolyzable tannin content in different extracts**

It is based on the non-stoichiometric oxidation of the molecules containing a phenolic hydroxyl group. The absorbance values measured at 700 nm using Folin-Ciocalteu reagent for different concentrations of tannic acid was used for the construction of the calibration curve. The absorbance values for different concentrations (125, 250, 500 and 1000 $\mu\text{g/mL}$) of each extract were also measured at 700 nm. The total hydrolysable tannin content in different extracts was calculated from the calibration curve using regression equation $Y = 0.006x - 0.027$, $R^2 = 0.9848$ followed by the formula $C = cV/m$ and expressed as mg tannic acid equivalents (TAE) per g of extract in dry weight (mg/g). The results of this investigation indicated that the highest amount of hydrolysable tannin was detected in the ethyl acetate extract (151.47 ± 3.57 CE/g) while the lowest amount was detected in the methanol extract (48.17 ± 6.44 CE/g extract). The results are shown in Table 2.

Tannins are known to possess antioxidative property, which is essential in protecting cellular oxidative damage, including lipid peroxidation. The scavenging activity of tannins increases

with an increase in the number of galloyl groups and molecular weight and the presence of an ortho-dihydroxy structure. The hydroxyl groups are responsible for the chelating and radical scavenging properties of these compounds (Yokozawa *et al.*, 1998). It was described that tannins enhance glucose uptake capacity and inhibit adipogenesis, thus being potential drugs for the treatment of non-insulin dependent diabetes mellitus (Muthusamy *et al.*, 2008).

Determination of total condensed tannin content in different extracts

The absorbance values measured at 500 nm using vanillin-hydrochloric acid reagent for different concentrations of catechin was used for the construction of the calibration curve. The absorbance values for different concentrations (125, 250, 500 and 1000 $\mu\text{g/mL}$) of each extract were also measured at 500 nm. The total condensed tannin content in different extracts was calculated from the calibration curve using regression

equation $Y = 0.0006x + 0.0072$, $R^2 = 0.993$ followed by the formula $C = cV/m$ and expressed as mg catechin equivalents (CE) per g of extract in dry weight (mg/g). The results of this investigation indicated that the highest amount of condensed tannin was detected in the ethyl acetate extract (337.50 ± 5.00 CE/g) while the lowest amount was detected in the methanol extract (115.42 ± 6.80 CE/g extract). The results are shown in Table 2.

It was reported that proanthocyanidins and flavan-3-ols of monomers aid in lowering plasma cholesterol levels, inhibit LDL oxidation, and activate endothelial nitric oxide synthase to prevent platelet adhesion and aggregation that contribute to blood clot formation (Aron and Kennedy 2008; Bagchi *et al.*, 2003).

In general, traditional spectrophotometric assays provide fast and straightforward screening methods to quantify classes of phenolic compounds in crude plant samples. However, due to the complexity of the plant phenolics and different reactivity of phenols toward assay reagents, a broad spectrum of methods is used for assay of the constituents, leading to differing and often non-comparable results. In addition to that, the methods are quite prone to interferences and consequently

Table 2. Total phenolic, flavonoid, tannin, sugar and antioxidant activity of different extracts

Analysis	EtOAc	MeOH	50% MeOH
Total phenolic content (mg GAE/g extract)	57.45±1.15	53.28±5.57	114.27±10.37
Total flavonoid content (mg CE/g extract)	151.47±3.57	48.17±6.44	111.18±7.95
Total hydrolysable tannin content (mg TAE/g extract)	26.62±2.89	19.11±3.56	31.21±2.14
Total condensed tannin content (mg CE/g extract)	337.50±5.00	115.42±6.80	235.83±6.76
Total sugar (mg GE/g extract)	207.44±4.81	809.74±7.86	293.66±6.10
IC ₅₀ (µg/ml) in DPPH assay	342.42±7.85	210.54±6.75	185.03±7.21

often result in over or underestimation of the contents.

Determination of total sugar content in different extracts

Plant extracts containing sugars were first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethylfurfural that with anthrone, an enol form of anthrone forms a green coloured product with an absorption maximum at 630 nm which was measured against a blank consisting of anthrone solution and distilled water. The absorbance values measured at 630 nm using anthrone reagent for different concentrations of glucose was used for the construction of the calibration curve. The absorbance values for different concentrations (100, 200, 400 and 600 µg/mL) of each extract were also measured at 630 nm. The total sugar content in different extracts was calculated from the calibration curve using regression equation $Y=0.0034x+0.023$ $R^2 = 0.972$ followed by the formula $C= cV/m$ and expressed as mg glucose equivalents (GE) per g of extract in dry weight (mg/g). The results of this investigation indicated that the highest amount of sugar was

detected in the methanol extract (809.74±7.86 GE/g) while the lowest amount was detected in the ethyl acetate extract (207.44±4.81 GE/g extract). The results are shown in Table 2.

