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Variations in Andrographolide Content, Phytochemical Constituents and Antioxidant Activity of Leaves of *Andrographis paniculata* (L.) Nees Collected from Different Locations of Southern India

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In present study, the samples collected from different locations of Southern India *viz.*, Yellapur Beltargadde, Siddapur, Joida, Ankola, Sirsi Kangod, Yellapur Shalabail, Sirsi Bairumbe, Karwar of Karnataka and Kasaragod from Kerala were analyzed for the andrographolide content, total phenolic and flavonoid content and screened for their antioxidant potential. The *A. paniculata* leaves were extracted with three different solvents (chloroform, methanol and water) and methanolic extract of Siddapur showed highest (8.82 ± 0.25 mg/g DW) amount of phenolic content whereas, aqueous extract of Ankola (3.00 ± 1.18 mg/g DW) showed the least amount. Chloroform extract of Yellapur Beltargadde village showed highest quantity *i.e.* 1.87 ± 0.50 mg/g DW of flavonoid content and aqueous extract of Yellapur Beltargadde showed 0.30 ± 0.20 mg/g DW which was least among all the tested samples. The sample collected from Karwar was found to have highest andrographolide content (9.36 ± 0.02 mg/g DW) followed by Yellapur Beltargadde sample with 7.29 ± 0.01 mg/g DW and Sirsi Kasaragod has the lowest contents of 1.54 ± 0.1 mg/g DW when analyzed through HPLC. Among the nine locations, methanol extract from Joida showed highest percentage of scavenging activity (91.95%) followed by methanol extract of Ankola (90.42%) and chloroform extract of Siddapur (77.31%) which was the lowest value of all samples tested.

Keywords: *Andrographis paniculata* andrographolide, HPLC, Total phenolic content, Antioxidant activity.

INTRODUCTION

Many plants containing high-value compounds are difficult to cultivate or are becoming endangered because of over-exploitation. One such highly valuable and over exploited medicinal plant is *Andrographis paniculata* L. Nees. It belongs to the family Acanthaceae and has been called by synonyms such as *Justicia latebrosa* Russ., *Justicia paniculata* Burm. f., *Justicia stricta* Lam. ex Steud. *A. paniculata* is commonly known by many regional names, such as Kalmegh (Sanskrit), Kirayat (Hindi), King of Bitters and The Creat (English), Nelabevu (Kannada), Nilavembu (Tamil) and Nelavemaa (Telugu) [1,2]. The herb is distributed in tropical Asian countries and also in China and West Indies but native to India and Srilanka [3,4].

Andrographis paniculata with its unique and wide spectrum of pharmacological activities attracted researchers all around the globe. *A. paniculata* is found to be representing 55 ent-labdane diterpenoids, 30 flavonoids, 8 quinic acid derivatives and 4 xanthones (only in roots) and 5 rare noriridoids [5-8]. Of these labdane diterpenoids andrographolide is the principal active secondary metabolite which constitutes about 2-3% of the total biomass of the plant. Andrographolide ($C_{20}H_{30}O_5$) is a colourless crystalline solid with melting point ranging from 230-239 ºC, extremely bitter in taste and found usually in aerial parts of the plant. Andrographolide is found to show various pharmacological activities like anticancer, antiretroviral, pro-apoptotic, anti-inflammatory, radio-sensitizer, cholestatic, immunomodulatory, antidiabetic, antiangiogenic, antithrombotic, antiurothelial, hepatoprotective, analgesic, antipyretic, antiulcerogenic, antioxidant, antileishmanial, antivenom, anti-HIV, vasorelaxant, beneficiary on intestine, effective on common cold and fever, anti-cancerous protective activity against alcohol-induced hepatic and renal toxicity, cardioprotective and inhibition of Epstein Barr-virus [9-17].

