Vol.1 No.1 (Apr 2012)

Indian Journal of Drugs and Diseases

Determination of antioxidant property of *Andrographis paniculata*

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Abstract

Antioxidant property of *Andrographis paniculata* is evaluated in this study by employing three methods viz. DPPH, Lipid Peroxidation and DNA cleavage protective assay. First two methods employed, gallic acid and α- tocopherol as standard antioxidant for comparing with the antioxidant property of *Andrographis paniculata*; while in the DNA cleavage protective assay pBS plasmid was used to check the protective activity of *Andrographis paniculata.* In the DPPH method the RSC (radical scavenging capacity) of the plant extract was measured spectrophotometrically at 512nm. The lipid peroxidation inhibition activity of plant extract was also evaluated by taking the absorbance of pink color complexed formed at 535nm. In the DNA cleavage protective assay, the electrophoretic pattern of DNA after UV-induced photolysed H_2O_2 - oxidative damage showed difference in the absence and presence of methanolic extracts of the plant. This study indicates the methanolic extracted of the *Andrographis paniculata* has higher antioxidant activity than water and methanolic extract.

Keywords: Antioxidant property, *Andrographis paniculata*, free radical, methanolic extract.

Introduction

Free radicals initiate oxidative stress as it contains unpaired electron. Free radicals exhibiting very reactive chemical bonds, stabilizes through electron pairing with biomolecules such as proteins, lipids and DNA in healthy human cells and resulting in damage of structural protein and DNA along with lipid peroxidation. Free radicals, also known as reactive oxygen species (ROS), play a role in the etiology of several major diseases, are including cancer, atherosclerosis, and diabetes (Kuyvenhoven *et al.*, 1999; Kebapci *et al.*, 1999).

Oxidative stress induced by oxygen radicals is believed to be a primary factor in various degenerative diseases of the central nervous system as well as in the normal process of aging (Ramarathnam *et al.*, 1997). All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase (SOD) and catalase (Niki *et al.,*1994), which are disrupted when formation of free radicals exceed its neutralization in the human body. So it is necessary to provide human being with antioxidant for protection from against action of free radicals. Antioxidant is defined as

substance that even at low concentration significantly delays or prevents oxidation of easily oxidisable substrate. The main role of antioxidant is to help the body to protect against damage caused by reactive oxygen species (Shahidi, 1997). Antioxidant can also decrease mutagenesis and induce carcinogenesis in two ways -by decreasing oxidative DNA damage and by decreasing cell division. Antioxidant agents are natural origin because of their protective effect on humans (Osawa, 1990).

Though criticized, the ancient system of medical treatment (plant based medicine) has survived through the ages and it is still catering to the health needs of million all over the world (Kapoor, 2001).

In the context of providing efficient and inexpensive medicine to the masses and to build up public health confidence, it is deemed necessary that they are evaluated by current concepts of modern research. With this view, this study has been designed to evaluate the medicinal plants yielding herbal drugs with potent antioxidant property. The aim of this study is the scientific evaluation of the antioxidant properties of *Andrographis paniculata* - commonly known as "King of bitter". It is known for its 26 Ayurvedic formations included in the Indian pharmacopoeia – as kalmegh (Hindi).

The plant belongs to the family Acanthaceae. *Andrographis paniculata* is an annual - branched, erect-running ½ to 1 meter in height. The aerial parts of the plant (leaves and stems) are used to extract the active phytochemicals (Kiritikar & Basu, 1975). It grows abundantly in south-eastern Asia: India, Sri Lanka, Pakistan and Indonesia (Jarukamjorn & Nemoto, 2008).

The primary medicinal component of *Andrographis* is known chemically as diterpene lactone" based on its ring like structure, having a very bitter taste and is colorless crystalline. Research conducted in '80's and '90's has confirmed that *Andrographis* administration has a surprising broad range of pharmacological effects. Its common use include liver disorders, bowel complaints of children and convalescence after fever (Puri *et al*., 1993). It is been reported that *Andrographis* beneficial for preventing heart diseases, helps protection from liver diseases, and stimulates gall bladder contraction (Zhang & Tan, 2000; Wang & Zhao, 1994).

Material and methods

Sample collection and preparation

Plant samples were collected from field survey and procured from a vegetable market of Kolkota (India). Botanical Survey of India (Kolkota) plant taxonomist identified the samples and vouchers specimens were kept for reference.

100gm of fresh plant's leaves were placed in Erlenmeyer flasks containing ethanol of 500 ml and in another flask 100gm of leaves containing mixture of water and ethanol (1:1) and kept overnight at the room temperature. The extraction of leaves were repeated thrice on three consecutive days. All the extracts were pooled together and evaporated to dryness in rotatory evaporator to a minimum volume of 5 ml. The residual extract was lyophilized in a lyophiliser to get dry sample. Finally samples were dissolved in water and extract concentrated by vacuum dry method.

