Research Article **www.ijcps.com**

International Journal of Chemical and Pharmaceutical Sciences
2017, Mar., Vol. 8 (1)

ISSN: 0976-9390
IJCPS

An *in-vitro* study of anti-oxidant, anti-inflammatory, anti-arthritic and anti-diabetic activity of ethanolic extract of *Andrographis paniculata leaves*

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Received: 26th Jan 2017, Revised and Accepted: 28th Jan 2017

ABSTRACT

The study on ethanolic extract of leaves of *Andrographis paniculata* shows that it possesses an antioxidant, anti-inflammatory, anti-arthritic and anti-diabetic activity. Ethanolic extract of leaf samples of *Andrographis paniculata* was qualitatively analysed for the presence of phytochemicals and it was confirmed that the plant extract contain metabolites such as alkaloids, flavonoids, terpenes and steroids. Hence further studies can be carried out using the ethanolic extract of *Andrographis paniculata* leaves. This study suggests that environmental features have variation in phytochemical content and accumulation in plants. This study provides scientific details to investigate the phytochemicals in the leaves of *Andrographis paniculata.*

Keywords: *Andrographis paniculata,* Anti-arthritic, Anti-diabetic, Anti-inflammatory, Antioxidant.

1. INTRODUCTION

Medicinal plants are an important source of producing valuable bioactive secondary metabolites which are of great importance for the health of individuals and societies. The medicinal values of the plants are due to the chemical substances that produce a definite physiological action on human body[1-3]. *Andrographis paniculata* belongs to the family Acanthaceae. *Andrographis paniculata* also known as the king of bitters and is traditionally used in Ayurveda and Chinese medicines. It is a perennial herb widely cultivated in tropical and subtropical areas such as South East Asia and India. It also forms the principle ingredient for several pharmaceutical preparations. The flowers have minute white petals bearing purplish spots. The stem is deep green, with diameter ranging from 2mm to 6mm or more. The flowers give rise to oblong capsules bearing numerous, minute brown seeds. It is also used as a folk medicine for fever, pain relief and disorders of the intestinal tract. In this study we have examined the promising activity against antioxidant, antidiabetic, antiarthritic and anti-inflammatory. Therefore this study has been undertaken to evaluate the effect of crude aqueous and ethanolic extracts of *Andrographis paniculata*.

2. MATERIALS AND METHODS

2.1. Collection and processing of plant *A.paniculata*

The leaves of *A.paniculata* were collected from the local area (Cuddalore). The leaves of *A.paniculata* were washed with water, shade dried at room temperature and powdered coarsely. Exactly 10g of the coarse powder of leaves was taken in 100mL of solvents such as ethanol and water. The extracts were allowed to evaporate in vaccum for 24hrs and 48hrs respectively. The extracts were stored in an air tight container for further studies.

2.2. Qualitative analysis of *A.paniculata*

2.2.1. Phytochemical screening

Qualitative tests for alkaloids, flavonoids, carbohydrates, glycosides, saponins, tannins, terpenes and phytosterols were performed according to the standard protocol.

2.2.2. Test for Alkaloids

The aqueous and ethanolic extracts were separately stirred with a 1mL of dilute hydrochloric acid and filtered. The filterate were tested with various alkaloidal reagents such as

Mayer's, Dragendroff's Wagner's and Hager's reagent which indicates the presence of alkaloids.

2.2.3. Test for Flavonoids

To 1mL of aqueous and ethanolic extracts were evaluated for flavonoids using alkaline reagent test, Ferric chloride test, Fluorescence test and reaction with alkali and acid which showed the presence of flavonoids.

2.2.4. Test for Tannins

To 1mL of aqueous and ethanolic extracts were treated with distilled water and filtered. The filterate were treated with copper sulphate solution indicating precipitation which shows the presence of tannins.

2.2.5. Test for Saponins

To 1mL of aqueous and ethanolic extracts were tested for saponins using foam test and hemolysis test which therefore indicates the presence of saponins.

2.2.6. Test of Terpenes

To 1mL of aqueous and ethanolic extracts were treated with copper acetate test which shows the presence of terpenes indicating green colour.

2.2.7. Test for Quinones

To 1 mL of both the extracts 1mL of concentrated sulphuric acid was added which shows the presence of Quinone indicating red colour.

2.2.8. Test for Phytosterols

To 1mL of aqueous and ethanolic extracts were refluxed with 0.5N alcoholic potassium hydroxide until the saponification was complete. The saponification mixture was diluted with distilled water and extracted with petroleum ether. The extracts were evaporated and unsaponification matter was subjected to Libermann's, Salkowski's test which indicates the presence of phytosterols.