Reactive oxygen species (ROS) such as singlet oxygen, superoxide ion, hydroxyl ion and hydrogen peroxide are highly reactive, toxic molecules which are generated normally in cells during metabolism. However, a higher amount of ROS leads

to severe damage to proteins, lipids, enzymes *etc.* by covalent bonding leading to tissue damage [18]. Natural antioxidants have been much of interest because of their ability to scavenge these free radicals. Free radicals have been involved in the development of number of diseases, such as cancer, neurodegeneration and inflammation, which lead to the study of antioxidants for the prevention and treatment of diseases. The presence of antioxidants such as phenolics, flavonoids, tannins *etc*. in plants may play a major role in providing protection against number of diseases [15,19]. Therefore, medicinal plants are being investigated for their antioxidant properties and the demand for these plants is increasing. In view of these characters, the present study is aimed at quantification of the active metabolites specially andrographolide and other natural antioxidants from *A. paniculata* collected from different locations of Southern India.

EXPERIMENTAL

All the chemicals were purchased from Merck (USA), the standards gallic acid, quercetin, α-tocopherol and DPPH were purchased from Hi-media, India. Standard andrographolide was purchased from Natural Remedies (India).

Andrographis paniculata was collected from different places of Southern India *viz.* Yellapur Beltargadde, Siddapur, Joida, Ankola, Sirsi Kangod, Yellapur Shalabail, Sirsi Bairumbe, Karwar of Karnataka and Kasaragod from Kerala, respectively. The collected plant materials (leaves) were cleaned and shade dried at room temperature. Once the plant materials were dried, they were powdered using mixer grinder and stored in air tight plastic bags at room temperature for further use.

Quantitative analysis

Quantification of andrographolide using HPLC: Extraction and HPLC analysis of andrographolide was carried out according to the method of Praveen *et al.* [20] with some modifications.

Sample preparation: Finely powdered dry leaf material (0.5 g) was extracted with 25 mL of methanol and incubated overnight at 25 ºC on a rotary shaker at 100 rpm. The extract was filtered and the filtrate was allowed to air dry. The obtained residue was redissolved in 2 mL of HPLC grade methanol. The sample was filtered using nylon membrane (0.45 µm pore size) before HPLC analysis.

Quantification of andrographolide: Authentic, HPLC grade (purity 96%) andrographolide was obtained from Natural Remedies Pvt. Ltd. (Bangalore, India). The HPLC system used was Waters 510 series equipped with Waters 486 series detector with column (250 mm × 4.6 mm). The mobile phase consists of Acetonitrile: Water (70:30 v/v) using isocratic program. A 10 min re-equilibrium was allowed between injections. The flow rate was 1.0 mL/min and aliquots of 20 µL were injected at 26 ºC column temperature into HPLC. The detector wavelength was 230 nm. HPLC was performed in triplicates for both standard and samples.

Preparation of extracts for phytochemical analysis and antioxidant activity: Approximately 5 g of dried leaf powder was subjected to Soxhlet extraction using different solvents such as chloroform, methanol and water. The extracts were collected in screw capped tubes and solvents were evaporated to dryness and stored in refrigerator at 4 ºC.

Determination of total phenolic contents: The total phenolic contents were determined by the Folin-Ciocalteau method with some modifications [21]. Distilled water (3.10 mL) was mixed with extract (100 µL) and 200 µL of Folin-Ciocalteau reagent was added and incubated at room temperature for 5 min. Then, 600 µL of 15% sodium carbonate was added and mixed well. The tubes were incubated at room temperature for 40 min. Absorbance of the developed blue colour was determined at 765 nm using UV-VIS spectrophotometer (Elico, India). The concentration of the total phenolic content was determined as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The estimation of phenolic compounds was done in triplicates and the results were averaged.

Determination of total flavonoid contents: Total flavonoid content was determined by using aluminium chloride method [22] with some modifications. Extract (100 µL) was mixed well with 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate and 4.70 mL of distilled water. The solutions were incubated at room temperature for 30 min. Absorbance was measured at 415 nm using UV-vis spectrophotometer (Elico, India). The concentration of total flavonoid content was determined as mg of quercetin equivalent by using an equation obtained from quercetin calibration curve. The estimation was done in triplicates and the results were averaged.

