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Polyphenol Composition and Antioxidant Activity of *Andrographis paniculata* L. Nees

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Abstract

The aim of the present study was to establish the polyphenolic profile and antioxidant activity of the leaves of *Andrographis paniculata* (kalmegh) that could be potentially used for the benefit of mankind. An ultra high performance liquid chromatographic method was used to identify and quantify the individual phenolic compounds of the kalmegh leaves. A total of 20 polyphenolic compounds were identified and quantified in kalmegh leaves, including hydroxybenzoic acids, hydroxycinnamic acids, flavonols and other group of phenolic compounds. The UPLC analysis of the phenolic compounds revealed that salicylic acid was the dominant phenolic compound present in the leaves extract followed by gallic acid, chlorogenic acid, veratric acid and protocatechuic acid. The kalmegh leaves were extracted with 4 different solvents (ethyl acetate, methanol, butanol and water) and screened for total phenolic content (TPC) and antioxidant activity. The antioxidant activity of kalmegh was assessed by evaluating the 1, 1-diphenyl-2-picrylhydrazyl (DPPH), reducing power and phosphomolybdenum assay. Ethyl acetate and butanol extract exhibited the highest phenolic

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content (32.29 and 32.27 mg gallic acid equivalent 100 g DW^{-1}). The ethyl acetate extract possesses highest antioxidant activity towards DPPH, reducing power and phosphomolybdenum assay. Ethyl acetate extract exhibited the highest antioxidant activity whereas water extract exhibited the least antioxidant activity.

Keywords: Andrographis paniculata, Antioxidant activity, DPPH activity, Reducing power, total phenolic content

Introduction

Andrographis paniculata Nees belonging to Acanthaceae family, commonly known as 'Kalmegh', is widely distributed in India, Thailand, China and Malaysia. It is also known as "The Creat" and "King of bitters" in English. Kalmegh is one of the most widely used herbs in various Ayurvedic formulations. The whole plant of kalmegh is used extensively as an anti-inflammatory and antipyretic drug for the treatment of fever, cold, laryngitis, diarrhea, and inflammation. The therapeutic value of kalmegh is due to its mechanism of action by enzyme induction. It is an important cold property herb, used in fevers and to dispel toxins from the body. It is used to treat gastrointestinal tract and upper respiratory infections, fever, herpes, sore throat, hepatitis and a variety of other chronic and infectious diseases [1]. It exhibits antibacterial, antimalarial, filaricidal, antidiarrhoeal, and cardiovascular activities, fertility effects and protective effects on liver and gall bladder. The herb and its isolates like andrographolide, neoandrographolide, dehydroandrographolide, isoandrographolide, etc. are reported to possess anti-inflammatory, hepatoprotective, astringent, anodyne, tonic, alexipharmic and anti-pyretic properties and helps in arresting dysentery, cholera, diabetes, influenza, bronchitis, swellings and itches, piles and gonorrhea [2].

In recent years, the trend has changed towards the utilization of natural phytochemicals present in natural resources like vegetables, fruits, oilseeds and herbs which serve as potential antioxidants and functional ingredients [3, 4]. Polyphenolic compounds are a group of low and moderate molecular weight secondary metabolites that are widely distributed in plants, which can be divided into two

major subgroups; phenolic acids and flavonoids. Phenolic acids include mainly hydroxybenzoic acids (e.g. benzoic, gentisic or p-anisic acids) and hydroxycinnamic acids (e.g. caffeic or ferulic acid conjugates, sinapic acid). There is a much higher quantity and diversity of hydroxycinnamates than hydroxybenzoates and they consist of p-coumaric, caffeic, ferulic and sinapic acids either glycosylated or esterified with quinic, shikimic or tartaric acids. In fruits and leaves, the main hydroxycinnamates result from the esterification of caffeic acid groups(s) with quinic acid, the most frequent and abundant caffeoylquinic acid isomer being 5-0-caffeoylquinic acid (chlorogenic acid). In cereal grains, ferulic acid esters are the most common hydroxycinnamates. Flavonoids, perhaps the most important single group of phenolics in foods, comprise a group of over 4000 aromatic plant compounds; they include anthocyanins, proanthocyanidins, flavonols and catechins [5]. Epidemiological studies have provided evidence of beneficial health effects of dietary fruits and vegetables, and the beneficial effects have been attributed at least in part to secondary metabolites, including flavonoids and hydroxycinnamic acids [6]. Phenolic compounds are associated with a high number of biological activities and one with special interest is the antioxidant capacity. The consumption of antioxidant compounds or foods with high levels of these compounds is associated with the prevention and reduction of the risk of diseases associated to free radical reactions. The increase of degenerative diseases such as coronary heart disease, diabetes, cancer and age related diseases has required the urgency to find new natural sources of non-toxic antioxidant compounds [7].

