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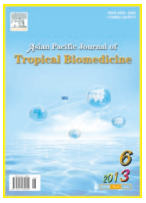

Document heading doi:

In vitro antioxidant, cytotoxic, thrombolytic activities and phytochemical evaluation of methanol extract of the *A. philippense* L. leaves

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PEER REVIEW

Peer reviewer

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Comments

It is a good work where the authors determine the antioxidant, thrombolytic and cytotoxic activities of methanol extract of leaves of *Adiantum philippense* L. The results are interesting and it has been found that the extract shows effective antioxidant and thrombolytic activities.

Details on Page 468

ABSTRACT

Objective: To study the leaves of *Adiantum philippense* L. for their antioxidant, cytotoxicity and thrombolytic activities and to perform phytochemical evaluation. **Methods:** *In-vitro* antioxidant activity of extract was studied using DPPH radical scavenging, reducing power, total phenol and total flavonoid content determination assays. The cytotoxic activity was determined using brine shrimp lethality bioassay, thrombolytic activity by clot disruption and phytochemical potential by qualitative analysis. **Results:** The antioxidant activity of the extracts was found promising. The reducing power of this crude extract increase with the increase of concentration; IC_{50} values of DPPH scavenging activity was (140.00±0.86) μg/mL as compared to ascorbic acid [IC_{50} (130.00±0.76) μg/mL]; Total phenol and total flavonoids content were (148.26±0.24) mg/mL and (163.06±0.56) mg/mL respectively. In cytotoxicity assay the LC_{50} values of the sample was (106.41±0.78) μg/mL where as for standard vincristin sulphate was (08.50±0.24) μg/mL as a positive control and the extract shows (12.86±1.02)% clot lytic whereas standard streptokinase shows (30.86±0.44% clot lytic activity in thrombolytic assay. The phytochemical evaluation indicates the presence of chemical constituents including carbohydrates, alkaloids, saponins, glycosides, flavonoids. **Conclusions:** This study shows that the methanol extract of leaves of *Adiantum philippense* L. has bioactivity but further compound isolation is necessary to confirm the activities of individual compounds.

KEYWORDS

Adiantum philippense L., Antioxidant, Cytotoxicity, Phytochemical evaluation

1. Introduction

The free radicals are the culprit for not only in support of aging but also many age-related diseases[1]. Free radical damage within cells has been linked to a range of disorders including cancer, arthritis, atherosclerosis, Alzheimer's disease, and diabetes[2,3]. There has been some evidence to suggest that free radicals and some reactive nitrogen species trigger and increase cell death mechanisms within the body such as apoptosis and in extreme cases necrosis[4]. Targeted antioxidants may lead to better medicinal effects such as mitochondria-targeted ubiquinone, for example, may prevent damage to the liver caused by excessive alcohol[5]. Some reviews suggest that antioxidant could reduce side effects or increase survival times[6].

Portal vein thrombosis is a form of venous thrombosis affecting the hepatic portal vein, which can lead to portal hypertension and a reduction of the blood supply

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Foundation Project: Supported by Department of Pharmacy, International Islamic University Chittagong, Bangladesh. Grant No. Pharm-97/06-2012.

Article history:
Received 25 Mar 2013
Received in revised form 29 Mar, 2nd revised form 4 Apr, 3rd revised form 18 Apr 2013
Accepted 23 May 2013
Available online 28 Jun 2013

to the liver[7]. It usually has a pathological cause such as pancreatitis, cirrhosis, diverticulitis or cholangiocarcinoma and may cause death. In the UK, the Parliamentary Health Committee revealed that the annual rate of death due to thrombosis was 25 000[8]. The oral anticoagulants are derived from coumarin, which is found in many plants like warfarin, heparin is used to treat patients with deep-vein thrombosis, pulmonary embolism (PE), atrial fibrillation and mechanical prosthetic heart valves[9,10]. Heparin also used as a prophylaxis which may decrease the risk of PE and deep vein thrombosis.

