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Original Research Article

In vitro Antifungal, Antioxidant and Cytotoxic Activities of a Partially Purified Protein Fraction from *Atlantia monophylla* Linn (Rutaceae) Leaf

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Abstract

Purpose: *To determine the in vitro antifungal and antioxidant activities of the aqueous extract and protein fraction of Atlantia monophylla Linn (Rutaceae) leaf.*
Methods: *Ammonium sulphate (0 – 80 %) precipitation method was used to extract protein from the leaves of A. monophylla Linn (Rutaceae). In vitro antifungal assays were performed by disc-diffusion and micro-broth dilution methods. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), superoxide and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities were performed to evaluate in vitro antioxidant activities. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to assess the molecular weight of the protein fractions. Protein concentration was determined by Bradford method. The cytotoxicity of these extracts was tested on Vero cell lines.*
Results: *Both the aqueous extract and protein fraction (AMP III) of Atlantia monophylla leaf exhibited higher antifungal activity on Candida albicans than on Aspergillus fumigatus. AMP III fraction showed greater in vitro antioxidant activity than the aqueous extract. SDS-PAGE analyses revealed the presence of two protein bands with molecular weight approximately of 16 and 67 KDa in AMP III. Protein concentration was 240 µg/ml in the aqueous extract and 670 µg/ml in AMP III fraction. The aqueous extract and protein fraction exhibited cytotoxicity at concentrations > 100 µg/ml on Vero cells.*
Conclusion: *Plant-derived proteins/peptides possessing antifungal and antioxidant properties would be a good alternative preparation for the treatment of infectious diseases.*

Keywords: *Atlantia monophylla, Antifungal, Antioxidant, Cytotoxicity, Proteins/peptides, Vero cell lines*

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INTRODUCTION

Fungal infections remain a significant cause of morbidity and mortality despite advances in medicine and the emergence of new antifungal agents. Increasing incidents of fungal resistance following opportunistic pathogen infections such as Human Immunodeficiency Virus (HIV), patients with immunosuppressive chemotherapy led researchers to look for new, safer and more effective antifungal agents as an alternative strategy to the available drugs in the market [1]. Due to these pathological conditions, there are alterations in normal metabolism that may generate reactive oxygen species and other free radicals in the body. Reactive oxygen species

(ROS) is a collective term that encompasses radical and non- radical derivatives of molecular oxygen that inflict injuries to the tissues through lipid peroxidation resulting in atherosclerosis, cancer, diabetes and other diseases [2]. The schematic search of higher plants for antifungal activity has shown that some plants extracts may have the ability to retard fungal growth or completely inhibit the fungus.

Many low molecular mass proteins or peptides have been reported to possess antifungal and antioxidant activities. Huynh et al [3] isolated low molecular mass proteins which are involved in a defense mechanism against fungi. Chen *et al* [4] studied the antioxidant activities of synthetic peptides based on an antioxidant peptide isolated from the soy protein hydrolizates.

Medicinal plants have been the basis for the treatment of various infectious diseases in traditional medicine, and possess a variety of compounds of known therapeutic properties. Much attention has been paid to plant-derived antifungal and antioxidant compounds, based on the knowledge that plants have their own defense system [5]. Only a small fraction of the known plant species of the world have been evaluated for the presence of antimicrobial and antioxidant compounds, and thus it is necessary to increase the efforts in collecting and screening for the development of novel and environmentally safe therapeutic agents.

Atlantia monophylla Linn (*A. monophylla*) belongs to the family of Rutaceae commonly called as Jungli Nimbu. The main objective of the present study was to determine the in vitro antifungal and antioxidant activities of a partially purified protein from the leaves of *A. monophylla* Linn (Rutaceae).

EXPERIMENTAL

Fungal strains

Candida albicans 10231 and *Aspergillus fumigatus* 1022 used in the present study are of American Type Cell Culture (ATCC) procured from IMTECH, Chandigarh, India.

Plant material

The leaves of *Atlantia monophylla* Linn (Rutaceae) were collected in the month of October 2008, from Tirumala hills, Chittoor District, Andhra Pradesh, India. The plant was authenticated by Prof N Yasadomma, Department of Botany, Sri Venkateswara University, India. A voucher specimen was deposited in the herbarium as SVU/B/AM-32.

