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Original Research Article

In vitro Cytotoxic and Antioxidant Activity of Leaf Extracts of Mangrove Plant, *Phoenix paludosa* Roxb

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Abstract

Purpose: *To investigate the anti-proliferative and antioxidant potentials of four different solvent extracts of Phoenix paludosa Roxb leaves.*
Methods: *Four different solvent (hexane, chloroform, ethyl acetate and methanol) leaf extracts of the plant were tested for cytotoxicity against four cancer cells, viz, MCF-7 (oestrogen positive breast cancer cell line), MDA-MB-231 (triple negative breast cancer cell line), SK-BR-3 (breast adenocarcinoma) and ACHN (renal adenocarcinoma) as well as two normal cell lines, namely, HEK-293 (embryonic kidney cells) and MCF-10A (normal mammary epithelial cells)]. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and 2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging assays were used to evaluate the antioxidant activity of the crude extracts.*
Results: *The methanol extract showed the highest antioxidant activity (DPPH, half maximal inhibitory concentration (IC_{50}) = 30.17 ± 6.21 µg/mL) and (ABTS, IC_{50} = 27.91 ± 3.21 µg/mL). Of the four extracts, methanol extract showed the strongest significant ($p < 0.05$) cytotoxicity to all four cancer cell lines at 24 and 48 h of incubation followed by the chloroform extract (IC_{50} of methanol extract (24 and 48 h): 36.71 ± 8.72 and 33.19 ± 5.53 µg/mL (MCF-7), 159.7 ± 32.09 and 141.9 ± 26.2 µg/mL (MDA-MB-231), 103.3 ± 18.9 and 75.39 ± 19.39 µg/mL (SKBR-3), 57.21 ± 3.72 and 43.16 ± 10.25 µg/mL (MCF-10A), 37.48 ± 5.75 and 26.99 ± 1.85 (ACHN) and 66.83 ± 14.26 and 60.34 ± 10.66 µg/mL (HEK-293)). Furthermore, the methanol extract was least cytotoxic to normal cell lines.*
Conclusion: *The results obtained indicate that the methanol leaf extract of P. paludosa exhibit potent antioxidant and cytotoxic activities and has the potential of being developed into an anti-cancer agent.*

Keywords: *Phoenix paludosa, antiproliferative, antioxidant, cytotoxicity*

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INTRODUCTION

Cancer is a dreadful disease affecting both developed and developing countries and is now a very serious health problem. Chemotherapy, radiotherapy, hormone therapy, immune therapy and surgery have shown limited success in the treatment of cancers [1]. Therefore, it is imperative to search for additional anti-cancer drugs in the treatment and management of cancers.

Plants play an important role in the development of anti-cancer drugs. Vincristine, vinblastine and paclitaxel are well known examples of clinically useful anti-cancer drugs derived from plants [2].

The discovery of new plant derived anticancer agents is a long term process and it contains several steps. Steps involved in the discovery of new anticancer agent from plants include cytotoxicity screening of plant extracts, bio-activity guided isolation of active compounds with anticancer properties, in-vitro testing, toxicity assessment of anticancer compounds and ultimately in vivo testing [3]. Free radical scavenging properties of different plant extracts are also of great importance because natural compounds with free radical scavenging activity can protect from damages due to free radicals and reactive oxygen species (ROS) in biological systems [4].

Phoenix paludosa, also known as Mangrove date palm (family: Aracaceae), is a flowering plant found in Sri Lanka, India, Bangladesh, Myanmar, Thailand and Malaysia. These trees are thorny and grow in clusters, to 6-7 m high and leaves are 2 to 3 m long [5]. This plant has been used as an antipyretic and an anti-inflammatory agent in Bangladesh [6]. A previous study has demonstrated that *P. paludosa* shoot has quinonereductase induction activity [7]. However, data available on possible cytotoxic activity or free radical scavenging activity of organic extracts of aerial parts of this mangrove plant are limited. Thus, the aim of this study was evaluate the cytotoxic and free radical scavenging activities of organic solvent extracts of the aerial parts of this plant.