Adiantum philippense L. (*A. philippense*) (Family-Adiantaceae) locally known as Goyalelata, is a small fern. Stipes 10-15 cm long, tufted, naked, wiry, polished dark chestnut-brown; fronds 15-30 cm long and 7.6 cm broad, simply pinnate, often elongated and rooting at the apex; pinnae subdimidiate, the lower edge nearly in a line or oblique with the petiole, the upper edge rounded.

The plant is cooling, alterative, useful in dysentery, ulcers, erysipelas, burning sensation and epileptic fits. Roots are good for strangury and for fever due to elephantiasis. It is also used for the treatment of febrile affections in children; the leaves are rubbed with water and given with sugar. It mainly contains triterpene, 3,4-epoxyfilicares, flavonoids, adiantone and other related compounds[11]. It is distributed in districts of Bangladesh like Chittagong, Chittagong Hill Tracts, Comilla, Cox's Bazar, and also found in India, Malawi, Mozambique, Zimbabwe and Zambia. The previous study of this plant shows that it has antidiabetic activity[13], the whole plant is used as a medicine for bronchitis and cough[12], but according to the best of our knowledge there is not any scientific detailed report on antioxidant, cytotoxic and thrombolytic activities. So we have selected the methanol extract of leaves of *A. philippense* L. to see the antioxidant, cytotoxic, thrombolytic potentials as well as phytochemical constituents.

2. Materials and methods

2.1. Chemicals

Lyophilised streptokinase vial (1 500 000 IU) was purchased from Square Pharmaceuticals Ltd, Bangladesh. DPPH (1,1-diphenyl, 2-picryl hydrazyl) trichloroacetic acid, ferric chloride, Gallic acid and quercetin were obtained from Sigma Chemical Co. USA. Ascorbic acid was obtained from SD Fine chem. Ltd. India and potassium ferricyanide from May and Backer, Dagenham, UK. Methanol, Folin-Ciocalteu reagent, sodium carbonate and potassium ferricyanide were purchased from Merck, Germany. All chemicals used were of analytical reagent grade.

2.2. Plant materials

Fresh leaves of *A. philippense* L. for this study were collected from the local area of Chittagong, Bangladesh and were authenticated by Dr. Sheikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Bangladesh. The collected leaves were dried at room temperature in the shade and away from direct sunlight for 5 d and were kept in hot air oven for 2 d.

2.3. Preparation of crude extract

After drying the leaves were coarsely powdered and extracted by dissolving with methanol for 7 d. The sediments were filtered and the filtrates were dried at 40 °C in a water bath. The solvent was completely removed by filtering with Hartman filter paper and obtained dried crude extract was used for experiment.

2.4. Antioxidant activity

2.4.1. DPPH radical scavenging activity

Free radical scavenging activity of the methanol extract of *A. philippense* leaves, based on the scavenging activity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Braca *et al*[14]. Crude extract (0.1 mL) was added to 3 mL of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated by using the equation: % scavenging activity=$[(A_0-A_1)/A_0]\times100$. Where A_0 is the absorbance of the control and A_1 is the absorbance of the extract. Lower the absorbance, the higher is the free radical scavenging activity[15]. The curves were prepared and the IC_{50} value was calculated using linear regression analysis.

2.4.2. Reducing power

Reducing power was determined according to the method described by Srinivas *et al*[16]. The different concentrations of extract (125, 250, 500 and 1 000 μg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide-$K_3Fe(CN)_6$ (2.5 mL, 1% w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3 000 r/min for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v) and the absorbance was measured at 700 nm. The increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference and phosphate buffer (pH 6.6) was used as blank solution.

2.4.3. Total phenol content

The total phenol content of the extract was determined

using Folin-Ciocalteu reagent using the method of Singelton *et al*[17]. Extract (200 μg/mL) was mixed with 400 μL of the Folin-Ciocalteu reagent and 1.5 mL of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 mL using distilled water and then allowed to stand for 2 h. Then the absorbance at 765 nm was taken. The concentration of total phenol content in the extract (10-200 μg/mL) was then determined as mg of gallic acid equivalent by using an equation that was obtained from the standard gallic acid graph.