2.3. Quantitative analysis of *A. paniculata*

2.3.1. Moisture content

About 1gm of the sample was weighed into a clean dry pre-washed petriplate. Then the sample was determined in the triplicate by drying at 120ºC in a hot air oven and then the plates were removed and cooled in a room temperature. Weight of the sample was taken after cooling process and it is expressed in percentage using the formula.

Moisture content % = (W_1-W_2/Weight of samples) x 100

2.3.2. Ash content

About 1gm of the sample was weighed into a clean dry pre washed silica dish. Then the sample was ignited slowly over a Bunsen flame in a fume cupboard until the fumes has been evaporated. Then the dish was transferred to muffle furnace and incinerated until it was free from black carbon particles and turns white in colour in about 3hrs. Dish was removed carefully and cooled in a desiccator. Weight was taken after cooling process has been completed and it is expressed in % using the formula.

Ash % = Weight of ash/Weight of samples X 100

2.4. Antioxidant activity

2.4.1. Determination of DPPH scavenging activity

DPPH scavenging activity of ethanolic extract was determined according to the standard protocol[4]. An aliquot of 0.5mL of sample solution was mixed with 0.5Mm of methanol and to this 2.5mL of DPPH reagent was added. The mixture was shaken vigorously and incubated for 30minutes in dark at room temperature. The absorbance was measured at 517nm using UV spectrophotometer. Ascorbic acid was used as a positive control. DPPH free radical scavenging activity % was calculated using the formula.

% of inhibition = absorbance of control (A_0)-Absorbance of Sample (As)/Absorbance of control X 100.

2.4.2. Determination of FRAP reducing assay

FRAP reducing activity was determined according to the standard protocol[5].The principle is based on the formation of O-Phenanthroline-Fe^{2+} complex and its disruption in the presence of chelating agents. The reaction mixture containing 1mL of 0.05% O-Phenanthroline in methanol, 2mL of ferric chloride(200µM) and 2mL of ethanolic extract at various concentrations ranging from 10 to 500µg was incubated at room temperature for 10min and the absorbance of the same was measured at 510nm. EDTA was used as a classical metal chelator.

2.4.3. *In-vitro* anti-inflammatory activity membrane stabilization

2.4.3.1. Preparation of red blood cell (RBSs) suspension

The blood was collected from healthy human volunteer who had not taken any NSAIDs for 2 weeks prior to the experiment and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3000 rpm for 20min and packed cells were washed three times with isosaline (0.85%, pH 7.20). The volume of the

blood was measured and reconstituted as 10% v/v suspension with isosaline[6,7].

2.4.3.2. Heat induced haemolysis

The reaction mixture 2mL consists of 1mL test sample of different concentrations ranging (100-500µg/mL) and 1mL of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubating in water bath at 56°C for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was read at 560nm[8]. The experiment was performed in triplicates for all the test samples.

The % inhibition of hemolysis was calculated using the formula

% Inhibition = (Optical density of test sample/Optical density of control) X 100

2.4.3.3. Hypotonicity induced haemolysis

The principle involves the stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The assay mixture contains 1mL phosphate buffer (pH 7.4, 0.15M), 2mL hypo saline (0.36%), 0.5mL HRBC suspension (10% v/v with 0.2mL of plant extracts and standard drug diclofenac sodium of various concentrations (100 and 200µg/mL) and control (distilled water instead of hypo saline to produce 100% hemolysis) were incubated at 37°C for 30min and centrifuged respectively. The haemoglobin content in the suspension was estimated using spectrophotometer at 560 nm[9].

The percentage of HRBC membrane stabilization can be calculated using the formula

% Protection = 100 - (Optical density of test sample/optical density of control) x 100.

2.5. Anti-arthritic activity

2.5.1. *In-vitro* anti-arthritic activity by inhibition of protein denaturation method

Samples containing Bovine Serum albumin (5% W/V Ethanol solution) and test solution (25mg/mL) was made upto 1mL. Similarly two duplicates were prepared and made upto 1mL.Test control contains Bovine serum albumin (5% W/V Ethanol solution) and distilled water which is made upto 1mL using distilled water. Product control consists of distilled water and test solution which is again made upto 1mL. A standard solution (1mL) consists of 0.9mL of Bovine serum albumin (5%W/V Ethanol solution) and 0.1mL of Aspirin (25mg/mL). All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°C for 20 min and the temperature was increased to 57°C for 3min. After cooling, add 2.5mL of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416nm[10,11]. The control represents 100% protein denaturation. The results were compared with Aspirin (25mg/mL) and percentage inhibition of protein denaturation can be calculated using the formula,

% Inhibition = 100 - (Optical density of test solution – optical density of product control/optical density of test control) X 100.