Antioxidant activity

Determination of radical scavenging activity by DPPH method: Radical scavenging activity of sample extracts were determined by α-diphenyl-β-picrylhydrazyl (DPPH) method as described by Katerere & Eloff [23] with some modifications. Extracts (0.1 mL) of concentration 250 µg/mL were taken in different test tubes and the volume was adjusted to 3 mL by adding methanol. Methanolic solution of DPPH (0.1 mM, 1 mL) was added to these tubes and mixed well. The control was prepared as above but without any extracts. The tubes were allowed to stand for 20 min at 37 ºC. Pure methanol was used for baseline correction in UV-VIS spectrophotometer (Elico, India). The changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Assay of reductive potential: The reductive potential of extracts was determined according to Oyaizu′s method [24]. Extracts (0.1 mL) of concentration 1 mg/mL were taken in different test tubes and the volume was adjusted to 1.0 mL using distilled water. Phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and $K_3[Fe(CN)_6]$ (2.5 mL, 1% w/v) were added to the reaction mixture and incubated at 50 ºC for 20 min. Trichloroacetic acid (2.5 mL, 10% w/v) was added to the mixture and mixed well. A portion of this mixture was pipetted out and mixed with 2.5 mL of distilled water and $FeCl_3$ (0.5 mL, 0.1% w/v) and the absorbance was measured at 700 nm in a spectrophoto-

meter. Increased absorbance of the reaction mixture indicated increased reductive potential.

RESULTS AND DISCUSSION

Quantification of andrographolide using HPLC: The HPLC analysis of *A. paniculata* leaves from natural plants of different locations of Southern India were carried out. Of the nine samples analyzed, the sample collected from Karwar was found to have highest andrographolide content (9.36 ± 0.02 mg/g DW) followed by Yellapur Beltargadde sample with 7.29 ± 0.01 mg/g DW contents and the lowest content of andrographolide was observed in the samples collected from Sirsi Kangod (1.54 ± 0.1 mg/g DW) (Table-1) Similar to present results, variations in the andrographolide content has been reported from the samples collected from Madhya Pradesh and Chattisgarh states of India, where the amount of andrographolide varied from 1.07 to 2.24% [25]. Sabu *et al.* [26] also reported variations in the andrographolide content ranging from 0.73 to 1.47% in leaves of 12 accessions collected from Southern regions of India and 3 accessions collected from tropical Asian countries. Variability at morphological, molecular and biochemical level of *A. paniculata* has been reported [27]. Similarly, Raina *et al.* [28] also reported variations in the andrographolide content in the dry leaves of kalmegh in the range of 1.14 to 2.60% amongst their collections. The better understanding of variation existing in the given populations helps in identifying better genotypes for crop improvement as well as to evolve strategies for the effective *in situ* and *ex situ* conservation programmes. The results obtained in the present study are important in the exploration of *A. paniculata* genotypes for commercial production of andrographolide due to its high andrographolide content.

TABLE-1
HPLC ANALYSIS OF ANDROGRAPHOLIDE CONTENT FROM DRIED LEAVES OF *Andrographis paniculata* L. NEES COLLECTED FROM DIFFERENT LOCATIONS

Location	Andrographolide content (mg/g DW)
Yellapur Beltargadde	7.29 ± 0.12
Siddapur	3.29 ± 0.05
Joida	2.43 ± 0.07
Ankola	2.04 ± 0.17
Sirsi Kangod	1.54 ± 0.08
Yellapur Shalabail	2.44 ± 0.33
Sirsi Bairumbe	2.88 ± 0.28
Karwar	9.36 ± 0.17
Kasaragod	6.85 ± 0.09

Determination of total phenolic content: Although most antioxidant activities from plant sources are derived from phenolic type compounds [29], these effects do not always correlate with the presence of large quantities of phenolics. Therefore, both sets of data need to be examined together. The total phenolic content of the different extracts of *A. paniculata* collected from different geographical locations were determined by Folin-Ciocalteau method. The concentration of total phenolic content was determined as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve (Fig. 1).

