British Journal of Pharmaceutical Research
4(17): 2106-2115, 2014
ISSN: 2231-2919

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Evaluation of Antioxidant, Cytotoxic, Antimicrobial, Membrane Stabilizing and Thrombolytic Activities of *Polianthes tuberose* Linn

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Authors' contributions

This work was carried out in collaboration between all authors. Author FR designed the study, wrote the protocol. Author MRK wrote the first draft of the manuscript and managed the analyses of the study. Author SCD managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2014/12414
Editor(s):
(1) Vasudevan Mani, Universiti Teknologi MARA (UiTM), Selangor, Malaysia.
Reviewers:
(1) Sundus Hameed Ahmed, Radio Biology Center, Ministry of Science and Technology Baghdad, Iraq.
(2) Anonymous, National Research Center, Egypt.
(3) Sophia Wan-Pyo Hong, Department of Biology, Chungbuk National University (CBNU), Cheongju City, South Korea.
Peer review History: http://www.sciencedomain.org/review-history.php?iid=633&id=14&aid=5938

Original Research Article

Received 30th June 2014
Accepted 30th July 2014
Published 4th September 2014

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ABSTRACT

Aims: In the present study, the crude methanol extract of tuber of *Polianthes tuberosa* Linn along with its all Kupchan fractions were investigated for antioxidant, cytotoxic, antimicrobial, membrane stabilizing and thrombolytic activities.
Place and Duration of Study: The study was carried out for one year in 2012 in the Department of Pharmacy, Manarat International University (MIU), Dhaka-1216, Bangladesh.
Methodology: The antioxidant activity was evaluated by using free radical scavenging (DPPH) assay. Here, butylated hydroxytoluene (BHT) was used as standard antioxidant. The total phenolic content was also determined and expressed in gallic acid equivalent. Cytotoxicity and antimicrobial activity of the plant fractions were determined by brine shrimp lethality bioassay as well as by the disc diffusion method, respectively. The membrane stabilizing activity was assessed by hypotonic solution and heat-induced methods and was compared with standard acetyl salicylic acid (ASA).
Results: In the free radical scavenging assay, the crude methanol extraxct showed significant free radical scavenging activity with IC_{50} value 71.23 µg/ml. The highest phenolic content was found in crude methanol extract (113.49 mg of GAE/gm of extractives). In the brine shrimp lethality bioassay, both the crude methanol extract and its carbon tetrachloride soluble fraction demonstrated strong cytotoxic activity with LC_{50} value of 3.56 and 9.31 µg/ml, respectively compared to that of 0.451 µg/ml exhibited by standard vincristine sulfate (VS). In the disc diffusion antibacterial assay, all the plant samples showed mild to moderate activity (zone of inhibition = 9.0-15.0 mm) against test pathogens. In membrane stabilizing activity test, the plant samples at 2.0 mg/ml inhibited the isotonic solution-induced hemolysis of RBC by 65.23% and heat-induced hemolysis of RBC by 35.61%. During assay for thrombolytic activity, the crude methanol extract revealed 52.6% lysis of clot while standard streptokinase (SK) used as positive control, demonstrated 66.8% lysis of clot.
Conclusion: The plant possesses significant bioactivities which rationalize its use as folk medicine.

Keywords: Polianthes tuberosa; antioxidant; total phenol content; DPPH; antimicrobial; membrane stabilizing; thrombolytic.

1. INTRODUCTION

Bangladesh is a developing country and it covers a large number of poor people having no access to modern medical support. Most of them are usually dependent upon the traditional practitioners for their health problem. The use of natural products is growing in the world especially in developing countries such as Bangladesh, India, China, Arabic countries and Iran. The chemical diversity of plants has made them one of the main sources for the isolation of bioactive organic compounds [1].

Polianthes tuberosa Linn (Family: *Amaryllidaceae*, Bengali name: Rajanigandha) is a flower plant distributed in hotter parts, mainly Mexico or the andes of South America. It is popular as an ornamental garden plants with beautiful blossoms. The tuberose is a night blooming plant thought to be a native of Mexico along with other species of *Polianthes*. Flowers are used in perfume industry and also diuretic and emetic activity [2]. Bulbs are used for antigonorrhoea, diuretic, emetic and for curing rashes in infant [3]. Bulb contains saponins

and sapogenins, tigogenin. Indole has been isolated from the cultivated tuberose varieties. Flowers contain an essential oil [4]. Phytochemical investigation of *P. tuberosa* led to isolation of Polianthoside B and C [5], spirostanol and furostanol glycosides [6], spirostanol pentaglycosides having cytotoxic activity against human carcinoma cells [7]. As part of our ongoing effect to study the medicinal plants of Bangladesh [8-10] we evaluated the antioxidant, cytotoxic, antimicrobial, membrane stabilizing and thrombolytic activities of *P. tuberosa* as well as to find out the logical evidence for its folk uses.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The tuber of *P. tuberosa* was collected from Jessore, Khulna in June, 2012. A voucher specimen (Accession number-37935) for this collection has been deposited in Bangladesh National Herbarium, Mirpur, Dhaka-1216. The samples were then cut into small pieces and sun dried for 7 days followed by oven drying for 24 hours at 40ºC to facilitate proper grinding.