2.4.4. Total flavonoids content

The total flavonoids content was determined using a method described by Kumaran *et al.* using quercetin as a reference compound[18]. A volume of 1 mL of the plant extract in methanol (200 μg/mL) was mixed with 1 mL aluminium trichloride in methanol (20 mg/mL) and a drop of acetic acid, and then diluted with methanol to 25 mL. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 mL of plant extract and a drop of acetic acid, and then diluted to 25 mL with ethanol. The total flavonoid content was determined using a standard curve of quercetin (12.5-100 μg/mL) and expressed as mg of quercetin equivalent (QE/mg of extract).

2.5. Cytotoxicity screening

The brine shrimp lethality bioassay was used to determine the cytotoxic compounds using simple zoological organism *Artemia salina* as a convenient monitor for the screening. The eggs of the brine shrimp were hatched in artificial seawater (3.8% NaCl solution) for 48 h to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using the method of Meyer *et al*. which also concurred with the method used by Hossain S *et al*[19,20]. The test sample was prepared by dissolving them in DMSO (not more than 50 μL in 5 mL solution) with sea water (3.8% NaCl in water) to attain concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL. A vial containing 50 μL DMSO diluted to 5 mL was used as a negative control. Standard vincristine sulphate was used as positive control. The matured nauplii were applied to each of all experimental vials and control vial. After 24 h, the vials were inspected using a magnifying glass and the number of surviving nauplii in each vial was counted. The percent (%) of mortality of the brine shrimp was calculated for each concentration using the formula: % Mortality=NtN0 ×100. Where, Nt=Number of killed nauplii after 24 h of incubation, N0=Number of total nauplii transferred, *i.e.* 20. The LC_{50} (median lethal concentration) was then determined using Probit analysis.

2.6. Thrombolytic activity

This test was performed according to the method described by Prasad *et al*[21]. In the commercially available lyophilised streptokinase vial (1 500 000 IU) 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock solution from which appropriate dilution was made. Five milliliter of venous blood was drawn from the healthy volunteers (n=10) without the history of oral contraceptive or anticoagulant therapy and was distributed (0.5 mL/tube) to each ten previously weighed sterile micro centrifuge tube and incubated at 37 °C for 45 min to form the clot. After the clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight. A volume of 100 μL of methanol extract (10 mg/mL) was added to each micro centrifuge tube containing pre weighed clot. As a positive control, 100 μL of streptokinase and as a negative control 100 μL of distilled water were separately added to the control tube numbered. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

2.7. Phytochemical screening

The preliminary phytochemical analysis of methanol extract of *A. philippense* L. leaves was done according to the method described by Harbrone[22]. Recently this method is also widely used[23-25].

2.8. Statistical analysis

All the results obtained by *in vitro* experiment were expressed as mean±SEM of three measurements followed by Dunnet's test where $P<0.01$ was considered as statistically significant.

3. Results

DPPH radical scavenging activity of *A. philippense* L. was found to increase with increasing concentration of the extract (Figure 1). This assay was based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The extract also displayed significant reducing power which was found to increase with the increasing concentration. During the determination of total phenol and flavonoid content (Figure 2). It was found that the amount of phenol and flavonoid were significant (Table 1). The results and methods of analysis of antioxidant activities concurred with other studies[23,25,26]. The lethality of the crude extract of *A. philippense* L. leaves to brine shrimp was determined on *Artemia salina* after 24 h of exposure the samples, the negative control and vincristine sulphate. This technique was applied for the determination

of general toxic property of the plant extract. The LC_{50} value (Figure 3) of the extract was 106.41 μg/mL and that for standard vincristine sulphate was 8.50 μg/mL. No mortality was found in the control group, using DMSO and sea water. The plant extract showed moderate clot lysis activity (12.86%) as compared to standard streptokinase's clot lysis (30.26%) activity (Figure 4). The percentage (%) of clot lysis was significant (P<0.01) when compared with control. The phytochemical screening indicates qualitative presence of carbohydrate, alkaloid, glycosides, saponins and flavonoids (Table 2).