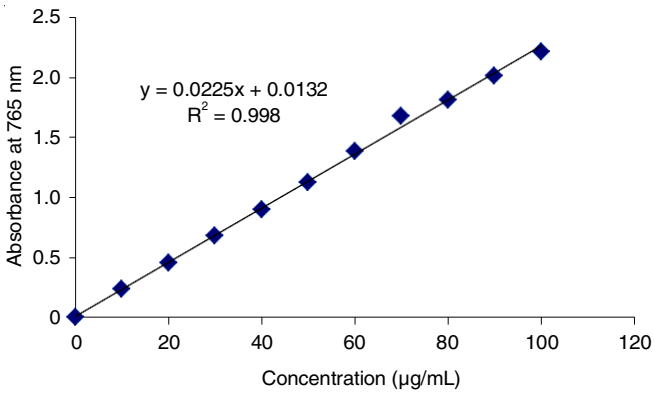

Fig. 1. Standard calibration curve of gallic acid for the estimation of total phenolic contents

Among all the extracts, methanol extract of Siddapur showed highest (8.82 ± 0.25 mg/g DW) amount of phenolic compounds followed by aqueous extract of Siddapur (8.78 ± 0.83 mg/g DW) and aqueous extract of Ankola (3.00 ± 1.18 mg/g DW) which was found to be the least value of all nine samples with three solvent extracts tested (Table-2). The levels of phenolic compounds determined through this method is not accurate as they are grouped based on the relative chemical reducing capacity compared to gallic acid [30].

TABLE-2
ESTIMATION OF TOTAL PHENOLIC CONTENTS OF LEAF EXTRACTS OF *Andrographis paniculata* L. NEES COLLECTED FROM DIFFERENT LOCATIONS

Location	Total phenolic content of extracts (mg/g ± SE)		
	Chloroform	Methanol	Water
Yellapur Beltargadde	4.83 ± 0.03	2.48 ± 1.26	3.29 ± 0.07
Siddapur	4.29 ± 0.41	8.82 ± 0.25	8.78 ± 0.83
Joida	4.81 ± 0.07	7.35 ± 0.64	3.27 ± 0.55
Ankola	5.62 ± 1.13	5.23 ± 0.05	3.00 ± 1.18
Sirsi Kangod	4.42 ± 0.05	3.45 ± 0.50	3.60 ± 1.63
Yellapur Shalabail	5.00 ± 0.71	6.44 ± 0.08	4.42 ± 1.01
Sirsi Bairumbe	4.72 ± 0.69	4.32 ± 1.59	4.17 ± 0.04
Karwar	4.50 ± 0.09	2.84 ± 0.87	7.24 ± 0.41
Kasaragod	4.68 ± 0.02	6.91 ± 0.21	3.96 ± 0.78

Determination of total flavonoid content: The total flavonoid content of the different extracts from leaves of *A. paniculata* were determined by $AlCl_3$ method. The concentration of total flavonoid content was determined as mg of quercetin equivalent by using an equation obtained from quercetin calibration curve (Fig. 2). The chloroform extract of Yellapur Beltargadde village showed highest quantity *i.e.* 1.87 ± 0.50 mg/g DW of flavonoid compounds followed by chloroform extract of Ankola (1.86 ± 0.94 mg/g DW) and aqueous extract of Yellapur Beltargadde showed 0.30 ± 0.20 mg/g DW, which was the least amount among all (Table-3). The activity of flavonoids mainly depends on the presence of hydroxyl groups [31].

Antioxidant activity

DPPH radical scavenging activity: The free radical scavenging activity of sample extracts were tested using DPPH

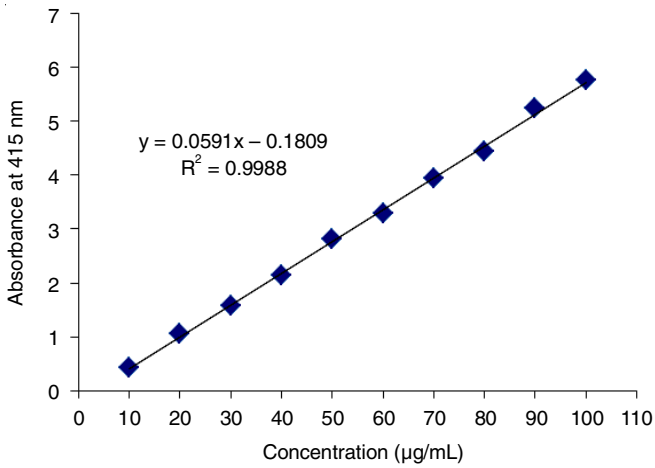

Fig. 2. Standard calibration curve of quercetin for the estimation of total flavonoid contents

