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EVALUATION OF *IN VITRO* ANTIOXIDANT POTENTIAL OF AERIAL PARTS OF *ATYLOSIA ALBICANS*

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ABSTRACT

*The aim of this study was to evaluate the antioxidant activities of aerial parts of Atylosia albicans (A. albicans). The extraction of polyphenols has been followed by the determination of total polyphenols and flavonoids. Several methods were used to evaluate the in vitro antioxidant activity such as DPPH (2, 2'-diphenyl-1-picrylhydrazy), NO (nitric oxide), H_2O_2 (hydrogen peroxide) and TBARS (thiobarbituric acid) assays. The results showed that the highest levels of polyphenols were recorded in **AAM** (85.63±0.26mg of GA/g of extract (Gallic Acid Equivalent))/g of extract). Total flavonoid contents of extracts/fractions showed that **AAM** (112.26±0.61mg of Qu/g of extract (Quercetin Equivalent))/g of extract) contains the highest level. The **AAM** extract shows more inhibition of DPPH (89.74), NO (133.20), H_2O_2 (104.73) and TBARS (171.55µg/ml) respectively when compared with other extracts. These findings suggest that the extracts obtained from A. albicans have active substances contributing to the increase in natural antioxidant potential.*

KEY WORDS

Atylosia albicans, Polyphenols, Flavonoids, DPPH, NO, $H_2O_2$and TBARS

INTRODUCTION:

The treatment of diseases began long ago with the use of plants. Plants form an important component of traditional medicine worldwide and are considered to be an important source of valuable medicines. It is estimated that>80% of world's population depends on traditional medicine to meet primary health care needs. Worldwide, the methods of folk healing commonly utilize plants and plant-based formulations as part of tradition. The practice of traditional medicine based on plants is most common in countries like China, India, Japan, Pakistan, Sri Lanka and Thailand. Besides, many plants are widely used in various systems of traditional medicine like Ayurveda, Unani, Homeopathy and Sidda. These plants are the sources of lead compounds for the development of several drugs [1].

Antioxidants are substances that delay or oppose oxidation of an oxidizable substrate in low concentrations in comparison with those of the substrate. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are formed because of normal cellular metabolisms which play a key role in pathogenesis of several oxidative stress induced diseases. This led to an increase in finding of various substances which are of great antioxidant potentials and can play a vital role in disease prevention due to oxidative stress [2, 3]. Antioxidants lower oxidative stress in the cells by two mechanisms: 1) stopping the free radical generation and 2) detoxifying ROS or RNS by up regulating the catalase (CAT), glutathione peroxidase (GPx) and super oxide dismutase (SOD) which are free radical scavengers and protect the cell membrane from injury [4]. Medicinal plants are the important source of antioxidants [5].

The genus Atylosia, a member of Fabaceae holds many species distributed throughout the tropical and

subtropical regions of the world. In India, these are in abundance in the broad leaf evergreen forest areas of the Westernghats and Malabar coast [6]. The species of Atylosia, a forage legume, by and large are hardy perennial [7] insect, disease resistant and possess high protein content. *Atylosia albicans*, as the leaves are broadly obovate and calyx lobes shorter than tube. But the crucial character is whether the pod is brown-golden hairy or white hairy. The main aim of this study was to evaluate the *in vitro* antioxidant activities of aerial parts of *Atylosia albicans*.

MATERIAL AND METHODS:

Chemicals

Petroleum ether, Ethyl acetate, Methanol, Gallic acid, Quercetin, Aluminium-chloride, 1,1-diphenyl-2-picrylhydrazyl (a,a-diphenyl-bpicryl hydrazyl; DPPH), H_2O_2, Griess reagent, Trichloroacetic acid, Thiobarbituric acid.

Plant material

The aerial parts of *Atylosia albicans* were collected during rainy season in regions of Kerala. The collected aerial parts were authenticated by Dr.V.Chelladurai, Research Officer-Botany (Scientist -C) Central Council for Research in Ayurveda & Siddha, AYUSH, Govt. of India.

Preparation of extract

The collected aerial parts were washed with distilled water and dried at room temperature to constant weights and ground to powder. The powder (UNIT Quantity) was packed in muslin cloth and extracted by Soxhlation with pet.ether, ethyl acetate and methanol as solvents respectively according to the increasing order of polarity [8]. Each extract was concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator [9].

