http://www.e-journals.net

ISSN: 0973-4945; CODEN ECJHAO
E-Journal of Chemistry
2010, **7(2)**, 363-368

Activity-Guided Isolation of Antioxidant Compounds from *Andrographis stenophylla* Leaf

NEELAVENI THANGAVEL* and JAYANTA KUMAR GUPTA

*Sri Vasavi Institute of Pharmaceutical Sciences,
Tadepalligudem, Andhra Pradesh - 534 101, India.
Department of Pharmaceutical Technology,
Jadavpur University, Kolkata- 700 032, India.
venipsgcop2004@yahoo.co.in

Received 13 July 2009; Accepted 5 September 2009

Abstract: The antioxidant potency of various extracts of *Andrographis stenophylla* leaf was evaluated *in vitro* using ferric thiocyanate method. Reductive ability and free radical scavenging activity of the extracts were also investigated. Amounts of phenolic compounds in each of the extracts were determined using Folin-Ciocalteau reagent and compared to observe the correlation between antioxidant activities and total phenolic content. Methanol extract exhibited maximum antioxidant activity and was found to contain 2% of total phenolic compounds. Methanol extract was subjected to column chromatographic separation over silica gel G using ethyl acetate: formic acid: acetic acid: water. Fractions thus obtained were screened for their antioxidant activity. Among the eleven fractions screened, fraction C was more active than the standard butylated hydroxyanisole. Fraction C on further fractionation with *n*-butanol: acetic acid: water afforded two flavanoids namely acacetine and isosakuranetine. Fraction A was also shown to possess good antioxidant activity which was developed using TLC and indicated the presence of a terpenoid, Andrographolide. The structures of the isolated compounds were confirmed by UV, IR, MS, ^{1}H and ^{13}C NMR spectral data. This is the first report wherein Andrographolide, Acacetine and Isosakuranetine are isolated from *Andrographis stenophylla* leaf.

Keywords: Antioxidant activity, *Andrographis stenophylla*, Flavanoids, Terpenoids, Free radical scavenging, reductive ability.

Introduction

Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions. Antioxidants can interfere with the

oxidation process by reacting with free radicals, chelating metals and also by acting as oxygen scavenger[1]. Antioxidants are of interest for the treatment of many kinds of cellular degeneration. Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis, hypertension, diabetes, alzheimer's disease, down's syndrome, parkinson's disease, hepatic damage[2] *etc*. Restrictions on the use of synthetic antioxidants are being imposed because of their hepatotoxicity and carcinogenicity. Thus the interest in natural antioxidants has been increased considerably. Especially India being a wealthy land of medicinal herbs and since only 5% of plant kingdom has been investigated it becomes essential to explore the medicinal value of herbs.

The genus andrographis belongs to the Acanthaceae family. There are about thirty eight species identified for the genus andrographis. Among these, twenty four species have been found to be distributed mainly in the hill areas of the districts of Tamilnadu, India[3]. There is so far no report available on the title plant. The leaves of *Andrographis stenophylla* are used as folklore medicine for the treatment of snake venom poisoning and diabetes. Since antioxidant principles may be used to combat various human diseases this work aims at evaluating the antioxidant potential of the plant. Attempts were also made to isolate the antioxidant compounds from the leaves of the plant.

Experimental

The plant was collected during the month of July, 2000 from Marudhamalai hills of Coimbatore district, Tamilnadu. The plant was authenticated at Botanical Survey of India, Southern Circle, Coimbatore and a voucher specimen was deposited, (No.BSI/SC/5/21/2000-358). The leaves were dried under shade, powdered and passed through 40 mesh sieve and stored in air tight container. The powdered leaf was extracted with petroleum ether (40-60 ^{0}C), benzene, dichloroethane, methanol and water successively in a soxhelet apparatus. The solvents were then evaporated under reduced pressure to obtain dry extracts. Preliminary phytochemical studies were also performed on all the extracts.