EXPERIMENTAL

Chemicals, cell lines and cell culture reagents

2,2'-Diphenyl-1-picrylhydrazyl (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox), Folin-Ciocalteau's phenol reagent, 2,2'-Azino-bis(3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), gallic acid, sodium carbonate, quercetin, aluminium chloride, potassium persulphate, insulin, streptomycin and penicillin were purchased from Sigma-Aldrich (St. Louis, MO). All the chemicals used in this study were of analytical grade. Human MCF-7, oestrogen positive breast cancer cells (ATCC, HTB-22TM); MDA-MB-231, triple negative breast cancer (ATCC, HTB-26TM); SK-BR-3, breast adenocarcinoma (ATCC® HTB-30™); MCF-10A, normal mammary epithelial (ATCC, CRL-10317TM); ACHN, renal adenocarcinoma (ATCC® CRL-1611™); HEK-293 embryonic kidney (ATCC® CRL-1573™), cell lines and fetal bovine serum (30-2020 TM) were purchased from American type cell culture (ATCC), Manassas, USA.

Collection of plant material

Healthy, fresh leaves of *Phoenix paludosa* (Family: Aracaceae) were collected from mangrove park, Kadolkele, Negombo in the Western Province of Sri Lanka (Lat. 7°11'54.03" Long. 79°50'30.94") during April, 2013 and authenticated by a taxonomist, A.M.A.S. Attanayaka of National Herbarium, Department of National Botanic Gardens, Peradeniya, Sri Lanka. A voucher specimen (no: S-10) was deposited in the herbarium of Institute of Biochemistry, Molecular biology and Biotechnology, University of Colombo, Sri Lanka.

Extraction of plant material

The collected leaves were brought to the laboratory, washed thoroughly under running tap water in order to remove dirt, and other contaminants. Leaves were then oven dried at 40 °C to reduce the moisture content and ground thoroughly into a powder. Pulverised sample (500 g) was subjected to cold sequential extraction using different organic solvents (i.e., hexane, chloroform, ethyl acetate and methanol). The liquid extracts were evaporated using Rotavapor® R-3(BUCHI) and stored at -20 °C for further analysis.

Determination of total phenolic content

Total phenolic content in the extracts were determined by the modified Folin-Ciocalteu method as described earlier [8]. An aliquot (100 µL) of the extracts/standard (diluted in distilled water) was mixed with 5 mL Folin- Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL (10 %) of sodium carbonate. The tubes were vortexed for 15 s and allowed to stand for 60 min at 37 °C for colour development. A standard curve was prepared using gallic acid with a concentration range from 0.5 to 25 µg/mL. Absorbance was recorded against reagent blank at 765 nm using the Synergy™ HT Multi-Mode Microplate Reader. Total phenolic content was expressed as mg/g gallic acid equivalents.

Determination of total flavonoids content

Total flavonoid content was determined using the Dowd method as adapted by Meda *et al* [9]. A volume of 5 mL of 2 % $AlCl_3$ methanol solution was added to 1 mL of sample diluted in methanol. After 1 h at room temperature, the absorbance was measured at 415 nm against the blank using the Synergy™ HT Multi-Mode Microplate Reader. A yellow colour indicated the presence of flavonoids in the extracts. Total

flavonoid content was calculated as quercetin equivalent (mg/g).

Determination of antioxidant activity

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and ABTS (2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging assays were used for the evaluation of the antioxidant activity of the crude extracts. Trolox (1.0 - 5.0 µg/mL) was used as the positive reference for both assays.

DPPH radical scavenging activity assay

DPPH free radical scavenging assay was carried out as previously described by Mothana *et al* [10]. The dried crude hexane, chloroform, ethyl acetate and methanol extracts were diluted in methanol. The reaction mixture, containing 100 µL of the desired extract concentration in methanol, 25 µL of 1 mM DPPH and 75 µL of methanol were added into a 96-well plate and incubated at 37 °C for 30 min. Decrease of absorbance was monitored at 517 nm using the Microplate Reader.

ABTS (2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay

The ABTS free radical-scavenging activity of each extract was determined according to the method described by Re *et al* [11]. Working solution of ABTS was prepared by mixing a 7 mM ABTS stock solution with a 2.4 mM potassium persulphate stock solution in equal quantities and allowing the mixture to stand in the dark at room temperature for 5 h. The mixture was then diluted with phosphate buffered saline (PBS, pH 7.4) and absorbance measured, at 734 nm. Each sample (diluted with PBS) was mixed with the solution of the radical cation $ABTS^{\bullet+}$ and the decrease of absorbance was measured at 734 nm after 10 min. Trolox (1.1, 1.7, 2.3, 2.9, 3.5 µg/mL) was used as positive reference.

