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Determination of total phenolic content, total flavonoid content and total antioxidant capacity of *Ageratina adenophora* (Spreng.) King & H. Rob

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Abstract

Petroleum ether, chloroform and methanol extracts of *Ageratina adenophora* leaves were screened for assessing bioactive phytochemical constituents. The preliminary phytochemical screening using the standard phytochemical tests detected the presence of alkaloids, steroids, flavonoids, reducing sugars, tannins, saponins, terpenoids, anthraquinones, cardiac glycosides and phenols in different solvent extracts. Total phenolic and flavonoid contents along with total antioxidant capacity of the polar methanolic extract of *A. adenophora* leaves were evaluated to explore the reliable and potential sources of novel natural antioxidants. The methanol extract leaves revealed a total phenolic content of 30.0 mg gallic acid equivalent/g and 510.0 mg quercetin equivalent/g. The total antioxidant capacity ranges from 16.98 to 94.87% of standard ascorbic acid at concentrations ranging from 0.05 to 1 mg/ml of the plant extract in methanol.

Key words: Ageratina adenophora; total phenol content; total flavonoid content; total antioxidant capacity.

Introduction

Natural product compounds, especially derived from plants, have been explored for their chemical attributes and bioactivity. Plants are one of the essential sources of reliable bioactive compounds such as secondary metabolites and antioxidants. Secondary metabolites are concentrated at different parts of plant, *viz.*, the leaves, flower, stem, bark, fruit, roots and seeds, de-

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pending upon the family of plant species. Leaves of plant absorb sunlight, in the visible region of the electromagnetic radiation for the oxidation of water, to produce oxygen by photosynthesis and the harvested electrons (e^-) and protons (H^+) are utilized for the biosynthesis of carbohydrates, amino acids, lipids and secondary metabolites. Secondary metabolites comprise an orbit of chemically diverse compounds, specific to a family of plant species, not strictly required for survival. Most of the secondary metabolites, sourced from plants, are commercially exploitable as they find application as the active pharmaceutical ingredients/ lead compounds for new medicines. Polyphenol compounds are one of the important group of secondary metabolites and bioactive compounds of plants.[1] They are low molecular weight compounds (mol. wt. <2000 amu) ubiquitously existing in all tissues of higher plants and play an important role during the development of a plant.

At present, most of the antioxidants are manufactured by synthesis and they, mostly, provoke undesirable side effects when consumed *in vivo*.[2] Recently, there is a general trend to find naturally occurring antioxidants, which are safe and effective, to supplement processed food (for value addition, in addition to extending the shelf life) or pharmaceuticals and replace synthetic antioxidants which are being restricted due to adverse side effects elicited by them.[3] Plants are one of the important sources of invaluable antioxidants. Natural antioxidants, derived from plants, are secondary metabolites, mainly plant phenolics[4] such as phenolic acids, flavonoids and carotenoids, which are amongst various antioxidants produced by plants for their sustenance.[5] Recently, phenolic acids and flavonoids have been considered as great antioxidants and proved to be more effective than Vitamin C, E and carotenoids.[4]

Phenolics can be extracted from fresh, frozen or dried plant samples. Usually before extraction, plant samples are treated by milling, grinding and homogenization, which may be preceded by air-drying or lyophilization.[6] Solvent extractions are the most commonly used procedures for the preparation of extracts from plant materials due to their ease of use, efficacy, and wide applicability. The solubility of phenolics is governed by the chemical structural features of the plant sample as well as the polarity of the solvents used. Plant materials may contain a range of chemically diverse phenolics varying from simple (e.g. phenolic acids, anthocyanins) to highly polymerized substances (e.g. tannins) in different quantities.[4]

Figure 1. *Ageratina adenophora* (Asteraceae)

Ageratina adenophora (Fig. 1) is a plant from *Asteraceae* family, a toxic weed that causes damage to farmlands, pasture fields and forests as it has been observed that other plants were rarely found in the proximity as it suppresses the growth of other plants in the vicinity due to its allelopathy, in addition to its strong adaptability under diverse environmental conditions.[7] It is a profusely branching undershrub, growing up to 90-120 cm in height, with ascending branches holding simple, lanceolate and glabrous type leaves, arranged opposite to each other. The leaves are used as an astringent agent, thermogenic and stimulant in folklore medicine in India.[8] Presence of flavonoidal glycosides in the leaves has been reported.[9,10] In addition, presence of quercetin was quantitatively estimated, by high performance liquid chromatography, in the leaves, using reversed phase (RP-HPLC) with stepwise gradient elution, on a reverse

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phase C-18 column.[11]

Materials and Methods

Chemicals and reagents

All Chemicals were obtained from commercial sources and were of analytical grade. Gallic acid and ascorbic acid were purchased from Loba Chemie Pvt. Ltd., Mumbai and quercetin, from Sigma Aldrich, Bangalore. Sodium carbonate, sodium hydroxide, ferric chloride, potassium dichromate, lead acetate, sodium nitrite, aluminium chloride, sodium phosphate and ammonium molybdate were purchased from Himedia Laboratories Pvt. Ltd., Mumbai. All solvents are used as purchased. Sulphuric acid was purchased from Merck Specialities Pvt. Ltd., Mumbai.