Antioxidant activity evaluation by ferric thiocyanate method

The antioxidant activity of dichloroethane, methanol and water extracts were determined using ferric thiocyanate method[1]. In this method 10 mg of each extract was dissolved in 1 mL of ethanol. 1.25 mL of each extract was pipetted into a reaction mixture containing 2.50 mL of 2.5% linoleic acid and 6.25 mL of 40 mM phosphate buffer in a vial. The vials were incubated at 40 °C for 60 h. After incubation 1.25 mL of each vial was diluted with 8.55 mL of 75% ethanol, 0.1 mL ammonium thiocyanate and 0.1 mL of ferrous chloride. The absorbance of samples was measured at 500 nm and the percent of inhibition was determined. Various concentrations of the extracts were screened. Ethanol without sample was used as negative control and butylated hydroxylanisole was used as positive control.

Evaluation of reducing power[4]

Ten mg of each extract, in 1 mL of distilled water was mixed with 2.5 mL phosphate buffer of pH 6.6 and 2.5 mL of 1% potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min. 2.5 mL of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.1% of 0.5 mL of ferric chloride and the absorbance was measured at 700 nm.

Evaluation of free radical scavenging activity[5]

1 mL of 0.04% solution of DPPH radical in ethanol was mixed with varying concentrations of extract solution in ethanol and the solution was made up to 5 mL with ethanol. After 30 min absorbance was measured at 517 nm.

Determination of amount of total phenolic compounds[6]

Various concentrations of the extracts were taken by pipetting out solutions of the extract in ethanol (100 mcg/mL) and diluted with distilled water to 8.5 mL. To each flask 0.5 mL of folin-ciocalteau reagent was added and mixed thoroughly. After 3 min 1 mL of saturated sodium carbonate solution was added to each flask and shaken thoroughly. The mixture was allowed to stand for 24 h to develop blue color. The absorbance was measured at 760 nm. Resorcinol was used as the standard to construct the calibration curve.

Activity guided isolation of anti-oxidant compounds

A glass column (18 x 450 mm) was packed with silica gel G (250 g) by suspending silica gel in ethyl acetate and allowed to settle. The dried methanol extract (10 g) was triturated with little amount of silica Gel and loaded into the column. The column was then developed using ethyl acetate: formic acid: glacial acetic acid: water. A total of 59 fractions were collected each of 10 mL volume and all the fractions were monitored by TLC. Alike fractions were combined together and finally eleven fractions were obtained named as A-K. Free radical scavenging activities of all the fractions were measured using DPPH method. The solvents were evaporated and the pure compound from fraction A was obtained by recrystallisation from suitable solvent. Fraction C was further eluted with *n*-butanol: acetic acid: water which yielded two compounds. The structures of these compounds were confirmed using UV, IR, MS, ^{1}H and ^{13}C NMR data.

Results and Discussion

Andrographis stenophylla demonstrated effective antioxidant activity. The effect of various extracts on per oxidation of linoleic acid is shown in Figure 1. High absorbance is an indication of high concentration of formed peroxides. Therefore low absorbance indicates high antioxidant activity. Methanol extract displayed strong antioxidant activity and the petroleum ether, benzene extracts did not show any antioxidant property. Petroleum ether and benzene extracts were shown to possess only steroids, dichloroethane extract contains phenolic compounds, flavanoids and terpenes, methanol extract contains carbohydrates, phenolic compounds, flavanoids and terpenes, whereas water extract indicated the presence of carbohydrates, phenolic compounds, flavanoids, terpenes and saponins. Absence of phenolic compounds, flavanoids and terpenes might be one of the reasons for the lack of antioxidant activity in petroleum ether and benzene extracts. Methanol extract exhibited high reducing power and free radical scavenging activity. Results are presented in Figures 2 & 3. Total phenolic compounds in dichloroethane extract, methanol extract and water extract were determined using folin-ciocalteau reagent which developed blue color after 24 h. The concentration of total phenolic compounds was determined as mcg of resorcinol equivalent by using the equation obtained from the calibration curve, $Y = 0.0037 x + 0.057$, where Y = absorbance and x = concentration of resorcinol in mcg. The results in Table 1 indicate that the methanol extract possesses high total phenolic content. This may be due to the greater solubility of the phenolics in methanol[7]. The phenolic compounds may contribute directly to antioxidant activity, reducing capability and free radical scavenging activity and hence the strong antioxidant activity of methanol extract may be correlated with the highest phenolic content. Because of this methanol extract was further subjected to column chromatography to isolate the active constituents responsible for anti-oxidant activity.