Protein extraction from aqueous extract

Leaf powder (100 g) of *A. monophylla* Linn (Rutaceae) was placed in a corning jar; 300 ml of water was added and soaked for 48 h. Protein fractions were isolated from the aqueous extract using ammonium sulphate precipitation from 0-20, 20-40, 40-60 and 60-80 % saturation, followed by centrifugation at 10000 x g for 10 min at 4 °C. The supernatants were dialyzed with 10 mM phosphate buffer, pH 7.4 and the resultant protein fractions designated as AMP I, AMP II, AMP III, and AMP IV were subjected to antifungal, antioxidant and cytotoxicity studies.

Protein concentration determination

The protein contents of the aqueous extract and dialyzed samples of the leaves were determined with Bradford method [6] using bovine serum albumin (BSA) as standard.

Evaluation of antifungal activity

Disc-diffusion method

Antifungal activity of the aqueous extract of *A. monophylla* Linn (Rutacea) fractionated with ammonium sulphate precipitation was tested using the diffusion method according to Bauer *et al* [7]. The fungal strains were maintained on potato dextrose agar (PDA) medium (Hi-Media). Sterile discs of Whatmann No.1 filter paper of about 6 mm diameter were impregnated on the surface with the media. Different concentrations of both aqueous and protein fractions were prepared and applied on the discs and incubated for 48–72 h at 28 °C. The results were recorded by measuring the inhibitory zone around the discs.

Micro-broth dilution method

Evaluation of the susceptibility of *Candida albicans* and *Aspergillus fumigatus* species were performed using the micro broth dilution method according to M27-A2 for yeast guidelines [8]. Yeast strains were grown aerobically overnight at 35 °C on Sabouraud dextrose agar plates. Yeasts were harvested and suspended in 1 % sterile saline and the turbidity of the supernatants measured with a spectrophotometer at 625 nm with an absorbance of 0.08 - 0.10 equivalent to no. 0.5 McFarland standard following the NCCLS M27-A2 guidelines. The working suspension was diluted 1:20 in a mixture containing RPMI 1640

medium and 0.165 M morpholinepropanesulfonic acid buffered to pH 7.0. The working suspension was further diluted with the medium (1:50) to obtain the final test inoculums, (1.0 – 5.0) x 10^3 CFU ml^{-1}. The microtiter plates were allowed to thaw and equilibrated to room temperature under aseptic conditions which contained different concentrations of the test extracts. Aliquots of working inoculum suspensions were dispensed into each well and the plates incubated in an aerobic environment at 35 °C for 24 h. After incubation, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-18sulfophenyl)-2H-terazolium salt (MTS, Promega Corporation, Madison, USA) was added directly to each well, incubated at 37 ºC for 4 h and the absorbance recorded at 490 nm on a 96-well plate reader (Vacutec). All analyses were performed in triplicate and data are reported as the mean ± standard error of the mean.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli and Favre [9]. It was carried out on Bio-Rad gels composed of stacking gel (5 % w/v) using 1.0 M Tris-glycine buffer containing 0.4 % SDS at pH 6.8 and resolving gel (12 % w/v) using 1.5 M Tris-glycine buffer containing 0.4 % SDS at pH 8.8. Protein sample was dissolved in phosphate buffer (5 mg/ml) and mixed with a solubilization buffer Tris – HCl 6.22 mµ (pH 6.8) which contains 2 % w/v) SDS, 50 % glycerol, a pinch of bromophenol blue and reduced with 0.9 mµ 2-mercaptoethanol in boiling water for 3 min. Protein sample was loaded onto each well and electrophoresis (Bio-Rad electrophoresis apparatus, Bio-Rad Laboratories, Hercules, CA) was conducted at constant current of 60 volts by a Bio-Rad electrophoresis constant power supply unit (Model 200/2, Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA). After electrophoresis, gels were stained with CBB. Molecular masses were determined using the molecular weight standard kit from GENEI, Bangalore, India.