Determination of total phenolic content

The Total phenolic content of three extracts was evaluated with Folin-Ciocalteu method [10]. Extracts containing polyphenols are reduced by the Folin-Ciocalteu reagent there by producing blue colored complex. For estimation of phenolic content of extracts was estimated from a gallic acid calibration curve. Calibration curve prepared from, 0.5mL aliquots of 12.5, 25, 50, 100, 200, and 400 µg/mL methanolic gallic acid solutions were mixed with 2.5 mL Folin-Ciocalteu reagent (diluted ten-fold) and 2.5 mL (75 g/L) sodium carbonate. After incubation at 25°C for 30 min, the quantitative phenolic estimation was performed at 765 nm against reagent blank by UV Spectrophotometer 1650 Shimadzu, Japan. The calibration curve was constructed by putting the value of absorbance vs. concentration. A similar procedure was adopted for the extracts as above described in the preparation of calibration curve. All determinations were performed in triplicate. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per g of extract.

Determination of total flavonoid content (TFC).

Aluminium-chloride colorimetric assay was used to determine the total flavonoid content in the extracts as previously reported [11]. Briefly, 1 ml of the extract (1 mg/mL) was mixed with 4 mL of distilled water in a 10 mL volumetric flask. 0.30 mL of 5% sodium nitrite was added to the flask. After 5 min, 0.30 ml of 10% $AlCl_3.6H_2O$ solution was added to the mixture, followed by addition of 2 mL of 1.0 M NaOH after another 5 min and diluted to the mark with distilled water. A set of standard solutions of quercetin (12.5, 25, 50,100,200 and 400 µg/ml) were prepared in the same manner as described for the extracts. The absorbances of the extracts and standard solutions were measured against the reagent blank at 510 nm with a UV/Visible spectrophotometer. The total flavonoid content was determined from the calibration curve and expressed as milligram of quercetin equivalent (QE) per gram of extracts [12]. The determinations of total flavonoid in the extracts and standards were carried out in triplicates.

In vitro antioxidant activity

DPPH radical scavenging activity

The molecule 1, 1-diphenyl-2-picrylhydrazyl (a, a-diphenyl-bpicryl hydrazyl; DPPH) is characterized as a stable free radically virtue of the delocalization of the spare electron over the

molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color.

In order to evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored.

According to Manzocco *et al*., the sample extract (0.2 mL) is diluted with methanol and 2 mL of DPPH solution (0.5 mM) is added. After 30 min, the absorbance is measured at 517 nm. The percentage of the DPPH radical scavenging is calculated using the equation as given below:

% inhibition of DPPH radical = {(Abr -Aar)/ Abr} × 100

where Abr is the absorbance before reaction and Aar is the absorbance after reaction has taken place [13].

Hydrogen peroxide scavenging (H_2O_2) assay

Human beings are exposed to H_2O_2 indirectly via the environment nearly about 0.28 mg/kg/day with intake mostly from leaf crops. Hydrogen peroxide may enter into the human body through inhalation of vapor or mist and through eye or skin contact. H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH) that can initiate lipid peroxidation and cause DNA damage in the body. The ability of plant extracts to scavenge hydrogen peroxide can be estimated according to the method of Ruch *et al* [14]. A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer.

Extract (20–60 lg/mL) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows:

% scavenged (H2O2) = {(Ai -At)/ Ai} × 100

where Ai is the absorbance of control and at is the absorbance of test.

Nitric oxide scavenging activity

NO is generated in biological tissues by specific nitric oxide synthases, which metabolizes arginine to citrulline with the formation of NO via a five-electron oxidative reaction. The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Griess reagent. Two (2) mL of 10 mM sodium nitroprusside dissolved in 0.5 mLphosphate buffer saline (pH 7.4) is mixed with 0.5 mL of sample at various concentrations (0.2–0.8 mg/mL). The mixture is then incubated at 25 °C. After 150 min of incubation, 0.5 mL of the incubated solution is withdrawn and mixed with0.5 mL of Griess reagent [(1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for5 min with 1 mL of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture is then incubated at room temperature for 30 min and its absorbance pouring into a cuvette is measured at 546 nm. The amount of nitric oxide radical inhibition is calculated following this equation:

% inhibition of NO radical ={(A0-A1)/ A0} × 100

where A0 is the absorbance before reaction and A1 is the absorbance after reaction has taken place with Griess reagent [15].

TBARS assay

TBA method, described by Ottolenghi *et al*, the final sample concentration of 0.02% w/v was used in this method. Two mL of 20% trichloroacetic acid and 2 mL of 0.67% of thiobarbituric acid were added to 1 mL of sample solution. The mixture was placed in a boiling water bath for 10 min and then centrifuged after cooling at 3000 rpm for 20 min. The absorbance activity of the supernatant was measured at552 nm and recorded after it has reached its maximum [15].