The aim of our study was to investigate the phenolic composition and antioxidant activity of extracts from different solvents from leaves of *A. paniculata*. According to the recommendations, the antioxidant effects in three different bioassays were studied, besides determination of total phenolic content. The study of polyphenolic composition is an important scientific agenda for food and nutritional sciences, which may contribute to the improvement of conventional foods with added health benefits being very useful to determine these chemicals in plants, in the field of nutrition, pharmacology and agronomy. To the best of our

knowledge, this is the first report on the phenolic composition and antioxidant activity from the leaves of *A. paniculata.*

Materials and methods

Chemicals

Methanol, acetonitrile, glacial acetic acid, hydrochloric acid and distilled water (HPLC grade) were purchased from Merck (USA), Dimethyl sulfoxide (DMSO), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, protocatechuic acid, β-resorcylic acid, chlorogenic acid, caffeic acid, syringic acid, vanillin, ρ-coumaric acid, salicylic acid, ferulic acid, veratric acid, rutin, o-coumaric acid, hesperedin, resveratrol, quercetin, naringenin, kaempferol formononetin and biochanin A were purchased from Sigma-Aldrich (USA).

Plant material

The *Andrographis paniculata* L. Nees leaves (500 gm) were collected from the botanical garden of Christ University, Bengaluru, India in July 2014. A voucher specimen is deposited in the Department of Botany, Christ University, Bengaluru, India.

Preparation of extracts

A. paniculata leaves were washed thoroughly in tap water to remove adhering mud particles, rinsed in distilled water, drained, and dried in a hot air oven at 40 ± 2°C for 2 days. The dried leaves were finely powdered. The dried powder was extracted with 5 x 2 L methanol for 24 h. After removal of the solvent in vacuo, the crude extract was suspended in 0.5 L distilled water and extracted with 0.2 L portions of ethyl acetate and butanol until the extracts were nearly colorless. Solvents were removed in vacuo, and extracts were obtained respectively.

Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu (FC) method [8]. Distilled water (3.16 ml) was mixed with a DMSO solution of the test compound (40 µl). Then, 200 µl of FC reagent was added. After 5 min, 600 µl of 20% sodium carbonate solution was added and the solutions were mixed again. The solutions were

left at room temperature for 2 h. Then the absorption of the developed blue colour was determined at 765 nm, using a UV-Vis spectrophotometer. The concentration of the total phenolic content was determined as mg of gallic acid equivalent by using an equation obtained from the gallic acid calibration curve. The estimation of phenolic compounds from the extracts was carried out in triplicate and the results were averaged.

Extraction of phenolic compounds for the UPLC analysis

One gram of dried leaf material was extracted with 10 ml of acetonitrile and 2 ml of 0.1 N hydrochloric acid. The mixture was stirred for 2 h at room temperature. The extract was filtered through No. 42 Whatman filter paper and was concentrated using a vacuum evaporator. The residues were dissolved in 10 ml of 80% aqueous methanol and filtered through a 0.45 µm membrane. The filtrate was used for the UPLC analysis.

UPLC analysis of the phenolic compounds

UPLC was performed using the Thermo Accela UPLC (Thermo, New York, USA) system. Separation was primarily achieved using a HALO C18 (2.7 µm, 2.1 x 100 mm) column and the absorbance were measured at 280 nm. The mobile phases were 0.1% glacial acetic acid in distilled water (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). The injection volume was 4 µl and the linear gradient of UPLC solvents was as follows: 0 min, 92% A : 8% B; 0-2.2 min, 90% A : 10% B; 2.2-5 min, 85% A : 15% B; 5-7.5 min, 84.5% A : 15.5% B; 7.5-8.5 min, 82.2% A : 17.8% B; 8.5-13 min, 55% A : 45% B; 13-14 min, 0% A : 100% B; and 14-15 min, 92% A : 8% B. The run time was 15 min and the flow rate was 500 µl min^{-1}.