Assessment of *in vitro* antioxidant activity

DPPH free radical scavenging activity

Free radical scavenging activity was determined using the 2, 2′-diphenly-1-picrylhydrazyl (DPPH) method of Burits and Bucar [10]. One ml of various concentrations of the extract in methanol was added to 4 ml of 0.004 % methanol solution of DPPH. After a 30-min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The inhibition of free radicals by the extract in percentage terms (I %) was calculated using equation 1.

$$I\ \% = \{(Ac - As)/Ac\} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

where Ac is the absorbance of the control reaction (containing all reagents except the test compound), and As is the absorbance of the reaction mixture containing the test compound.

Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity was carried out according to Gulcin et al. [11]. Tris-HCl buffer (1.5 ml, pH 8.0, 32 mM), 0.5 ml of nitroblue tetrazolium (NBT, 300 µM), 0.5 ml of reduced nicotinamide adenine dinucleotide (NADH) solution of 468 µM and different concentrations of extract (10 - 250 µg/ml) were taken. The reaction was initiated by the addition of 1 mL phenozine methosulphate (PMS) (30 µM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and absorbance was measured at 560 nm. Decreased absorbance of the reaction mixture indicates the increased superoxide anion scavenging activity. Ascorbic acid was used as a standard and inhibition calculated.

H_2O_2 scavenging activity

H_2O_2 scavenging activity was determined according to the method of Ruch *et al* [12]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentrations of both plant extracts in 3.4 ml phosphate buffer were added to a H_2O_2 solution (0.6 mL, 40 mM). The absorbance of the reaction mixture was recorded at 230 nm. Percent inhibition was calculated.

ABTS radical scavenging activity

The antioxidant activity was determined with stable 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS+) cation radical method of Re *et al* [13]. ABTS (2 mM) was prepared by dissolving in 50 ml of phosphate buffered saline (pH 7.4). ABTS+ was produced by reacting 50 ml of stock solution with 200 µl of 70 mM potassium persulfate ($K_2S_2O_8$) water solution. The mixture was left to stand in the dark at room temperature for 15-16 h before use. For the evaluation of antioxidant activity, the ABTS+ solution was diluted with PBS to obtain the absorbency of 0.8 ± 0.03 at 734 nm. Different concentrations of both the extracts were prepared and mixed with 3 ml of ABTS solution. The absorbance was read at room temperature after 10 min at 734 nm.

PBS solution was used as a blank sample. The percentage of inhibition was calculated.

Cytotoxicity assay

Vero cell lines were maintained in RPMI 1640 medium supplemented with 10 % FBS, 1 mM sodium pyruvate and were grown in an incubator at 37 ºC, 5 % CO_2 in a humidified atmosphere. Aqueous extract and protein fraction (AMP III) were dissolved in water and its final concentration was made to 2 % in each well. The confluent cell culture was trypsinized and the cell count was adjusted to 5.4 x 10^5 cells/ml. To each well of the 96-well microtiter plate, 0.1 ml of the diluted cell suspension (25000 cells/well) was added and kept for 24 h in an incubator at 37 ºC in 5 % CO_2 atmosphere for cell monolayer formation. After 24 h, the media was flicked off, washed and 100 µl of different concentrations of the compounds were added to the cells in microtiter plates. The plates were then incubated at 37 ºC for 24 h in 5 % CO_2 atmosphere. After incubation, 20 µl of MTS solution was added to each well and incubated for 3-4 h and OD was read at 490 nm. Cells without compounds served as blank and cadmium as the positive control. The experiment was carried out in triplicates.

Statistical analysis

Data reported were mean ± standard error of the mean. The statistical analysis was carried out using one-way analysis of variance (ANOVA) using Graphpad Instat version 3.0 software.