Statistical analysis

The experimental results were expressed as Mean ± Standard Deviation (SD). The data was analyzed using one-way analysis of variance (ANOVA) and the differences between samples were determined by Duncan's Multiple Range test using Graph Pad Prism software version 5.0. P values ≤0.05 were regarded as significant.

RESULTS

Total phenolic and flavonoid content:

The total phenolic and flavonoid contents of the petroleum ether, ethyl acetate and methanolic extracts of *Atylosia albicans* **(AA)** were done by Folin-Ciocalteu colorimetric and $AlCl_3$ methods, respectively. The total phenolic contents were expressed as mg gallic acid equivalent per gram of dry extract. In Table **1** phenolic content in the examined whole plant extracts of **AA** ranges from 61.08 to 85.63. The **AAM** have very high values of total phenolic contents (85.63) when compared with compared with remaining extracts like **AAP** (69.80) and **AAE** (61.08).

The concentration of flavonoids in **AA** whole plant extracts were determined using spectrophotometric method with $AlCl_3$. The content of flavonoids is expressed in terms of Quercetin equivalent (QuE), mg of Qu/g of extract. The summary of quantities of

flavonoids identified in the tested extracts is shown in Table **2**. The flavonoids content in the examined different extracts ranges from 85.45 to 112.26 mg Qu/g. The **AAM** extract (112.26) have very high values of total flavonoids contents when compared with **AAP** (99.48) and **AAE** (85.45).

DPPH radical scavenging activity:

The comparison of the antioxidant activities of the extracts and ascorbic acid is shown in Table **3**. The IC_{50} values for scavenging of free radicals were 32.00, 89.74, 235.20 and 347.60 µg/ml for ascorbic acid, methanol, ethyl acetate and petroleum ether extract respectively, which indicate the efficient DPPH scavenging activity. The order of DPPH scavenging activity was in the order of methanol> >ethyl acetate > Pet. ether extracts.

Hydrogen peroxide (H_2O_2) radical scavenging assay

The comparison of the antioxidant activities of the extracts and ascorbic acid is shown in Table **4**. The IC_{50} values for scavenging of free radicals were 52.07, 104.73, 272.20 and 384.62 µg/ml for ascorbic acid, methanol, ethyl acetate and petroleum ether extract respectively, which indicate the efficientH_2O_2 scavenging activity.

Nitric oxide (NO) radical scavenging:

The comparison of the antioxidant activities of the extracts and ascorbic acid is shown in Table **5**. The IC_{50} values for scavenging of free radicals were 53.18, 133.20, 157.05 and 378.24 µg/ml for ascorbic acid, methanol, ethyl acetate and petroleum ether extract respectively, which indicate the efficient NO scavenging activity.

Thiobarbituric acid reactive substances (TBARS) Assay

The comparison of the antioxidant activities of the extracts and ascorbic acid is shown in Table **6**. The IC_{50} values for scavenging of free radicals were 38.89, 171.55, 176.32 and 334.83 µg/ml for ascorbic acid, methanol, ethyl acetate and petroleum ether extract respectively, which indicate the efficient TBARS assay.

Table 1: Total phenolic contents in the plant extracts expressed in terms of Gallic acid equivalent (mg of GA/g of extract)

Test Samples	mg of GA/g of extract
AAP	69.80±0.14
AAE	61.08±0.72
AAM	85.63±0.26

Table 2: Total flavonoid contents in the plant extracts expressed in terms of Quercetin equivalent (mg of Qu/g of extract)

Test Samples	mg of Qu/g of extract
AAP	99.48±0.27
AAE	85.45±0.44
AAM	112.26±0.61

Table 3: DPPH radical scavenging activity of *Atylosia albicans* extracts

S.No	Conc.(µg/ml)	% Inhibition	IC_{50}(µg/ml)
Ascorbic Acid			
1	5	12.52	
2	10	28.78	
3	20	42.56	32.00
4	40	55.83	
5	80	68.89	
AAP			
1	25	20.84	
2	50	29.82	
3	100	38.29	
4	200	45.67	347.60
5	400	52.26	
AAE			
1	25	19.35	

S.No	Conc.(µg/ml)	% Inhibition	IC_{50}(µg/ml)
2	50	25.58	
3	100	36.91	235.20
4	200	48.59	
5	400	59.46	
AAM			
1	25	19.29	
2	50	39.42	
3	100	53.99	
4	200	65.77	89.74
5	400	74.56	

Table 4: Hydrogen Peroxide (H_2O_2) scavenging Assay of *Atylosia albicans* extracts