Solutions of available pure known compounds, gallic acid, protocatechuic acid, β-resorcylic acid, chlorogenic acid, caffeic acid, syringic acid, vanillin, ρ-coumaric acid, salicylic acid, ferulic acid, veratric acid, rutin, o-coumaric acid, hesperedin, resveratrol, Quercetin, naringenin, kaempferol formononetin and biochanin A were chromatographed as external standards. All standards were dissolved in methanol before injections in the analytical UPLC system. Their ranges of concentration used were 25, 50, 100, 150 µg mL^{-1}. Phenolic compounds of leaf extract were identified by

comparing their retention times with those of pure compounds. The results were expressed as μg g^{-1} of each compound from the total phenolic compounds.

Antioxidant activity

DPPH radical scavenging assay

The antioxidant activity of the extracts from kalmegh leaves, based on the scavenging activity of the stable α, α-diphenyl-2-picrylhrdrazyl (DPPH-) free radical was determined by the method described by Katerere and Eloff (2005) [9] with some modifications. Briefly, 500 μg ml^{-1} concentrations of the extracts were taken in different test tubes with 4 ml of a 0.006% MeOH solution of DPPH-. Water/methanol in place of the extracts was used as control. Absorbance at 517 nm was determined after 40 min of incubation at room temperature. Radical scavenging activity was expressed as the inhibition percentage, and was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control at 40 min reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the sample at 40 min.

Assay of reductive potential

The reducing power of the extracts was determined according to the method of Oyaizu (1986) [10] with some modifications. Briefly, 500 μg ml^{-1} concentration in 1 ml of distilled water was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$], and the mixture was incubated at 50°C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 650 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture indicates increased reducing power. All analyses were carried out in triplicate and averaged.

Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of kalmegh leaf extracts was evaluated by the method of Prieto et al. (1999) [11] and the results are expressed as equivalents of ascorbic acid (mg g^{-1} DW of extract).

Expression of data and statistical analysis

All analysis were carried out in triplicate and averaged and the data is presented as mean ± standard deviation (SD) for the three determinations.

Results and discussion

Evaluation of total phenolic content

The total phenolic content of the extracts from leaves of *A. paniculata* was determined by Folin-Ciocalteu (FC) method and the results were expressed as equivalents of gallic acid (Table 1). Among the four extracts, methanol and butanol extract had the highest (32.29 and 32.27 mg 100g DW^{-1} respectively) amount of phenolic compounds followed by methanol (27.90 mg 100g DW^{-1}), and water (15.47 mg 100g DW^{-1}) (Table 1). The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to gallic acid [4]. The phenolic compounds are the dominant antioxidants that exhibit scavenging efficiency on free radicals, and reactive oxygen species are numerous and widely distributed in the plant kingdom [12].

UPLC separation and determination of phenolic compounds in kalmegh leaf extract

The presence of flavonols, hydroxybenzoic acid and hydroxycinnamic acid compounds in plants have been considered a therapeutic agent due to their beneficial health effects, such as their supposed protection against certain cancers, cardiovascular diseases and aging [13]. Moreover, some of these phenolic compounds have also been used as a source of colors for food products, mainly anthocyanins, as alternative to synthetic dyes whose harmful effects upon human health have often been assumed and, in some cases demonstrated. Therefore qualitative

and quantitative analysis of the kalmegh leaf extract was made using UPLC as described in the experimental part and the results are presented in Table 2. The phenolic compounds in the kalmegh leaves extract were identified by comparisons to the retention time and UV spectra of authentic standards while the quantitative data were calculated from the calibration curves. Salicylic acid was the dominant phenolic compound in kalmegh leaf extract; it constituted about 528.57 μg g^{-1}, followed by gallic acid (56.50 μg g^{-1}), chlorogenic acid (44.49 μg g^{-1}), veratric acid (38.04 μg g^{-1}) and protocatechuic acid (34.12 μg g^{-1}). Similar variations in the phenolic compounds were reported in different species of *Artemisia* [14]. The two flavonols identified in the analysis were quercetin and kaempferol. Quercetin was the most dominant flavonol in the kalmegh leaves studied as it accounted for the largest proportion of the total flavonols content (Table 2). In the hydroxycinnamic acid group, ferulic acid was the most dominant hydroxycinnamic acid followed by caffeic acid and o-coumaric acid. Salicylic acid was the dominant compound in the hydroxybenzoic acid group followed by gallic acid and protocatechuic acid. Previous work has established that the antioxidant properties of some plants are partly due to low molecular mass phenolic compounds, particularly flavonoids, which are known to be potent antioxidants [15]. The results suggest that flavonols like quercetin, together with hydroxybenzoic acid, hydroxycinnamic acid and other group of phenolic acids play a predominant role in the leaves of kalmegh. In humans, the presence of flavonoids may contribute to the neutralization of cell-damaging free radicals and the maintenance of heart health [13]. The presence of hydroxycinnamic and hydroxybenzoic acids in our diets may also contribute to bolster cellular antioxidant defenses and to maintain a healthy vision. Although flavonoids are increasingly recognized as playing important roles as antioxidant, further work is necessary to uncover the full potential of these compounds in the improvement of human health.