2.6. Anti-diabetic activity

2.6.1. Alpha Glucosidase inhibition assay

The alpha glucosidase inhibitory activity was performed with a set of eppendorf tubes which was labelled as blank, control and samples at different concentrations were arranged in a stand. The reaction mixture containing 150µL of 0.1M PBS (pH 7.4), 75µL of 20mM p-nitrophenyl a-D-glucopyranoside (PNPG) solution and 25µL of the sample were added and the mixture was pre-incubated at 37°C for 10 mins. The reaction was initiated by adding 50µL of rat intestinal alpha-glucosidase enzyme solution (0.17U/mL) and incubated at 37°C for 10mins. The reaction was terminated by adding 1mL of 0.1mL of Na_2Co_3 and the amount of p-nitrophenol (PNP) released was determined by measuring the absorbance at 405nm. For blank the reagents was added in a reverse order. The control samples were free of test solution. A set of colour control was done for the test samples without enzymes and PNPG but maintain the volume with buffer. The absorbance of all the samples was measured at 405nm using UV visible spectrophotometer[12,13]. The percentage inhibition of enzyme activity by the test sample was calculated.

3. RESULTS

Table - 1: Qualitative analysis of carbohydrates on ethanol and aqueous extracts of *A.paniculata*

Test Name	Ethanol	Aqueous
Molisch's test	+	+
Fehling's test	+	+
Barfoed's test	+	+

Table1 Shows the qualitative analysis of carbohydrates in aqueous and ethanolic extracts of *A.paniculata*. It is observed that aqueous and ethanolic extracts has shown the presence of carbohydrates when tested with Molisch's, Fehling's and Barfoed's reagent.

Table 2 shows the qualitative phytochemical screening of aqueous and ethanolic extracts of *A.paniculata*. It is observed that aqueous extract shows the presence of alkaloids whereas ethanol extract shows the presence of flavonoids, tannins, glycosides and quinones. It also shows the presence of high amount of saponins and steroids. This confirms that the majority of the phytochemicals is present in ethanolic extract of *A.paniculata* leaves.

Table - 2: Phytochemical screening of ethanol and aqueous extracts of *A.paniculata*

Tests	Ethanol	Aqueous
Alkaloids	-	+
Flavonoids	+	-
Tannins	+	-
Saponins	++	-
Glycosides	+	-
Terpenes	-	-
Quinone	+	-
Steroids	++	-

Table - 3: Quantitative analysis of ash content

Moisture Content				
Leaf	T1	T2	T3	T4
Fresh leaf	201.3	138.1	67.5	135.63±66.93
Dried leaf	16.4	12.3	11.3	13.3±2.74

Table 3 shows the quantitative analysis of moisture content. This confirms that the moisture content was found to be high in fresh leaves when compared with the dried leaves.

Table - 4: Quantitative analysis of ash content

Ash Content				
Leaf	T1	T2	T3	T4
Fresh leaf	68	14.3	85	55.766±36.90
Dried leaf	268	266	303.2	279.063±20.92

Table 4 shows the quantitative analysis of Ash content. In this the ash content was found to be high in dried leaves when compared to the fresh leaves.

Table 5 shows the DPPH radical scavenging activity and FRAP of ethanolic extract of *A.paniculata*. In this the highest scavenging activity was found to be in 2mg of the extract and it is compared with the standard Ascorbic acid. This confirms that the antioxidant potential is found to be high as the concentration increases.

Table 6 shows the effect of ethanolic extract of *A.paniculata* on HRBC membrane by heat induced method. In this the % inhibition was found to be high in 200µg/mL concentration for the duration 1hr and it is compared with the standard Aspirin.