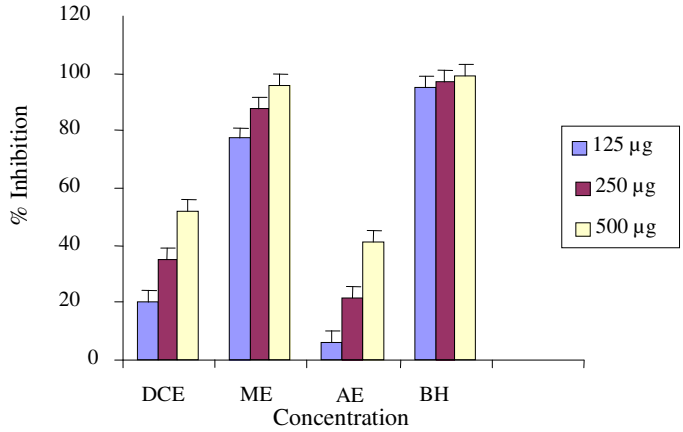


Figure 1. Antioxidant activity of extracts of *Andrographis stenophylla* after 60 h of Incubation

DCE = Dichloroethane extract, ME = Methanol extract, AE = Aqueous extract BHA = Butylated hydroxyanisole.

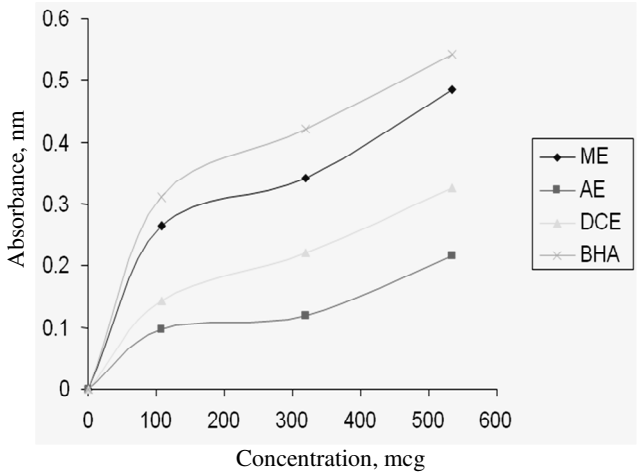


Figure 2. Reducing power of different extracts of *Andrographis stenophylla.*

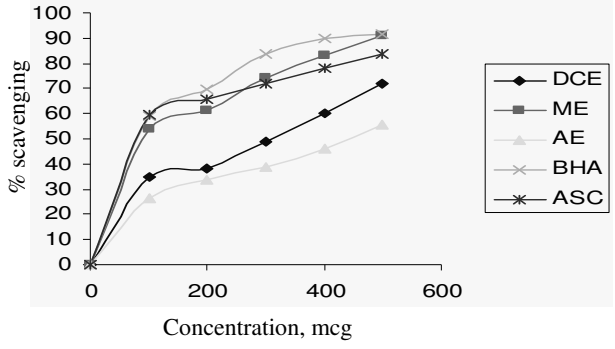


Figure 3. DPPH radical scavenging activity of *Andrographis stenophylla* leaf (ASC – Ascorbic acid).

Table 1. Total phenolic content of extracts of *Andrographis stenophylla* leaf (Readings are average of three determinations).

Extract	Total phenolic content mg RE/g of extract
DCE	100.4 ± 1.02
ME	200.1 ± 1.16
AE	98.6 ± 1.05

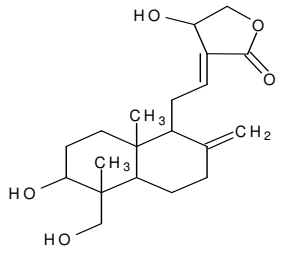
Antioxidant activities of all the fractions obtained were measured using DPPH method at the concentration of 125 mcg. Out of the eleven fractions obtained (A – K), six fractions were found to possess free radical scavenging property. The antioxidant activity of the fractions was in the order of C>BHA>A>K>E>F>D. Fraction C was further eluted with *n*-butanol: acetic acid: water which afforded two compounds namely acacetine and isosakuranetine. Fraction A when developed using TLC gave a single spot corresponding to the terpene andrographolide. The physiochemical data of the reported compounds are provided in Table 2.