2.1.1 Extraction and isolation

The powdered material (300 g) was soaked in 1.0 L of methanol in a large conical flask for 7 days with occasional shaking and stirring. The whole mixture was then filtered off through a cotton plug followed by Whatman filter paper no.1 and the filtrate thus obtained was concentrated by evaporation at room temperature. A portion (5.0 g) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning protocol [11], which afforded *n*-hexane (550 mg), carbon tetrachloride (900 mg), chloroform (650 mg) and aqueous (1.3 g) soluble materials.

2.2 Total Phenolics Analysis

Total phenolic content of *P. tuberosa* was measured using Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as a standard [12]. The Folin-Ciocalteu method is a relatively straight forward procedure that is useful for determining the total phenolic content of an extract [13-15]. To 0.5 ml of extract solution (2 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5 % w/v) solution were added. After 20 min of incubation at room temperature, the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolic content was quantified by calibration curve obtained from measuring the known concentrations of gallic acid (0-100 µg/ml). The phenol content of the sample was expressed as mg of GAE (gallic acid equivalent)/gm of the dried extract.

2.3 Free Radical Scavenging Activity

The free radical scavenging activity (antioxidant capacity) of the extracts was estimated on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) [16]. 2.0 ml of a methanol solution of the sample (extract/standard) at different concentrations (500 µg/mL to 0.977 µg/ml) were mixed with 3.0 ml of a DPPH (20 µg/ml) in methanol. After 30 min of reaction at room temperature in dark place, absorbance was measured at 517 nm against methanol as blank by a UV-Visible spectrophotometer (Brand name-Shimadzu, Japan). Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$(I\%) = (1 - A_{sample}/A_{blank}) \times 100$$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test material) and A_{sample} is the absorbance of the sample. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted with inhibition percentage against extractive/standard concentration.

2.4 Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay of *P. tuberosa* [17-18] was applied for the determination of general toxic property of the plant extracts. DMSO solutions of the samples were applied against *Artemia salina* in a 24 hours *In vivo* assay. For the experiment, 4.0 mg of each of the *n*-hexane, carbon tetrachloride and chloroform soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution technique using DMSO. Vincristine sulfate was used as positive control.

2.5 Antimicrobial Activity

The preliminary antibacterial activity of *P. tuberosa* was determined by the disc diffusion method [19] against a number of Gram positive and Gram negative bacteria (Table 2) using the concentration of 400 µg/disc of extract. The bacterial strains used in this experiment were collected as pure cultures from the Biomedical Research Centre (BRC), Faculty of Pharmacy, University of Dhaka. Kanamycin (30 µg) disc was used as the reference standard.

2.6 Membrane Stabilizing Activity

The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane [20]. Therefore, as membrane stabilizes, it interferes with the release and/or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc. The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced mice erythrocyte haemolysis [21]. To prepare the erythrocyte suspension, whole blood was obtained using syringes (containing anticoagulant EDTA) from mice through cardiac puncture. The blood was centrifuged and blood cells were washed three times with 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g.

2.6.1 Hypotonic solution-induced haemolysis

The test samples consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (2.0 mg/ml) or acetyl salicylic acid, ASA (0.1 mg/ml). The control sample consisted of 0.5 ml of RBCs mixed with hypotonic buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation-

$$\% \text{ Inhibition of haemolysis} = 100 \times (OD_1 - OD_2/OD_1),$$

where, OD_1 = optical density of hypotonic-buffered saline solution alone (control) and OD_2 = optical density of test sample in hypotonic solution.

2.6.2 Heat-induced haemolysis

Isotonic buffer containing aliquots (5 ml) of the different extractives were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µl) was added to each tube and mixed gently by inversion. A pair of the tubes was incubated at 54ºC for 20 min in a water bath. The other pair was maintained at 0-5ºC in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of haemolysis in tests was calculated according to the equation:

$$\%\text{Inhibition of haemolysis} = 100 \times [1 - (OD_2 - OD_1 / OD_3 - OD_1)]$$

where, OD_1 = optical density of unheated test sample, OD_2 = optical density of heated test sample and OD_3 = optical density of heated control sample.