TABLE-3
ESTIMATION OF TOTAL FLAVONOID CONTENTS OF LEAF EXTRACTS OF *Andrographis paniculata* L. NEES COLLECTED FROM DIFFERENT LOCATIONS

Location	Total flavonoid content of extracts (mg/g ± SE)		
	Chloroform	Methanol	Water
Yellapur Beltargadde	1.87 ± 0.50	0.64 ± 0.25	0.30 ± 0.20
Siddapur	1.45 ± 0.87	1.17 ± 0.18	0.52 ± 0.35
Joida	1.66 ± 1.12	1.06 ± 0.63	0.32 ± 0.08
Ankola	1.86 ± 0.94	1.02 ± 0.47	0.30 ± 0.88
Sirsi Kangod	1.73 ± 0.05	0.81 ± 0.06	0.41 ± 0.55
Yellapur Shalabail	1.59 ± 0.26	0.58 ± 0.37	0.45 ± 0.83
Sirsi Bairumbe	1.63 ± 1.77	0.50 ± 0.06	0.37 ± 0.33
Karwar	1.55 ± 0.59	1.10 ± 0.51	0.67 ± 0.07
Kasaragod	1.69 ± 1.18	0.84 ± 0.49	0.48 ± 0.62

method and the results were obtained as percentage of radical scavenging (Fig. 3). Among the nine locations, methanol extract from Joida showed highest percentage of scavenging (91.95%) followed by methanol extract of Ankola (90.42%) and chloroform extract of Siddapur (77.31%), which was the lowest value of all samples tested. The antioxidant activity of the plant is mainly due to the presence of phytomolecules [32]. Many reported works explain the antioxidant activity by taking different solvent extracts and it has been observed that aqueous extract shows the better results [33].

Assay of reductive potential: In this experiment, all the extracts showed effective reducing capacities. The reducing power of the chloroform extract of Yellapur Beltargadde village was the highest followed by the chloroform extract of Yellapur Shalabail and aqueous extract of Joida was the least among all. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [34].

Conclusion

Plant secondary metabolites such as alkaloids, flavonoids, flavones and antioxidant compounds have gained significant importance due to their myriad of activities including antimicrobial, anti-inflammatory, antidiabetic to name a few. In the present study, the presence of different phenolic and flavonoid compounds in *A. paniculata* were tested and also quantified the andrographolide from the crude extract.

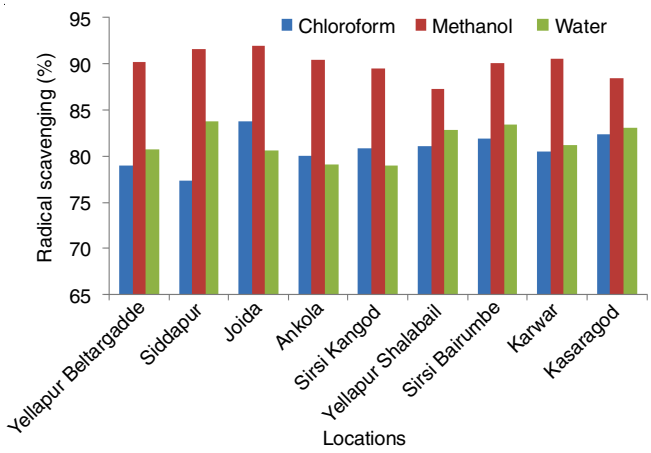

Fig. 3. Radical scavenging activity of leaf extracts of *Andrographis paniculata* L. nees collected from different locations by α-diphenyl-β-picryl-hydrazyl (DPPH) method

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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