Evaluation of antioxidant capacity by DPPH- radical scavenging activity

The free radical scavenging activity of the extracts was tested through DPPH method [9] and the results were compared with

BHT (Figure 1). DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH- solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The kalmegh leaf extracts were able to reduce the stable radical DPPH- to the yellow colored diphenylpicrylhydrazine. The IC_{50} values of the extracts were ethyl acetate (391.35 µg ml^{-1}), butanol (393.94 µg ml^{-1}), methanol (473.39 µg ml^{-1}), and water (509.58 µg ml^{-1}) respectively. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid) and aromatic amines (e.g., p-phenylene diamine, p-aminophenol), reduce and decolorize 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability [16]. The positive correlation between polyphenolic content of the extracts and its antioxidant activity is well documented [17]. Therefore, the content of total phenolic compounds in the extracts might explain their high antioxidant activities. In this study, the extracts exhibited antiradical activity by inhibiting DPPH- radical (Figure 1). Of the different extracts, ethyl acetate and butanol extract exhibited the highest antioxidant activity of 63.88% and 63.46% at 500 µg ml^{-1} concentration, followed by methanol (52.81%) and water (49.06%) respectively at the same concentration and it indicates that compounds with strong radical-scavenging capacity are of medium polarity (Figure 1). One of the possible mechanisms is polyphenolic associated compounds. Those kinds of phenolic compounds show antioxidant activity due to their redox properties, which play an important role in absorbing and neutralizing free radical, quenching singlet and triple oxygen or decomposing peroxide [18]. Butylated hydroxytoluene (BHT) showed similar degree of free radical scavenging activity with that of the extracts at low concentration points. The DPPH activity of BHT exhibited 92.04% at 50 µg ml^{-1} concentration.

Evaluation of reductive potential

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities [19, 20]. The reducing capacity of a

compound may serve as a significant indicator of its potential antioxidant activity. In the present study, the extracts exhibited effective reducing capacity (Figure 2). The reducing power of the extracts followed the order of ethyl acetate > butanol > methanol > water extract. The reducing properties are generally associated with the presence of reductones [21], which have been shown to exert antioxidant action by breaking the free radical chain, by donating a hydrogen atom [22]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation [18]. Our data on the reducing capacity of extracts suggested that reductone-associated and hydroxide groups of compounds can act as electron donors and can react with free radicals to convert them to more stable products, and thereby terminate radical chain reactions.

Evaluation of antioxidant capacity by phosphomolybdenum method

The antioxidant capacity of the kalmegh leaf extracts was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte, and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the extracts was found to decrease in the order: ethyl acetate > butanol > methanol > water extract (Table 3).

Conclusions

In our present study, the decreasing order of antioxidant activity among the *A. paniculata* leaves extracts assayed through all the three methods was found to be ethyl acetate > butanol > methanol > water extract. This order is similar to the phenolic contents of the extract that showed the extent of antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract. The UPLC analysis of the phenolic compounds profile revealed that salicylic acid was the dominant phenolic compound present in the kalmegh leaves extract followed by gallic acid, chlorogenic acid and veratric acid. The knowledge of the phenolic compound profile, occurring in the kalmegh holds great significance from the pharmaceutical point of view.

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Table 1. Total phenolic content (expressed as gallic acid equivalents) from leaves extracts of *A. paniculata.*

Extract	**Total phenolic content (mg 100g DW^{-1})**	
Ethyl acetate	32.29 ± 2.65	
Methanol	27.90 ± 0.98	
Butanol	32.27 ± 2.19	
Water	15.47 ± 0.22	

Data represents mean values ± SD of three replicates.

Table 2. Major phenolic compounds identified in the *A. paniculata* leaf extract by UPLC.