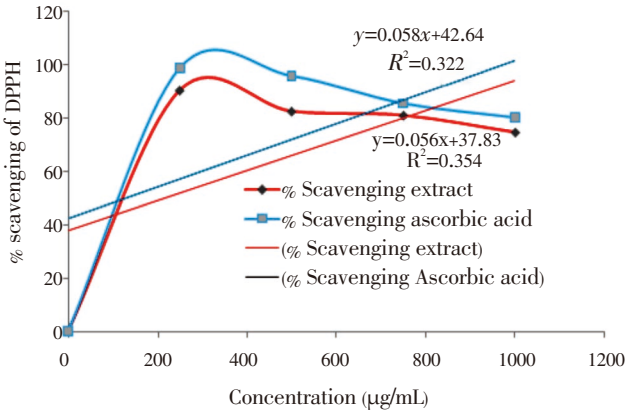

Figure 1. The free radical scavenging activity of *A. philippense* and ascorbic acid by DPPH.
Results are mean±SEM of three measurements.

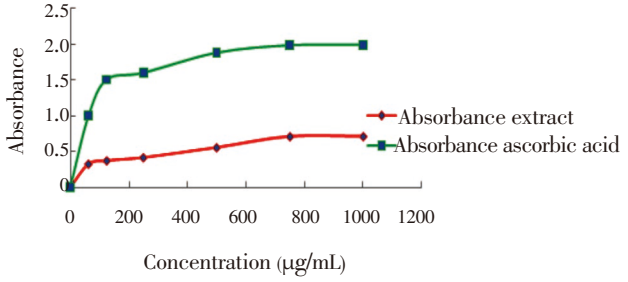

Figure 2. Reducing capacity of *A. philippense* extract.
Values are mean±SEM of three experiments.

Table 1
The total amount of phenol and flavonoid which are obtained in separate experiment have been shown.

Extract	Total phenol (mg/gm, gallic acid equivalent)	Total flavonoid (mg/gm, quercitn equivalent)
A. philippense	148.26±0.24	163.06±0.56

The results are expressed as mean±SEM of three consecutive experiments.

Table 2
Phytochemical constituents identified in the plant extracts of *A. philippense* L.

Phytochemicals	Methanol extract
Carbohydrates	(+)
Glycosides	(+)
Alkaloids	(+)
Tannins	(−)
Flavonoids	(+)
Saponins	(+)

+: indicate presence; −: indicate absence.

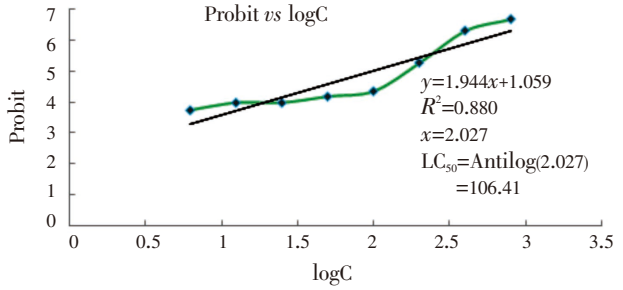

Figure 3. Toxicity assay of *A. Philippense* on brine shrimp.
The results are expressed as mean±SEM of three measurements.