Anti-oxidant assay

Free radical scavenging activity by DPPH method: Gallic acid was used as a standard antioxidant. The free radical scavenging

Table 1. Experimental set up for standard curve of Gallic acid

S. No.	Amount of Gallic acid	Concentration (µg/µl)	DPPH (ml)	Ethanol (ml)
1	20 µl	5	1	2.980
2	40 µl	15	1	2.960
3	60 µl	20	1	2.940
4	80 µl	25	1	2.920
5	100 µl	25	1	2.990

Vol.1 No.1 (Apr 2012)

activity of Gallic acid was determined by DPPH(1,1,diphenyl-2-picryl-hydrazyl) method after modification as described by Blois (1975).

Different concentrations (i.e. 5, 10, 15, 20µg/µl etc.) of Gallic acid were taken in 1 ml of DPPH and 2ml ethanol was added in such amount that its total reaction volume would be 3ml (Table 1), after vortexing it was incubated at room temperature for 20 minutes and its absorbance at 517 nm was noted.

Table2 .Experimental set up for plant (Andrographis paniculata) extract

Plant extracts (10 µl)	Ethanol	DPPH
1. Methanol (M)	1.990 ml	1.0 ml
2. Methanol: water (M:H)	1.990 ml	1.0 ml
3. CONTROL	2.0 ml	1.0 ml

The free radical scavenging activity of *Andrographis paniculata*, was determined using a test tube containing 1 ml of DPPH and 2 ml of absolute ethanol kept as control. In another set of test tubes 1 ml DPPH were taken and then added 10µl of plant extract (PE) sample, 1.990 ml ethanol was added to make total reaction volume of 3 ml (Table 2). Thoroughly mixed by vortexed and incubated for 20 minutes and the absorbance were taken at 517 nm. The percentage of reduction in absorbance was calculated from the absorbance of control (A_0) and absorbance of plant extract containing test sample (A_{20}). The reducing activity (RA %) was determined as follows, RA % reducing activity = $(A_0 - A_{20} / A_0) \times 100$

Antioxidant assay by lipid peroxidation method

A modified Thiobarbituric acid reactive species (TBARS) assay was used to measure the potential antioxidant capacity of the plant extract using egg yolk as lipid rich medium (Buege & Aust, 1978). Homogenate was prepared from egg yolk in 0.9% NaCl saline. 10 µl of plant extract was added to the homogenate then 35 µl of the distilled water was added.5 µl of the $FeSO_4$:H_2O_2(3:2) was added to induce the lipid peroxidation (Table 3).

150 µl of glacial acetic acid and 150 µl of TBA were added to the reaction mixture and mixed by vortexing then the mixture was incubated at 95°C in the hot water bath for one hour. 50 µl of butanol was added to checked the reaction and centrifuged at 120000 rpm for 5 minutes and absorbance noted at 532nm. The α-tocopherol was used as standard antioxidant (Table 4) to compare the results of the test sample.

All the values are based on the percentage antioxidant index (AI %), by using the formulation: AI % = (1 - t/c) x 100 where

Table 4 .Experimental set-up for standard curve of the α-Tocopherol

α-Tocoherol (µl)	Conc. of α-Tocoherol	Homogenate (µl)	$FeSO_4$: H_2O (3:2µl)	Distill H_2O(µl)
4 µl	100 ppm	50 µl	5 µl	41 µl
10 µl	250 ppm	50 µl	5 µl	35 µl
20 µl	500 ppm	50 µl	5 µl	25 µl
30 µl	750 ppm	50 µl	5 µl	15 µl
40 µl	1000 ppm	50 µl	5 µl	5 µl

Table 3. Experimental set-up for lipid peroxidation assay of Andrographis paniculata extracts

Plant extracts (10 µl)	Homogenate (µl)	D H_2O	$FeSO_4$:H_2O	Gla. Acetic Acid	TBA	Butano1
CONTROL		45 µl	3µl: 2µl (5µl)	150µl	150µl	50µl
Methanol (M)	50 µl	35µl	3µl: 2µl (5µl)	150µl	150µl	50µl
Methanol water (M:H)	50 µl	35µl	3µl: 2µl (5µl)	150µl	150µl	50µl

c is the absorbance value of the control (not treated with the extract) and t is the absorbance of the test sample.

DNA cleavage protective activity

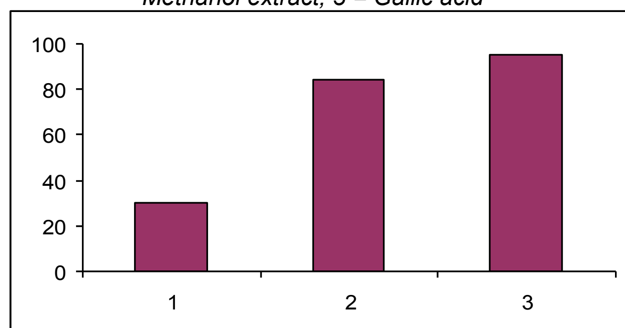
Isolation of DNA from pBS plasmid was done adopting modification of the methods of Birnboim & Doly (1979) ; Ish-Horowicz & Burke (1981).