Phenolic Compounds	**Concentration (µg/g)**
Gallic acid	56.50 ± 0.028
Protocatechuic acid	34.12 ± 6.314
β-Resorcylic acid	12.74 ± 0.627
Chlorogenic acid	44.49 ± 3.627
Caffeic acid	28.27 ± 2.828
Syringic acid	12.92 ± 0.318
Vanillin	12.25 ± 0.014
p-coumaric acid	11.57 ± 0.975
Salicylic acid	528.57 ± 11.25
Ferulic acid	33.04 ± 0.169
Veratric acid	38.04 ± 3.639
Rutin	32.51 ± 0.021
o-coumaric acid	14.96 ± 0.190
Hesperedin	13.56 ± 0.813
Resveratrol	16.27 ± 0.395
Quercetin	30.74 ± 0.197
Naringenin	21.75 ± 0.685
Kaempferol	10.69 ± 0.014
Formononetin	19.06 ± 0.473
Biochanin A	11.09 ± 0.197

Data represents mean values ± SD of three replicates.

Table 3. Antioxidant capacity of *A. paniculata* leaf extracts by phosphomolybdenum method.

Extract	Antioxidant capacity [as equivalent to α-tocopherol (mg g^{-1})]
Ethyl acetate	73.01 ± 3.44
Methanol	85.28 ± 2.01
Butanol	74.29 ± 2.70
Water	89.75 ± 2.05

Data represents mean values ± SD of three replicates.

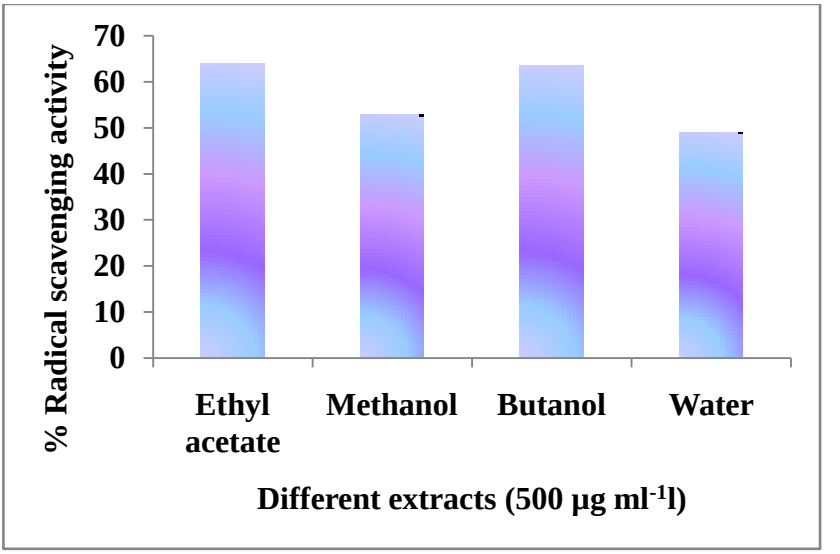

Fig. 1. Free radical-scavenging activity of the leaves extracts from *A. paniculata* by DPPH method. Each sample was assayed in triplicate. Experimental results were means ± SD of three parallel measurements.

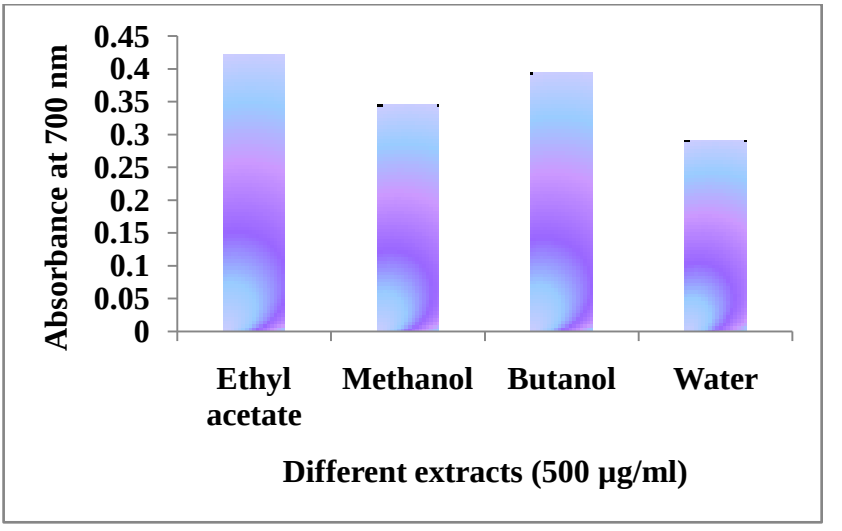

Fig. 2. Reducing power of different extracts from *A. paniculata* leaves at 500 µg ml^{-1} concentrations. Each sample was assayed in triplicate. Experimental results were means ± SD of three parallel measurements.