Preparation of plant extract

Leaves of *A. adenophora* were collected along the road sides of Tanhril campus of Mizoram University, Aizawl, Mizoram and was identified by Botanical Survey of India, Shillong, Meghalaya (No. BSI/ERC/2012/Plant identification/ dated 28-8-2013/coll no.1). Harvested leaves were rinsed with tap water, air dried under the shade with occasional shifting, ground to powder by employing an electrical blender and stored in air tight containers.

The dried powder material was defatted with petroleum ether (60–80°C) and to remove chlorophyll, it was further extracted with chloroform. The remaining soft pasty material was finally extracted with methanol to extract the phenolics.[12] Each extraction was carried out exhaustively by maceration, followed by soxhlet extraction and solvents were recovered by distillation under reduced pressure using a rotary evaporator. The recovered solvents were distilled twice prior to their reuse.

Phytochemical evaluation of the extract

Petroleum ether, chloroform and methanol extracts were tested for the presence of phytochemical constituents by performing the standard methods such as Mayer's test, Dragendorff's test, Wagner's test and a Hager's test for Alkaloids,[13] Libermann-Burchard test and H_2SO_4 test for sterols,[14, 15] Shinoda's test, H_2SO_4 and alkaline reagent test for Flavonoids,[16] Fehling's test and Benedict's test for reducing sugars, potassium dichromate test and lead acetate test for tannins, foam test for saponins , $CHCl_3$ test for terpenoids, modified Borntrager's test for anthraquinones, Raymond test and Keller-Killiani tests for cardiac glycosides and the ferric chloride ($FeCl_3$) test for phenols.[14, 15]

Determination of total phenolics content

Total phenolic content of methanol extract of *A. adenophora* leaves was determined with Folin-Ciocalteu method.[17] The Folin–Ciocalteu (F–C) reagent is sensitive to reducing compounds, polyphenols and thus produces a blue colour complex. The F-C assay relies on the transfer of reducing equivalents (electrons), in the alkaline medium, from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, manifested in the formation of blue colour complexes [possibly $(PMoW_{11}O_{40})^{4-}$] that are determined on a UV-visible spectrophotometer (Thermo Fischer model Evolution 201) by monitoring the absorbance at 765 nm.[18,19] Gallic acid was used as the reference compound for comparison and values are evaluated as the mg equivalent of gallic acid per g of extract. Briefly, a mixture containing 0.1 g of extract, 0.8 ml of deionised water and 0.1 ml of Folin-Ciocalteu reagent was first incubated at room temperature for 3 min. After adding 0.3 ml of Na_2CO_3 (20% w/v), the mixture was further incubated at room temperature for 30 min. To obtain a calibration curve, various concentration of gallic acid solutions (0.05, 0.04, 0.03, 0.02, 0.01, 0.008, 0.005 and 0.001 mg/ml) were prepared. Appropriate volume of sodium carbonate solution was added in each flask and the final volume was adjusted with distilled water. Measurements were carried

out after 1 h at 765 nm on a UV-visible spectrometer against the reagent blank. The calibration curve of concentration against the absorbance was plotted. 1 mL of stock solution of extracts was transferred in a 25 mL flask; similar procedure (*vide supra*) was adopted for the preparation of calibration curve. With the help of the calibration curve, the phenolic concentration of extracts was determined.

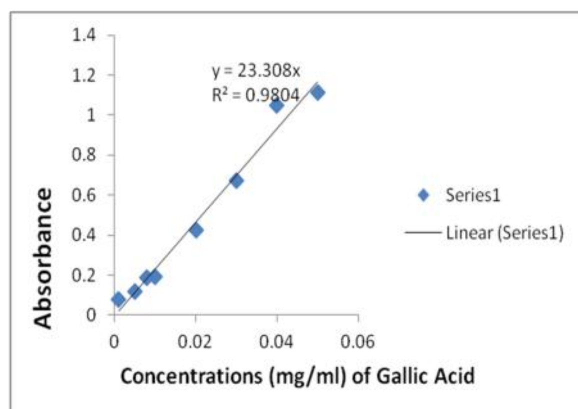

Figure 2. Standard calibration curve of gallic acid for the determination of total phenolic content.