RESULTS

Antifungal activity

The aqueous extract and protein fraction (AMP III) isolated from the leaves of *Atlantia monophylla* Linn (Rutaceae) leaves exhibited antifungal activity on *C. albicans* and *A. fumigatus*. Using disc diffusion method, *C. albicans* exhibited 19 mm of inhibitory zone at 500 µg/ml concentration whereas *A. fumigatus* exhibited 11 mm at the same concentration. AMP III exhibited more antifungal activity than the crude aqueous extract. The aqueous extract showed minimum inhibition concentration on *C. albicans* at 125 and on *A. fumigatus* at > 200 µg/mL using micro broth dilution method. Out of four fractions (designated as AMP I, AMP II, AMP III and AMP IV) collected from ammonium sulphate protein precipitation method, only AMP III exhibited antifungal activity. AMP I, II, and IV did not exhibit activity on both strains of fungi (Table 1). AMP III fraction exhibited 22 mm inhibitory zone on *C. albicans* and 16 mm on *A. fumigatus*.

SDS-PAGE was performed to identify the proteins in fraction III. Two protein bands were identified at 16 KDa and 67 KDa (Fig 1). The protein concentration was determined by using Bradford assay and found to be 240 and 670 µg/mL in crude extract and AMP III.

Antioxidant activity

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of 2, 2′-diphenyl-1- picryl hydrazyl (DPPH). At 250 µg/mL concentration the aqueous extract of *Atlantia monophylla* Linn (Rutaceae) exhibited 79 % of DPPH radical scavenging activity and AMP III exhibited 90 % (Table 2). Superoxide scavenging activity of both aqueous extract and AMP III were evaluated and the data obtained are presented in the Table 2. At 250 µg/ml concentration, the aqueous extract of *A. monophylla* Linn (Rutaceae) exhibited 69 % and fraction III exhibited 81 %. At 250 µg/ml concentration, the inhibition of H_2O_2 scavenging activity of the aqueous extract of *A. monophylla* Linn (Rutaceae) was 82 % and AMP III exhibited 91 % as shown in Table 2.

Table 1: Antifungal activity and MIC of aqueous extract and AMP III fraction of *A. monophylla* Linn (Rutaceae) leaves

Extract/fraction	*C. albicans*			*A. fumigatus*		
	250*	500*	MIC*	250*	500*	MIC*
Aqueous extract	10.37±0.23	19.28±0.12	125.11±0.11	8.19±0.17	11.15±0.11	>200.07±0.08
Fraction-I	-	-	-	-	-	-
Fraction-II	-	-	-	-	-	-
Fraction-III	16.22±0.07	22.18±0.08	50.20±0.01	12.13±0.06	16.24±0.011	90.06±0.12
Fraction-IV	-	-	-	-	-	-
Control (Ketconazole)	nt	nt	12.52±0.12	nt	nt	30.54±0.23

*Data reported are mean ± standard error of mean (n = 3); * expressed in µg/ml; 'nt' = not tested; '–' = no activity*

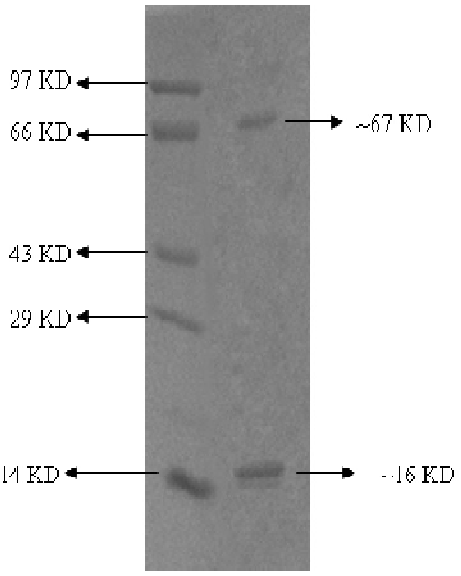

Fig 1: SDS-PAGE of AMP III of *A. monphylla* Linn

The inhibition of ABTS scavenging activity was found to be 79 % in the aqueous extract of *A. monophylla* Linn (Rutaceae) and 94 % in AMP III at 250 µg/ml concentrations (Table 2). Cytotoxicity was found to be greater than 100 µg/ml (IC_{50}) for both the aqueous and AMP fraction III when tested on Vero cell lines. Compared to the ethanol extracts of *A. monophylla* Linn (Rutaceae) reported by Reddy *et al* [14], the aqueous and protein fraction exhibited greater activity in both antifungal and antioxidant activities.