Table 2. Physicochemical data of isolated compounds.

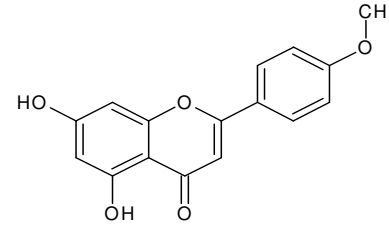
Parameter	Andrographolide	Acacetine	Isosakuranetine
Phys. Appearance	Colorless powder	Yellow needles	Colorless needles
% yield	1.4	0.2	0.25
Solubility	Acetone,$CHCl_3$, Ether,Ethylacetate	Alcohol, water	Alcohol, water
Melting Point	221	263	152
R_f value (Solvent system)	0.7 ($CHCl_3$:CH_3OH 70:10) Vanillin in H_2SO_4	0.54($CHCl_3$:CH_3OH 10:1) Ammonia vapor	0.34 ($CHCl_3$:CH_3OH 10:1) Ammonia vapor
Visualizing reagent			
λ_{max}	225	271 nm & 333 nm	286 nm & 315 nm shouldered.

Their structures were confirmed by comparing the UV, IR, MS, ^1H and ^{13}C NMR data with the previously reported spectral data. The data obtained are in accordance with the following structures for the compounds (Scheme 1).

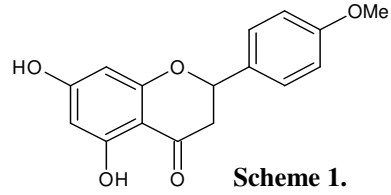
Andrographolide



Acacetine



Isosakuranetine



Scheme 1.

Among the isolated compounds, the highest antioxidant activity was observed with acacetine and the activity was higher than that of butylated hydroxyl anisole (Table 3). As such the *in vitro* antioxidant activities of the three isolated compounds have not been reported so far. The order of antioxidant activity of isolated compounds is in the order, acacetine > butylated hydroxyl anisole (standard) > andrographolide > isosakuranetine. From the results we could suggest that acacetine, isosakuranetine and andrographolide mainly contribute to antioxidant activity of methanol extract of *Andrographis stenophyll*a leaf. It has been reported that free hydroxyl groups in phenolic compounds are mainly responsible for antioxidant activity. But the results indicate that terpenoid lactone Andrographolide also possess antioxidant activity. In conclusion, the antioxidant activity of *Andrographis stenophylla* may be due to the presence of flavanoids acacetine, isosakuranetine and terpenoid andrographolide. These compounds are reported for the first time from the title plant.

Table 3. Free radical scavenging activities of isolated compounds.

Compound	Absorbance, nm	Percentage
Andrographolide	0.028 ± 0.001	97.35± 0.53*
Acacetine	0.005±0.001	99.52±0.55*
Isosakuranetine	0.095±0.001	91.03±0.64*
Butylated hydroxyl anisole	0.006±0.002	99.43±0.55*

** P< 0.001 using one way ANOVA compared to control.*

References

1. Ilhami Gulein, Munir Oktay, Irfan K O and Ali Aslan, *J Ethnopharmacol*., 2002, **79**, 325.
2. Malencic D J, Gasic O, Popovic M and Boza P, *Phytother Res*., 2000, **14**, 546.
3. Gamble J S, Flora of Presidency of Madras, Ad lard and Son Ltd, London, 1982, 1045.
4. Odabasoglu F, Aslan A, Cakir A and Halici M, *Fitotherapia*, 2005, **76,** 216.
5. Cakir A, Mavi A, Yildrim A and Kazaz C, *J Ethnopharmacol*., 2003, **87**, 73.
6. Sagar B P S and Zafar R, *Indian Drugs*, 2003, **40**, 112.
7. Vijaya C, Ramanathan M, Subburaju T and Suresh B, *Indian Drugs,* 2002, **39,** 453.