DPPH/ABTS free radical-scavenging activity (F) was calculated as in Eq 1.

$$F\ (\%) = \{(A_0 - (A_1 - A_2)/(A_0)\}100 \quad \ldots\ldots\ldots\ldots\ldots\ (1)$$

where A_0, A_1 and A_2 are the absorbance of the DPPH/ABTS (without the crude extract), crude extract with DPPH/ABTS and the crude extract without DPPH/ABTS, respectively.

Each measurement was in triplicate, and mean and standard deviation were calculated. Linear regression was used to observe the performance to indicate the relationship between the total phenolic content, flavonoid content and data from the antioxidant assays.

Cell culture and cytotoxicity assays

In vitro cytotoxic activity of the crude extracts was determined using sulphorhodamine (SRB) cytotoxic assay as previously described by Samarakoon *et al* [12]. Four human cancer cell lines (SKBR-3, MCF-7, MDA-MB-231, ACHN) and two non-carcinoma cell lines (MCF-10A and HEK-293) were used to assess cytotoxicity. Cells were cultured in a humidified environment (37 ºC, 95 % air; 5 % CO_2) to confluence in complete culture medium according to the manufacturer's instructions. Triple negative breast cancer cells were cultured without CO_2. Cells trypsinized and seeded ($5x10^3$ cells/well) in 96-well cell culture plates were exposed to different concentrations of leaf extracts (25, 50, 100, 200 and 400 µg/mL) for 24 and 48 h time intervals. Paclitaxel was used as the positive control.

At the end of the incubation periods cells were fixed with 50 µL of ice-cold 50 % trichloroacetic acid solution by gently adding on top of the medium overlaying the cells. The plates were then incubated for 60 min at 4 ºC. Wells were then rinsed five times with tap water and stained with 0.4 % SRB solution (100 µL stain/well) for 15 min at room temperature. After staining, unbound dye was removed by washing five times with 1 % acetic acid solution and left to air dry. The bound SRB dye was then solubilized by adding unbuffered Tris-base solution (200 µL/well), and the plates were placed on a plate shaker for 1 h at room temperature. Plates were then read at a wavelength of 540 nm using a microplate reader and the results expressed as percent of control values

Statistical analysis

All the experiments in the study were carried out for at least three times in triplicate and results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out with the help of the Graph Pad Prism (V5.0, Graphpad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by multiple comparisons by Tukey's test was used to assess IC_{50} values and cytotoxicity data. $P < 0.05$ was considered as statistically significant.

RESULTS

Total polyphenol and flavonoid content and DPPH/ABTS radical scavenging activity

The results of total phenolic content, total flavonoids content and the antioxidant activity of *P. paludosa* extracts are shown in Table 1 and Figures 1 and 2. The methanol extract had the highest content of phenolic compounds as well as the best antioxidant activity in both DPPH and ABTS assays, whereas chloroform extract showed highest content of flavonoids.

Cytotoxic effects of extracts

Cytotoxic effect of the four organic extracts of *P. paludosa* in four cancer cell lines and two normal cell lines are given in Table 2. Of the four solvent extracts tested chloroform and ethyl acetate extracts showed less cytotoxicity to cancer cells and more cytotoxicity to normal cells whereas methanol extract showed a higher cytotoxicity to cancer cells and less cytotoxicity to normal cells. Hexane extract did not show significant cytotoxicity to either cancer or normal cells.