Determination of total flavonoid content

Total flavonoid content of the methanolic extract of *A. ageratina* leaves were determined according to a modified colorimetric method.[20] Briefly, 1.5 ml of plant extract was taken and 75 µl of 5% $NaNO_2$ solution was added. After 6 min, 150 µl of 10% $AlCl_3.6H_2O$ was added to the mixture, which was kept at room temperature for 6 more minutes, followed by the addition of 0.5 ml of 1M NaOH and the total volume was made up to 2.5 ml with the addition of deionised water. The resulting solution was mixed well and immediately, the absorbance was measured at 510 nm on a UV-VIS spectrophotometer. For the blank, the extracts were replaced with an equal volume of deionised water. A standard calibration curve was prepared with 0.01, 0.05, 0.1, 0.2, 0.4 and 0.6 mg/ml of quercetin (in deionised water). The total flavonoid content was expressed as the mg equivalents of quercetin (QE) per g of extracts.

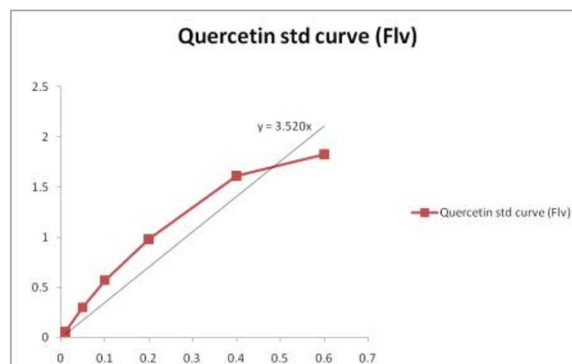

Figure 3. Standard calibration curve of quercetin for the determination of total flavonoid content .

Phosphomolybdate assay (total antioxidant capacity)

The total antioxidant capacity of the methanolic fraction was determined by phosphomolybdate method using ascorbic acid as a standard.[21] A stock solution of 1 mg/ml (plant extract in methanol) was prepared and diluted to concentrations ranging from 0.05 to 1 mg/ml. An aliquot of 0.1 ml of sample solution was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Sample tubes were capped and incubated in a water bath at 95°C for 90 min. Once after the sample has been cooled down to room temperature, absorbance of the mixture was measured at 695 nm against a blank on a UV-visible spectrophotometer. A typical blank contained 1 ml of the reagent solution along with an appropriate volume of the solvent and incubated under similar conditions. The antioxidant capacity of plant extract solution was estimated using following formula:

Total antioxidant capacity, TAC (%) = [(control absorbance – sample absorbance)/(control absorbance)] x 100

RESULT AND DISCUSSION

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Table 1. Phytochemical evaluation parameters of various solvent extracts of *A. adenophora* leaves.

S. No.	Plant Constituents	Test/Reagent	*A. adenophora*		
			Petroleum ether extract	Chloroform extract	Methanol extract
1.	Alkaloids	Mayer	-	-	-
		Dragendorff	-	-	+
		Wagner	-	-	+
		Hager	-	-	+
2.	Steroids	Salkowski	+	-	-
3.	Flavonoids	Shinoda	+	+	+
		Zinc-HCl	-	-	+
		Alkaline reagent	-	-	+
4.	Reducing sugars	Fehling	-	+	+
		Benedict	-	-	+
5.	Tannins	Potassiun Dichromate	-	-	+
		Lead acetate	-	-	+
6.	Saponins	Foam	-	-	+
7.	Terpenoids	$CHCl_3$	+	+	+
8.	Anthraquinones	Borntrager			
		Upper layer	+	+	+
		$CHCl_3$ layer	-	-	+
9.	Glycosides	Raymond	-	-	+
		Keller-Killiani	+	+	+
10.	Phenols	$FeCl_3$	-	-	+

Phytochemical screening

Phytochemical screening of different solvent fractions of *A. adenophora* leaves demonstrated the presence of alkaloids, steroids, flavonoids, reducing sugars, tannins, saponins, terpenoids, anthraquinones, cardiac glycosides and phenols as summarised in Table 1. Although, flavonoids, anthroquinones, terpenoids and glycosides are present in all the fractions, existence of steroids is confirmed only in the petroleum ether extract. Tannins, saponins, and phenols are present only in the methanol extract, while, reducing sugars are present in both chloroform and methanol fractions (Table 1).