2.7 Thrombolytic Activity

The thrombolytic activity of all extractives was evaluated using streptokinase as standard [22]. The plant extracts (100 mg) were suspended in 10 ml of distilled water and then kept overnight. Then the soluble supernatant was decanted and filtered. Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in five different pre weighed sterile micro centrifuge tube (1 ml/tube) and incubated at 37ºC for 45 min. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone).

To each micro-centrifuge tube containing pre-weighed clot, 100 µl aqueous solutions of different partitionates along with the crude extract was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control tubes. All the tubes were then incubated at 37ºC for 90 min and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The differences in weights taken before and after clot lysis were expressed as percentage of clot lysis as shown below:

$$\%\text{ of clot lysis} = (\text{wt of released clot} / \text{clot wt}) \times 100$$

2.8 Statistical Analysis

Results are expressed as the mean ± SEM. Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnett's multiple comparisons. The results obtained were compared with the vehicle control group; p=0.05 was considered as statistical significant.

3. RESULTS AND DISCUSSION

The present study was undertaken to evaluate the antioxidant, cytotoxic, antimicrobial, membrane stabilizing and thrombolytic activities of the organic soluble materials of a methanol extract of *Polianthes tuberosa* Linn and the results have been summarized in (Tables 1-3).

The amount of total phenolic content differs in different extractives and ranged from 42.37 to 113.49 mg of GAE/gm of extractives of *P. tuberose* (Table 1). Among all extractives, the highest phenolic content was found in crude methanol extract (113.49 mg of GAE/gm of extractives). The hexane and chloroform soluble fraction have significant amount of phenolic compounds. The crude methanol extract along with its different Kupchan partitionates *i.e.* hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates were subjected to free radical scavenging activity by the method of Brand-Williams et al. [16]. In this investigation, crude methanol extract and its hexane soluble fraction showed significant free radical scavenging activity with IC_{50} value 71.23 and 84.27 µg/ml, respectively as compared to IC_{50} value 27.50 µg/ml exhibited by standard BHT (Table 1).

The crude methanol extract of tubers and it's different partitionates *i.e.* hexane, carbon tetrachloride, chloroform and aqueous soluble fraction were also tested for brine shrimp lethality bioassay. The lethality of the extractives to brine shrimp was determined and the results are given in (Table 1). The lethal concentration LC_{50} of the test samples after 24 hr was obtained by a plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. Among all the samples, both crude methanol extract and its carbon tetrachloride soluble fraction demonstrated strong cytotoxic activity with LC_{50} value of 3.56 and 9.31 µg/ml, respectively compared to that of 0.451 µg/ml exhibited by standard vincristine sulfate. The significant cytotoxic activity by the sample suggests the presence of spirostanol pentaglycosides like compounds in the plant extract are active against human carcinoma cells.

Table 1. Total phenolic content, free radical scavenging activity (IC_{50} µg/ml) and cytotoxic activity (LC_{50} µg/ml) of different Kupchan fractions of *P. tuberosa*

Sample	Total phenolic content (mg of GAE/gm of dried extract)	Free radical scavenging activity (IC_{50} µg/ml)	Cytotoxic activity (LC_{50} µg/ml)
BHT	ND	27.50±0.23	ND
VS	ND	ND	0.451±1.21
CME	113.49±1.13	71.23±0.78	3.56±0.58
HSF	86.23±0.96	84.27±1.14	32.26±1.29
CTSF	42.37±1.18	112.05±0.67	9.31±1.16
CSF	58.18±1.07	93.82±0.64	18.65±0.63
AQSF	45.19±0.85	128.79±0.97	16.23±0.76

Here, BHT= Butylated hydroxy toluene, VS = Vincristine sulfate, ND = Not determined; CME = Crude methanol extract; HSF = n-hexane soluble fraction; CTSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = aqueous soluble fraction of the methanol extract of P. tuberose

The plant samples were subjected to antimicrobial screening with a concentration of 400 µg/disc in every case. All the test samples showed mild to moderate antimicrobial activities against most of the organisms used in the assay (Zone of inhibition = 9.0-15.0 mm)

(Table 2). Among the all samples, the chloroform soluble fraction exhibited moderate antimicrobial activity against *Bacilllus megaterium* (15.0 mm), *B. cereus* (14 mm), *Sarcina lutea* (14 mm). It is well recognized that infectious diseases report for high percentage of health problems, especially in the developing countries. Microorganism has developed resistance to several antibiotics and this has produced vast clinical problem in the management of infectious diseases [23]. This resistance has increased due to unsystematic use of commercial antimicrobial, antibiotics drugs commonly used in the treatment of infectious diseases. This situation enforced scientists to explore for new antimicrobial substances from diverse sources, such as medicinal plants [23,24]. In the present investigation the results for zone of inhibition for extracts of *P. tuberosa* were found to be moderate against *Bacilllus megaterium*, *B. cereus*, *Sarcina lutea, Salmonella paratyphi*, *Vibrio mimicus* (Table 2) which evaluates the traditional folk medicines by modern methods which are currently available for evaluation of natural products.