DNA cleavage protective activity was checked on 1% agarose gel on pBS plasmid DNA. In this experiment four eppendorff tubes were taken .In the first tube, 4 µl of pBS plasmid which did not undergo UV treatment and 1 µl of bromophenol blue was added and kept as control. In the second tube 4 µl of pBS plasmid which underwent H_2O_2- UV treatment following the protocol of Russo *et al.,* (2001) after modification and 1 µl of bromophenol blue were added. In the third tube same composition as in the second tube with 8 µl of methanolic extract was added. In the fourth tube instead of methanolic extract, water and methanolic extract was added. Then the sample were loaded in 1% agarose gel and run at 70V for one and half hour and stained with ethidium bromide and observed under UV transilluminator.

Result and discussion

Free radical scavenging activity of *Andrographis paniculata* is identified by the reduction of methanolic solution of colored free radicals DPPH. The decrease in absorption of DPPH at adsorption maxima 517 nm is proportional to the concentration of free radical scavenger added to the DPPH reagent. This study was carried out by measuring the disappearance of the free radical DPPH to quantify the RSC (Radical Scavenging Capacity) with the help of spectrophotometer.

Fig.1. Determination of % radical scavenging capacity (RSC) of Andrographis paniculata. Gallic acid was used as standard. 1 = Methanol: water extract; 2 = Methanol extract; 3 = Gallic acid



As shown in Fig.1 the RSC of methanolic extract is much more potent than the water and methanolic extract. Antioxidant activity of plant extracts is probably because of the presence of several polyphenols in this extract . The gallic acid was used as a standard antioxidant for comparison, as its free radical scavenging activity is much greater than that of Ascorbic acid (D'Mello *et al*., 2000).

When the antilipidperoxidant of plant extracts was measured in egg yolk as source of lipid homogenate, differences in term of antioxidant activity was observed between methanolic and water - methanolic extract (Fig. 2). The α-tocopherol was used as standard antioxidant. Similar to the %RSC in DPPH assay, methanolic extract showed the highest activity, which may be attributed to the variation of chemical composition of the extracts (Chattopadhyay *et al*., 1994).

In the DNA cleavage protective assay (figure not shown), results of electrophoretic pattern of DNA after UV-H_2O_2 treatment in the presence and absence of methanolic extract of the plant is different. DNA derived from pBS plasmid showed two bands on agarose gel electrophoresis, the faster moving band correspond to the native form of super coiled circular DNA (scDNA) and the slower moving band was the open circular form (ocDNA). The UV irradiation of DNA in the presence of H_2O_2 resulted in the cleavage of the scDNA to ocDNA and linear form (linDNA), indicating that OH generated from UV photolysis of H_2O_2 caused excision of DNA. The addition of methanolic extract of *Andrographis paniculata* to the reaction mixture suppressed the formation of linDNA and induced a partial recovery of sc DNA.

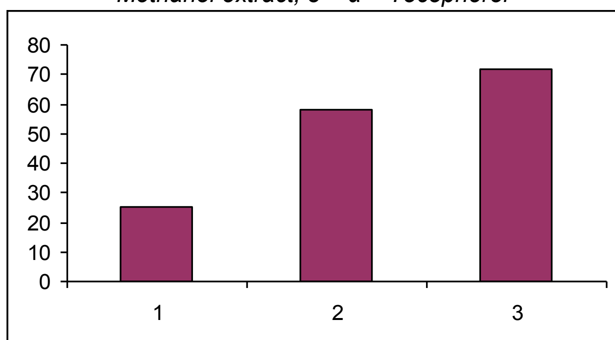
But this activity was not observed in methanol and water extract.

Conclusion

The main purpose was to evaluate the antioxidant property of *Andrographis paniculata* by employing three different methods (i) DPPH (ii) Lipid Peroxidation and (iii) DNA cleavage protective assay. In both the DPPH and Lipid Peroxidation methods, gallic acid and α- tocopherol were used as a standard antioxidant for comparison to the antioxidant property of *Andrographis paniculata*. Whereas in the DNA cleavage, pBS plasmid was used to assess the protective activity of *Andrographis paniculata.* In the DPPH method the RSC (radical scavenging capacity) of the plant extract was measured spectrophotometrically at 512nm. The lipid peroxidation inhibition activity of plant extract was also evaluated by taking the absorbance of pink colored complex formed at 535nm. In the DNA cleavage protective assay, the electrophoretic pattern of DNA after UV-induced photolysed H_2O_2 – oxidative damage were different in the absence and presence of methanolic extracts of the plant. In the above mentioned antioxidant assays the methanolic extract of *Andrographis paniculata* showed higher antioxidant activity than water - methanolic extract (Fig. 2.).

The results of potent antioxidant property from the above study provides fundamental data for the development of antioxidant products from methanolic extract of *Andrographis paniculata* .

Fig.2. Determination of % antilioperoxidant activity of the plant extracts Andrographis paniculata. α -tocopherol was used as standard. 1 = Methanol water extract; 2 = Methanol extract; 3 = α – Tocopherol



Acknowledgement

The first author gratefully acknowledges Dr. Sharmila Chattopadhyay, (Department of Cellular Biochemistry, Indian Institute of Chemical Biology (IICB), Kolkata) for guidance during the present investigation.

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