Total Phenolic Content

The concentration-absorbance calibration curve for 8 sequentially and separately prepared stock standards of gallic acid solution is illustrated in Figure 2. The measured absorbance values at 765 nm for the indicated concentration of gallic acid solutions are in the range of 0.08 to 1.16. Within this range of concentrations (0.001 to 0.05 mg/ml), the calibration curve of gallic acid has clearly exhibited linearity (Figure 2). The antioxidant activities of several natural polyphenol compounds present in fruits and vegetables has been reported.[22] Moreover, the polyphenolic compounds have exhibited inhibitory effects on mutagenesis and carcinogenesis in humans.[23] Total phenol content in the methanolic extract of *A. adenophora* leaves, using the calibration curve, was found to be 30.0 mg gallic acid equivalents/g dry weight of extract.

Total Flavonoid Content

The calibration curve for 6 sequentially and

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independently prepared stock standard solutions of quercetin that depicts the concentration of quercetin against the absorbance, as presented in Figure 3. The absorbance value increased proportionally upon increasing the concentration of quercetin from 0.05 to o.6 mg/ml. A slight deviation from the linearity seemingly occurred at the higher concentration region of quercetin calibration plot. Nevertheless, for our estimation purposes, the calibration plot was employed to ascertain the total flavonoid content of the polar methanolic extract. Total flavonoid content in the methanolic extract of *A. adenophora* leaves was found to be 510.0 mg/g equivalent to quercetin (QE), *i.e.*, 1 g of the extract contains 510 mg of quercetin equivalent.

Total Antioxidant Capacity

The phosphomolybdate method is a quantitative method for determining the total antioxidant capacity (TAC), which is expressed as ascorbic acid equivalents. This method gives a combined measure of the antioxidant activity of the range of chemically diverse phenolics and flavonoids present in the methanolic extract of *A. adenophora* leaves as determined by the formation of the reduced phosphomolybdate complex (695 nm) as indicated in Table 2. The total antioxidant capacity of *A. adenophora* leaves ranges from 16.98 to 94.87% of standard ascorbic acid at concentrations ranging from 0.05 to 1 mg/ml of the plant extract in methanol.

Table 2. Determination of total antioxidant capacity using ascorbic as standard.

Concentration mg/ml	Absorbance		% TAC
	Control	Sample	
0.05	0.312	0.016	94.87
0.1		0.019	93.91
0.25		0.041	86.85
0.50		0.107	65.70
0.75		0.186	40.38
1.00		0.259	16.98

It is important to substitute synthetic antioxidants with naturally occurring safer antioxidants as the synthetics have been suspected of causing or provoking unfavourable side effects, while stronger restrictions are encountered on their application.[24,25] Several reports have conclusively shown a correlation between the antioxidant activity and amount of total phenolics or total flavonoids.[26,27] Plant extracts with high phenolic contents also possess high flavonoid content as reported for other plant species.[28,29]

Aerobic organisms consume a large amount of molecular oxygen to maintain cellular metabolic processes. Reactive oxygen species (ROS) are the ramification of various metabolic processes for which the terminal electron acceptor is molecular oxygen (O_2) that acts as a thermodynamic sink. ROS (reactive oxygen species) have been generally accepted to cause harm to living organisms and thus, the induced oxidative stress due to the formation of ROS is attributed to the damage of biological systems in the body, promoting the development of various diseases such as cancer, atherosclerosis, myocardial infarction, diabetes mellitus, arthritis and also accelerating the aging process.[30,31] The regular intake of natural antioxidants, however, has been associated with the reduced risks of cancer, cardiovascular disease, diabetes, and other degenerative diseases associated with aging, have the advantage of being almost devoid of harmful side effects.[32]

Conclusion

In this study, an exploratory extraction was performed on a small amount of *A. adenophora* leaves to obtain an extract of varying polarity using petroleum ether, chloroform and methanol to gain a preliminary knowledge on the exact nature and amount of metabolites present in the biomass. Furthermore, the determination of total phenolics and flavonoid content along with the total antioxidant capacity of methanolic extract (polar extract) of *A. adenophora* leaves showed that this plant can be one of the potential sources of safer natural antioxidants. Thus,

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replacement of synthetic antioxidants with secondary metabolites exhibiting safe and effective antioxidant activities (because of their manifestations on human health) from abundantly available plant sources such as *A. adenophora* may be advantageous. To further characterize the antioxidative properties of methanolic extract of *A. Adenophora* leaves, quantification of various phenoics and flavonoids will be required as the next step.

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