S.No	Conc. (µg/ml)	% Inhibition	IC_{50}(µg/ml)
Ascorbic Acid			
1	5	13.56	
2	10	27.77	
3	20	44.65	52.07
4	40	56.81	
5	80	69.99	
AAP			
1	25	22.52	
2	50	30.53	
3	100	39.24	384.62
4	200	45.77	
5	400	52.53	
AAE			
1	25	18.82	
2	50	24.73	
3	100	32.99	272.20
4	200	46.65	
5	400	57.53	
AAM			
1	25	23.28	
2	50	31.54	
3	100	49.83	104.73
4	200	58.93	
5	400	76.68	

Table 5: Nitric Oxide (NO) scavenging Activity of *Atylosia albicans* extracts

S.No	Conc. (µg/ml)	% Inhibition	IC_{50}(µg/ml)
Ascorbic Acid			
1	5	24.66	
2	10	32.32	
3	20	39.55	53.18
4	40	47.88	
5	80	56.35	
AAP			
1	25	26.65	
2	50	31.62	
3	100	38.28	378.24
4	200	42.68	
5	400	51.54	
AAE			
1	25	29.52	157.05

S.No		Conc. (µg/ml)	% Inhibition	IC_{50}(µg/ml)
2		50	34.63	
3		100	42.69	
4		200	56.61	
5		400	67.50	
AAM	1	25	25.98	
2		50	38.34	
3		100	45.88	133.20
4		200	60.39	
5		400	78.86	

Table 6: Thiobarbituric Acid (TBARS) Assay of *Atylosia albicans* extracts

S.No	Conc. (µg/ml)	% Inhibition	IC_{50}(µg/ml)
Ascorbic Acid			
1	5	20.52	
2	10	29.41	
3	20	33.52	38.89
4	40	50.82	
5	80	66.58	
AAP			
1	25	24.67	
2	50	37.52	
3	100	42.22	334.83
4	200	46.63	
5	400	52.53	
AAE			
1	25	28.55	
2	50	32.43	
3	100	40.65	176.32
4	200	53.32	
5	400	64.63	
AAM			
1	25	21.87	
2	50	37.58	
3	100	45.83	171.55
4	200	52.59	
5	400	73.82	

DISCUSSION:

In the analysis of results for the concentrations of total phenolic compounds extracts, it is noticed that the highest concentration of phenolic compounds is in the extracts obtained using solvents of high and moderate polarity. The high dissolubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction [16, 17]. Based on the obtained values for concentration of flavonoids in the observed leaf, bark and fruit extracts, it has been found that the highest concentration of these compounds is in the extracts obtained using solvents of moderate polarity and nonpolar solvents. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation. [18]. The DPPH radical scavenging activity is based on the ability of DPPH (A stable free radical), to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance [19]. Hydrogen peroxide though a weak oxidizing agent is important because of its ability to penetrate biological membranes, once inside the cell it can probably react with Fe^{2+} and Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of

its toxic effects. Like superoxide anion, all extract showed excellent H_2O_2 scavenging activity. Hydrogen peroxide enters the human body through inhalation of vapor or mist and through eye or skin contact. In the body, H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage [20]. The toxicity and damage caused by NO and O_2 is multiplied as they react to produce reactive peroxynitrile (ONOO–), which leads to serious toxic reactions with bio molecules. It is well documented that NO plays a crucial role in the pathogenesis of inflammation where it is secreted as a mediator, this may explain the use of extract for the treatment of inflammatory diseases [21]. Lipid peroxidation is an important marker of oxidative stress. Thiobarbituric acid (TBA) reaction was used to detect lipid oxidation. When lipids are oxidized, malondialdehyde is formed. TBA reacts with malondialdehyde in hot acid to form a red complex of malonaldehyde: TBA (1:2) with an absorbance maximum of 532nm [22].

CONCLUSION:

The *Atylosia albicans* whole plant methanolic extract showed a strong antioxidant activity by inhibiting DPPH, NO, H_2O_2 and TBARS activities when compared with the standard L-ascorbic acid. In addition, the *Atylosia albicans* was found to contain a noticeable amount of total phenols and flavonoids, which play a major role in controlling oxidation. The results of this study show that the *Atylosia albicans* root can be used as an easily accessible source of natural antioxidant. Therefore, a further study is needed to determine the mechanism behind the antioxidant activity of this plant. Further studies are needed to elucidate whether *Atylosia albicans* whole could be useful in the management of human diseases resulting from oxidative stress.

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Conflict of Interests

No conflict of interests

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