Table 2. Antibacterial activity of *P. tuberosa* extractives at 400 µg/disc

Test microorganisms	Diameter of zone of inhibition (mm)					
	CME	HSF	CTSF	CSF	AQSF	Ciprofloxacin
Gram positive bacteria						
Bacillus cereus	10	9	11	14	9	42
B. megaterium	10	12	11	15	10	42
B. subtilis	12	12	9	10	10	41
Staphylococcus aureus	11	11	9	14	11	42
Sarcina lutea	11	13	14	12	9	42
Gram negative bacteria						
Escherichia coli	9	10	9	12	9	41
Pseudomonas aeruginosa	12	10	13	12	12	41
Salmonella paratyphi	11	9	14	10	11	42
Salmonella typhi	11	13	10	12	11	48
Shigella boydii	9	13	11	13	9	42
Shigella dysenteriae	10	9	11	10	10	42
Vibrio mimicus	14	10	10	13	10	41
V. parahemolyticus	13	10	10	14	9	42

CME = Crude methanol extract; HSF = n-hexane soluble fraction; CTSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = aqueous soluble fraction of the methanol extract of P. tuberose

The methanol extract and its different partitionates at concentration 2.0 mg/ml were tested to know the activity against lysis of human erythrocyte membrane induced by hypotonic solution as well as heat, as compared to the standard acetyl salicylic acid (0.10 mg/ml) (Table 3). At 2.0 mg/ml in hypotonic solution induced condition the methanol extract inhibited 65.23% while chloroform and hexane soluble fraction inhibited 46.34% and 39.27% haemolysis of RBC as compared to 71.9% revealed by acetyl salicylic acid (0.10 mg/ml), respectively. The aqueous and carbon tetrachloride soluble fraction showed mild to moderate inhibition of haemolysis of RBCs. During heat-induced condition the crude methanol extract also demonstrated highest 35.61% haemolysis of RBC. The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. Therefore, as membrane stabilizes that interfere in the release and or action of mediators like histamine, serotonin, prostaglandins, leukotrienes etc. [20-21].

In order to identify the drugs with the ability to promote lysis of blood clot from natural sources, the extractives of *P. tuberosa* were assessed for thrombolytic activity. Addition of 100 µl streptokinase (SK), a positive control (30,000 I.U.) to the clots of human blood and subsequent incubation for 90 minutes at 37ºC, showed 66.8% lysis of clot. On the other hand, distilled water when treated as negative control, showed negligible lysis of clot (3.62%). The mean difference in percentage of clot lysis between positive and negative control was found to be statistically significant. In the study of thrombolytic activity, the crude methanol extract exhibited significant thrombolytic activity (52.6%). Although the precise mechanism of this membrane stabilization is yet to be elucidated, it is thought that the plant may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation.

Table 3. Membrane stabilizing and thrombolytic activities of extractives of *P. tuberosa*

Sample	Concentration (mg/ml)	Haemolysis inhibition (%)		Thrombolytic activity (% Clot lysis)
		Hypotonic solution induced	Heat induced	
Hypotonic medium	50 mM	- -	- -	ND
CME	2.0 mg/ml	65.23	35.61	52.6±1.16
HSF	2.0 mg/ml	39.27	32.40	22.3±1.14
CTSF	2.0 mg/ml	24.42	17.19	37.8±0.72
CSF	2.0 mg/ml	46.35	29.56	12.34±1.05
AQSF	2.0 mg/ml	18.85	9.68	20.61±1.16
ASA	0.1 mg/ml	71.9	42.12	20.24±0.42

ASA= Acetyl salicylic acid; CME = Crude methanol extract; HSF = n-hexane soluble fraction; CTSF = carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = aqueous soluble fraction of the methanol extract of P. tuberosa

4. CONCLUSION

Currently there has been an increased interest worldwide to identify bioactive compounds from natural sources which are pharmacologically potent and have small or no side effects for use in protective medicine. The present study demonstrates that secondary metabolites with biological properties such as antioxidant, cytotoxic, antimicrobial, membrane stabilizing and thrombolytic activities are present in this plant. This could provide a rationale for traditional uses of this plant and suggests for further investigation and isolation of biologically active constituents responsible for the activity.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=633&id=14&aid=5938