DISCUSSION

From the results it is evident that the crude extract and the protein fraction (AMP III) extracted from the leaves of *A. monophylla* Linn (Rutaceae) possess good antifungal and antioxidant activities. With the rise in the incidence of opportunistic fungal infections, attempts are being made to develop new chemotherapeutic agents for treatment and management. Medicinal plants possess a mixture of compounds of known therapeutic properties [15]. Natural peptides/proteins or their analogs have been found with varying activities against pathogenic fungi with potential pharmaceutical utility [16]. Holetz *et al* [17] classified the potential of plant extracts and fractions based on MIC as potent (< 100 µg/ml), moderate (ranging from 100-500 µg/ml), weak (500 - 1000 µg/ml) and inactive (> 1000 µg/ml). From the results it is evident that the crude aqueous extract is moderately active (ranges from 125 - 250 µg/ml) and the AMP III fraction is very active (50 - 90 µg/ml) on fungal strains as shown in Table 1.

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm.

Table 2: *In vitro* antioxidant activities of the aqueous extract and AMP III protein fraction extracted from the leaves of *A. monophylla* Linn

DPPH scavenging activity					
	25	***50***	***75***	***100***	***250***
Ascorbic acid	47.16±0.12	81.21±0.18	88.14±.0.25	91.18±0.15	95.22±0.29
BHT	30.10±0.31	39.15±.0.27	51.24±0.39	58.09±0.37	67.18±0.26
Aqueous extract	20.21±0.27	36.32±0.35	58.25±0.31	64.19±0.20	79.15±0.36
Fraction III	36.11±0.22	47.16±0..31	69.26±0.37	78.35±0.37	90.14±0.28
H_2O_2					
	25	***50***	***75***	***100***	***250***
BHT	29.45±0.45	30.24±0.38	35.34±0.39	40.22±0.42	47.28±0.54
Aqueous extract	33.32±0.39	45.15±0.49	57.32±0.33	64.22±0.42	82.35±0.45
Fraction III	54.26±0.32	63.28±0.45	78.19±0.41	84.37±0.39	91.31±0.56
Superoxide					
	25	***50***	***75***	***100***	***250***
BHT	27.06±0.65	48.12±0.71	58.15±0.75	76.09±0.55	85.11±0.67
Aqueous extract	15.16±0.73	28.12±0.56	39.16±0.67	57.15±0.75	69.18±0.65
Fraction III	31.19±0.57	59.14±0.65	65.18±0.76	74.13±0.62	81.21±0.59
ABTS					
	25	***50***	***75***	***100***	***250***
BHT	68.21±0.28	81.19±0.15	92.42±0.24	94.26±0.21	95.18±0.32
Aqueous extract	52.11±0.21	61.24±0.29	69.33±0.34	74.24±0.24	79.17±0.21
Fraction III	59.18±0.36	67.11±0.24	76.38±0.22	89.22±0.30	94.19±0.22

Sanchez-Moreno *et al* [18] classified the kinetic behavior of antioxidants as follows: < 5 min (rapid), 5 - 30 min (intermediate) and > 30 min (slow). Based on this kinetics, both the crude aqueous and AMP III fraction exhibited radical scavenging activity differing in percentage activity. It is well known that superoxide anions damage biomolecules directly or indirectly by forming H_2O_2, .OH, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation [19].

Protein fraction III partially purified from *A. monophylla* Linn leaves exhibited greater scavenging activity than the crude extract. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects [11]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

ABTS is a relatively stable free radical which involves the direct generation of ABTS radical mono cation without any involvement of intermediary cation. Here, the radical cation is formed prior to addition of the antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant. This method, used for the screening of antioxidant activity, is applicable to both lipophilic and hydrophilic antioxidants [20]. The free radicals were scavenged in a concentration-dependent manner.

CONCLUSION

From the results it is evident that both the crude aqueous and protein fraction extracted from the leaves of *Atlantia monophylla* Linn possess both antifungal and antioxidant activities. Individual proteins from the protein fraction AMP III thus need to be isolated, purified, characterized and further evaluated for *in vitro* and *in vivo* antifungal and antioxidant activity.

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