Table - 5: Determination of antioxidant activity of DPPH and FRAP against standard ascorbic acid

Concentrations	% of scavenging and reducing activity								
	DPPH			FRAP			Ascorbic acid		
250µg	0.092	0.123	0.108	1.092	0.546	2.459	75.595	77.389	76.492
500µg	0.866	0.773	0.819	3.551	3.005	4.918	79.771	80.080	79.925
750µg	1.268	1.175	1.221	6.557	6.010	7.923	83.575	83.359	83.467
1mg	2.350	2.257	2.304	8.196	7.650	9.562	85.740	85.524	85.632
2mg	4.113	4.021	4.067	12.021	11.475	13.387	90.194	90.318	90.256

Table - 6: Effect of *A.paniculata* on HRBC membrane by heat induced method

Sample	% Inhibition of hemolysis		
	5min	1/2hr	1hr
Sample 1	14.99	43.57	7.77
Sample 2	43.15	26.47	56.56
Sample 3	24.00	53.69	5.24
Sample 4	15.13	48.71	18.53
Sample 5	48.71	34.10	13.52
Aspirin	69.80	71	70.09

Table - 7: Effect of *A.paniculata* on HRBC membrane by hypotonicity method

Sample	% Stabilization of hemolysis		
	5min	1/2hr	1hr
Sample 1	23.86	40.63	47.98
Sample 2	6.17	8.66	22.22
Sample 3	4.98	8.39	23.75
Sample 4	16.24	18.30	29.79
Sample 5	18.63	35.62	42.64
Aspirin	48	50.87	51

Table 7 shows the effect of ethanolic extract of *A.paniculata* on HRBC membrane by hypotonicity method. It is observed that highest stabilisation of hemolysis was found to be in 1hr duration (42-64%) for the concentration 500µg/mL respectively when compared with standard Diclofenac.

Table - 8: Anti-arthritic activity of ethanolic extract of *A.paniculata*

Conc (mg/mL)	Ethanolic extract of *A.paniculata*			Aspirin
	Sample 1	Sample 2	Sample 3	
250	45.35	42.47	33.13	70.59

Table 8 shows the anti-arthritic activity of ethanolic extract of *A.paniculata* leaves. In this 250mg/mL concentration is used for this study and its inhibition was found to be high in sample 1 when compared with other two samples as compared to the standard Aspirin.

Table - 9: Anti-diabetic activity of ethanolic extract of *A.paniculata*

Conc (µg/mL)	OD at 405nm	% of inhibition
250	0.269	9.866
500	0.248	17.056
750	0.235	21.237
1000	0.207	30.602
2000	0.190	36.454

Table 9 shows the Anti-Diabetic activity of ethanolic extract of *A.paniculata* leaves. In this the inhibition of alpha glucosidase was found to be high in 2000µg/mL concentrations which reveals that it could act as a most effective approaches to control diabetes.

4. DISCUSSION

This study was carried out to evaluate the qualitative analysis, quantitative analysis, antioxidant, anti-inflammatory, anti-arthritic and anti-diabetic activity of *A.paniculata*. The phytochemical tests of crude extracts revealed the presence of carbohydrates, alkaloids, flavonoids, saponins, tannins and phytosterols respectively. Plant steroids are the most naturally occurring phyto constituents that have found therapeutic application as cardiac drugs[14].

DPPH radical scavenging activity and FRAP assay also revealed the antioxidant activity of ethanolic extract of *A.paniculata* in a dose dependent manner. Antioxidants are the agents that reduce the damage of cells by neutralizing the free radicals[15]. Ethanolic extract of *A.paniculata* also revealed the anti-inflammatory activity by inhibiting the hemolysis and also by stabilizing hemolysis. Eugenol (1-hydroxy-2-methoxy-4-allylbenzene) a naturally occurring phenolic compound is a major component of basic oil and exists to a lesser extent in oil of several other plants [16].

Rheumatoid arthritis is a chronic inflammatory systemic autoimmune disease characterized by the development of a chronic inflammatory proliferation of the synovial linings of diarthrodial joints, which leads to aggressive cartilage destruction and progressive joint destruction, disability and premature death. Ethanolic extract of *A.paniculata* also shows the invitro anti-arthritic activity by inhibiting protein denaturation. Alpha-glucosidase is a membrane bound enzyme located on the epithelium of the small intestine, catalysing the cleavage of disaccharides to form glucose. Inhibitors can retard the uptake of dietary carbohydrates and suppress post-prandial hyper glycemia. It also revealed the antidiabetic activity by obeying alpha glucosidase test and thus confirms that it can also be used for the treatment of hypoglycemic property.

5. CONCLUSION

In the present investigation ethanolic extract of *A.paniculata* leaves were assessed for its pharmacologic activity and it is concluded from this study that the leaves of *A.paniculata* have antioxidant property, anti-inflammatory, anti-arthritic and anti-diabetic activity. The activity was found due to the presence of phytochemicals and phytosterols present in it. *A.paniculata* can be considered as a herbal drug for the treatment of free radical induced disorders like cancer, cardiac and many other diseases.

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