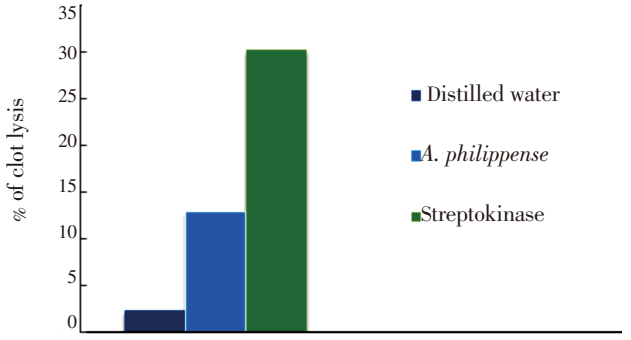

Figure 4. The clot lysis activity of *A. Philippense* extract and streptokinase.
All results are mean±SEM of three consecutive experiments.

4. Discussion

Antioxidants are helpful in reducing and preventing damage from free radical reactions because of their ability to donate electrons which neutralize the radical without forming another. Ascorbic acid, for example, can lose an electron to a free radical and remain stable itself by passing its unstable electron around the antioxidant molecule. Other antioxidant like alpha-tochopherol efficiently protects glutathione peroxidase 4-deficient cell from cell death[27]. The antioxidant chemicals found

in many foods are frequently cited as the basis of claims for the benefits of a high intake of vegetables and fruits in the diet[28,29]. In a 2010 survey of 1 000 plants, 356 had clinical trials published evaluating their "pharmacological activities and therapeutic applications" available in the Western market[30,31].

Most thrombolytic agents work by activating the enzyme plasminogen, which clears the cross-linked fibrin mesh. This makes the clot soluble and subject to further proteolysis by other enzymes, and restores blood flow over occluded blood vessels. Thus thrombolytic agents are useful for the treatment of myocardial infarction, thromboembolic strokes, deep vein thrombosis and PE to clear a blocked artery and avoid permanent damage to the perfused tissue (*e.g.* myocardium, brain, leg).

During the development of a drug the cytotoxicity should be taken into consideration. The experiment shows that the methanol extract of leaves of *A. phillippense* has considerable antioxidant and thrombolytic activities with minimum cytotoxicity. The phytochemical screening show the presence of glycosides, alkaloids, flavonoids and saponis. These compounds show these activities because the biological activities of plants may be due to the presence of these diverse group of chemical compounds[32-34]. Hence this study was conducted by crude extract, further advanced studies should be carried out for compound isolation and it is necessary to observe which compounds are actually responsible for specific effects.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are very much grateful to the authority of International Islamic University Chittagong, Bangladesh for timely providing all the research facilities and other logistic supports. Grant No. Pharm-97/06-2012.

Comments

Background

Cells are susceptible in the presence of excessive free radical which cause many many diseases like cancer, thrombosis *etc*. The scientists of the world are trying their level best to discover the suitable medicine to treat these diseases. So, it is a great interest to conduct a research on this plant to determine the potential activities like antioxidant, thrombolytic, cytotoxic activities and phytochemical evaluation.

Research frontiers

This study which includes the potential evaluation of *A. philippense* L. leaves, were collected from the local area of Bangladesh. The authors have performed antioxidant, thrombolytic, cytotoxic activities test and phytochemical screening with crude methanol extract of this plant.

Related reports

Data are available on the research of this plant. Paul T *et al*. (2011) shows it has antidiabetic activity. On the other hand it has also capability to cure bronchitis and cough (Pallay G, 2011).

Innovations and breakthroughs

This careful study showed that the methanol extract of leaves of this plant has antioxidant, thrombolytic and cytotoxic activities. The phytochemical evaluation also confirms some biologically active compounds like carbohydrates, glycosides, alkaloids, tannins, flavonoids and saponins.

Applications

This study will be great attention for isolation and characterization of new antioxidant, thrombolytic and cytotoxic agents as well as making a relation among these activities. Subsequent clinical trials will help to establish a new medicine for human use.

Peer review

It is a good work where the authors determine the antioxidant, thrombolytic and cytotoxic activities of methanol extract of leaves of *A. philippense* L. The results are interesting and it has been found that the extract shows the effective antioxidant and thrombolytic activities.

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