Plant polysaccharides produce stimulating/suppressing effect on the immune system (Xu 2011). Plant polysaccharides have been used extensively as pharmacological supplements to aid weight control, to regulate glucose control for diabetic patients and to prevent acute and chronic diseases (Mann 2007).

DPPH assay for antioxidant activity

The absorbance values were measured at 517 nm for different concentrations of extracts and the control against blank. These values were used to calculate the percentage inhibitions of DPPH radicals against the samples. The IC₅₀ values were calculated from the plotted graph of radical scavenging activity against the concentration of extracts. The IC₅₀ values in DPPH assay are given in Table 2. The results indicated that all the tested extracts showed radical scavenging capacity. The lowest IC₅₀ value was shown by the 50% methanol extract (185.03 µg/mL). This may be

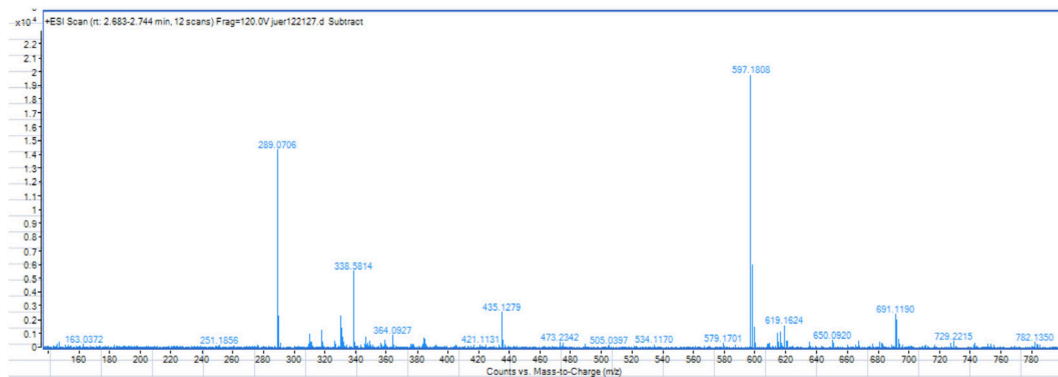


Fig. 1a Mass spectrum of rutin in positive ion mode

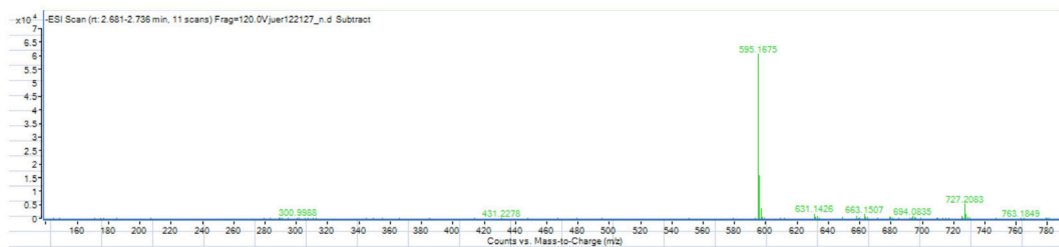


Fig. 1b Mass spectrum of rutin in negative ion mode

due to the highest amount of phenolic content in 50% methanol extract (114.27 ± 10.37 mg GAE/g extract) than other extracts. This confirms the role of phenolics in the antioxidant activity of *A. prolifera*.

LC-MS analysis of methanol extract of *A. prolifera*

The chemical composition of methanol extract of *A. prolifera* was determined using the LC-ESI-MS technique both in positive and negative ionization modes. All investigated compounds showed strong protonated molecular ion $[M+H]^+$ in (+)-ESI-MS mode and deprotonated molecular ion $[M-H]^-$ in (-)-ESI-MS mode. The molecular ions were subjected to MS/MS analysis to learn the basic cleavage patterns. Compounds were characterized based on their mass spectra, using the precursor ion, fragment ions, and comparison of the fragmentation patterns with molecules described in the literature.

In positive ion mode, a peak eluting at 0.901 min with precursor ion of m/z 189 with the fragment ion of m/z 171 was tentatively identified as peganin, a quinazoline alkaloid. The formation of fragment ion of m/z 171 was due to the loss of H_2O , $[M+H-[H_2O]]^+$ (Liu *et al.*, 2013). Although in phytochemical screening, alkaloid was not detected, peganin was tentatively identified. This may be due to the low concentration of alkaloids in extracts to make it visible by colour reaction.

In positive ion mode, a peak eluting at 1.15 min with precursor ion of m/z 219 with the single fragment ion of m/z 159 was identified as 1-methyltryptophan. Tryptophan fragment gave rise to an ion at m/z 159 due to the consecutive loss of NH_3 and CO_2 groups. Its particular indole group (m/z 116 C_8H_6N) was the most abundant fragment generated after consecutive losses of NH_3 , CO_2 and C_2H_2 (Taamalli *et al.*, 2015).

In positive ion mode (Fig 1a), a peak was eluted

Table 3. Identification of compounds by LC-ESI-MS in positive in mode

Retention Time	Precursor ion (m/z) $[M+H]^+$	Fragment ions (m/z)	Molecular mass	Molecular formula	Tentative assignment
0.901	189.1026	171, 153, 139	188.0953	$C_{11}H_{12}N_2O$	Peganine
1.150	219.1127	199, 159, 128	218.0791	$C_{12}H_{14}N_2O_2$	1-methyl tryptophan
2.705	611.1603	597, 435, 338	610.1529	$C_{27}H_{30}O_{16}$	Rutin

Table 4. Identification of compound by LC-ESI-MS in negative-ion mode

Retention time	Precursor ion (m/z) $[M-H]^-$	Fragment ions (m/z)	Molecular mass	Molecular formula	Tentative assignment
2.703	609.1469	595, 431, 301	610.1538	$C_{27}H_{30}O_{16}$	Rutin

at 2.705 min with precursor ion of m/z 611 with fragment ion of m/z 597, 435 and 338. In the negative ion mode (Fig 1b) with precursor ion of m/z 609 with fragment ion of m/z 595, 431 and 301 was identified as rutin. The fragment at m/z 431 is due to the loss of *O*-rhamnose moiety. The fragment at m/z 301 is due to the formation of quercetin daughter ion with the loss of glucose moiety (Ramirez *et. al.*, 2014). Rutin was detected better in negative mode due to the acidic nature of these compounds, which made the ions more abundant and easily detected in this ionization mode. It was reported that the use of ESI in the negative mode has proven to be more selective and efficient in the characterization of phenolic compounds (Gouveia and Castilho 2009). Our result is in agreement with the literature (Gouveia and Castilho 2009). The results are shown in Table 3 and 4.

Conclusion

The results of this study revealed that the ethyl acetate, methanol and 50% aqueous methanol extracts of *A. proliferata* contains pharmacologically active substances like phenolics, flavonoids, tannins as well as carbohydrates. These extracts also exhibited antioxidant activity against DPPH. The LC-MS analysis of the methanol extract showed the presence of many phytochemicals. Among them, peganin, 1-methyl tryptophan and rutin were tentatively identified based on the fragmentation pattern in the mass spectrum. Therefore, plant *A. proliferata* could be a new source for the development of phytopharmaceuticals for management of several diseases.

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References

Aron, P.M., Kennedy, J.A. 2008. Flavan-3-ols: Nature, occurrence and biological activity. *Molecular Nutrition & Food Research* **52**: 79-104.

Bharti, M., 2018. Studies on phytochemical analysis and screening for active compounds in some ferns of Ranchi and Latehar districts. *International Journal of Academic Research and Development*

3: 33-41.

Bagchi, D.B., Sen, C.K., Ray, S.D., Das, D.K., Bagchi, M., Preuss, H.G., Vinson, J.A. 2003. Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutation Research* **523**: 87-97.

Brand-Williams; W., Cuveiler, M.E., Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Food Science and Technology* **28**: 25-30.

Cos, P., Ying, L., Calomme, M., 1998. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *Journal of Natural Products*. **61** :71–76.

Cushnie, T.P.T., Lamb, A.J. 2005. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents***26**: 343-356.

Culie, I. 1982. Methodology for the analysis of vegetable drugs, Practical manuals on industrial utilization of medicinal and aromatic plant. *Phytochemistry* **63**: 97-104.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D. D., and Guo, Z.G., 1985. Medicinal plants in therapy. *Bulletin of WHO* **63**:965–981.

Gouveia, S.C., Castilho, P.C., 2009. Analysis of phenolic compounds from different morphological parts of *Helichrysum devide* by liquid chromatography with on-line UV and electrospray ionization mass spectrometric detection. *Rapid Communication and Mass Spectrometry* **23**: 3939–3953.

Hollman, P. C., Katan, M.B., 1999. Dietary flavonoids: intake, health effects and bioavailability. *Food Chemistry and Toxicology* **37**: 937-942.

Hedge, J.E. and Hofreiter, B.T. 1962. In: Carbohydrate Chemistry. Whistler R.L. BeMiller, J.N., Academic Press, New York, pp. **420**.

Kähkönen, M.P., Hopia, A.I., Vuorela, H.J. 1999. Antioxidant activity of plant extracts containing phenolic compounds *Journal of Agricultural and Food Chemistry* **47**:3954–3962.

Katoch, R., 2011. Methods for Nutritional Quality Evaluation of Food Materials. In: Analytical Techniques in Biochemistry and Molecular Biology. Springer, New York, NY, pp 251-322.

Laughton, M.J., Evans, P.J., Moroney, M.A., Hout, J.R.S., Halliwell, B. 1991. Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and iron ion-reducing ability. *Biochemical Pharmacology* **42**:1673–1681.

Liu, F., Kim, J., Li, Y., Liu, X., Li, J., Chen, X. 2001. An extract of *Lagerstroemia speciosa* L. has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3- L1

- cells. *Journal of Nutrition* **131**: 2242-2247.
- Liu, L., Zhao, T., Cheng, X.M., Wang, C.H., Wang, Z.T., 2013. Characterization and Determination of Trace Alkaloids in Seeds Extracts from *Peganum harmala* Linn Using LC-ESI-MS and HPLC. *Acta Chromatographica* **25**: 221-240.
- Mann, J., Cummings, J. H., Englyst, H. N., Key, T., Liu, S., Riccardi, G., Summerbell, C., Uauy, R., Dam, R.M., Venn, B., Vorster, H.H., Wiseman, M. 2007. *European Journal of Clinical Nutrition* **61**: S132-S137.
- Muthusamy, V.S., Anand, S., Sangeetha, K.N., Sujatha, S., Lakshmi, B.A.B.S. 2008. Tannins present in *Cichorium intybus* enhance glucose uptake and inhibit adipogenesis in 3T3-L1 adipocytes through PTP1B inhibition. *Chemico Biological Interaction* **174**: 69-78.
- Naczek, M., Shahidi, F. 2006. Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis* **41**: 1523-1542.
- Pan, S. Y., Zhou, S. F., Gao, S. H., Yu, Z. I., Zhang, S. F., Tang, M. K., Sun, J. N., Ma, D. L., Han, Y. F., Fong, W. F., Ko, K. M., 2013. New Perspectives on How to Discover Drugs from Herbal Medicines: CAM's Outstanding Contribution to Modern Therapeutic. *Evidence-Based Complementary and Alternative Medicine*. doi.org/10.1155/2013/627375
- Patwardan, B., Vaidya, A.D.B., Chorghade, M., 2004. Ayurveda and natural products drug discovery. *Current Science* **6**:789-799.
- Patwardan, B. and Mashelkar, R.A., 2009. Traditional medicine inspired approaches to drug discovery: can Ayurveda show the way forward? *Drug Discovery Today* **14**: 804.
- Ramirez, J. E., Zambrano, R., Sepulveda, B., Simiriotis, M.J. 2014. Antioxidant properties and hyphenated HPLC-PDA-MS profiling of Chilean Pica mango fruits (*Mangifera indica* L. Cv.piqueno). *Molecules* **19**: 438-458.
- Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., Pridham, J.B. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research*, **22**: 375-383.
- Rice-Evans, C.A., Miller, N.J., Paganga, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine* **20**: 933-956.
- Scalbert, A., Manach, C., Morand, C., Remesy, C., Jimenez, L. 2005. Dietary polyphenols and the prevention of diseases. *Critical Review in Food Science and Nutrition* **45**: 287-306.
- Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants using Folin-Ciocalteu reagent. *Methods in Enzymology* **299**: 152-178.
- Singleton, V.L., Rossi, J.A., 1965. Colourimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* **16**: 144-158.
- Sugihara, N., Arakawa, T., Ohnishi, M., Furuno, K. 1999. Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with α -linolenic acid. *Free Radical Biology and Medicine* **27**:1313-1323.
- Taamalli, A., Arraez Roman, D., Abaza, L., Iswaldi, I., Fernandez Gutierrez, A., Zarrouk, M., Segura Carretero, A. 2015. LC MS based metabolite profiling of methanolic extracts from the medicinal and aromatic species *Mentha pulegium* and *Origanum majorana*. *Phytochemical Analysis* **26**: 320-330.
- Waterhouse, A., 2002. Determination of total phenolics. In: *Current protocols in food analytical chemistry* (Ed Wrolstad, R. E). John Wiley and Sons, New York, Units 11.1.111.1.8.
- Winslow, L.C. and Kroll, D.J. 1998. Herbs as medicines. *Archives of Internal Medicine* **158**: 2192-2199.
- Xu, Z., Chen, X., Zhong, Z., Chen, L., Wang, Y. 2011. Ganoderma lucidum polysaccharides: immunomodulation and potential anti-tumour activities. *The American Journal of Chinese Medicine* **39**: 15-27.
- Yokozawa, T., Chen, C.P., Wong, E., Tanaka, T., Nonaka, G.I. Nishioka, I. 1998. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochemistry and Pharmacology* **56**: 213-222.
- Zhishen, J., Mengcheng, T., Jianming, W., 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry* **64**: 555-559.