Table 1: DPPH/ABTS radical scavenging activity of different extracts of *P. paludosa*

Extract	DPPH (IC_{50}, µg/mL)	ABTS (IC_{50}, µg/mL)
HE	440.3 ± 13.21	403.4 ± 46.1
CE	1439.9 ± 93.7	402.3 ± 13.39
EAE	101.3 ± 16.34	177.6 ± 19.81
ME	30.17 ± 6.21	27.91 ± 3.21
Positive reference	4.319 ± 1.398	2.902 ± 1.628

Hexane extract (HE), chlorofrom extract (CE), ethyl acetate extract (EAE), methanol extract (ME); positive reference for DPPH and ABTS =Trolox

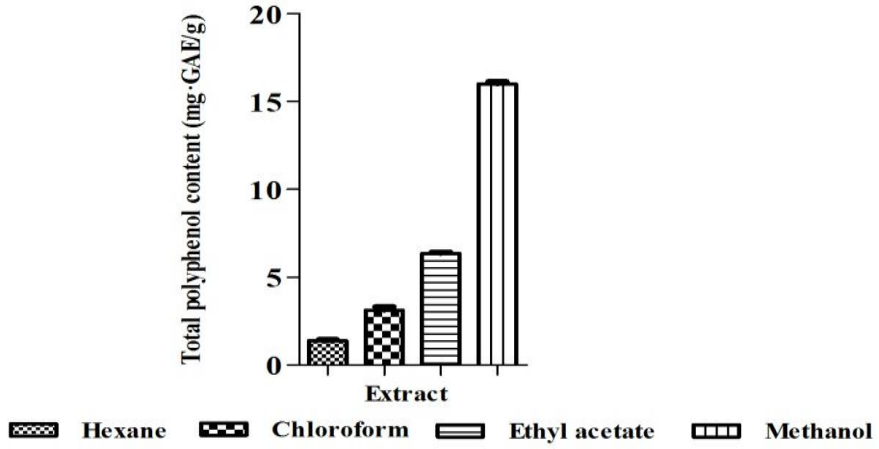

Figure 1: Total flavonoid content of *P. paludosa* leaf extracts

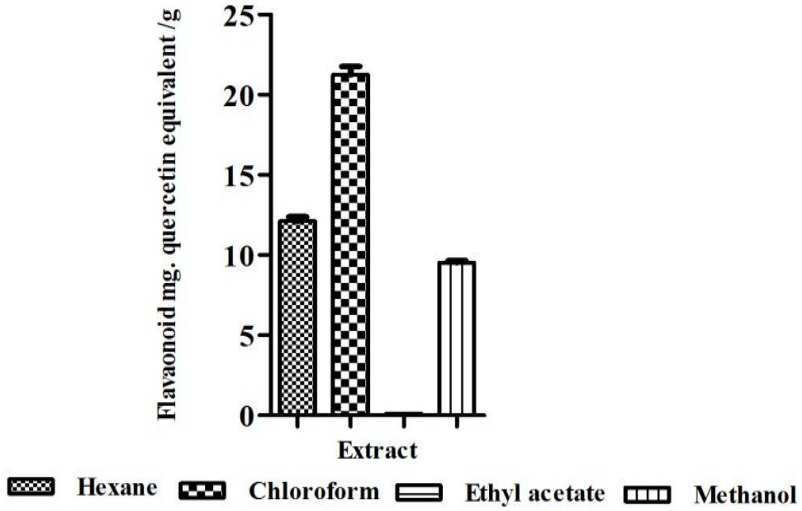

Figure 2: Total polyphenol content of *P. paludosa* leaf extracts

Table 2: Cytotoxic effects of four different leaf extracts of *P. paludosa*

Cell culture	Hexane (HE)		Chloroform (CE)		Ethyl acetate (EAE)		Methanol (ME)		Paclitaxel	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MCF -7	369.2 ± 76.7	319.9 ± 54.5	>500	>500	487.2 ± 102.9	465.2 ± 151.2	36.71 ± 8.72	33.19 ± 5.53	4.754 ± 1.103	3.827 ± 0.437
MDA-MB-231	>500	>500	>500	>500	>500	>500	159.7 ± 32.09	141.9 ± 26.2	3.442 ± 0.989	2.918 ± 0.748
SK-BR-3	325.4 ± 136.5	>500	>500	>500	>500	>500	103.3 ± 18.9	75.39 ± 19.39	7.583 ± 2.886	4.266 ± 1.469
MCF-10A	>500	>500	>500	>500	>500	293.2 ± 37.6	57.21 ± 3.72	43.16 ± 10.25	16.25 ± 6.65	12.6 ± 2.45
ACHN	>500	442.9 ± 67	321 ± 56	280.3 ± 61	132 ± 10.9	122.7 ± 9.6	37.48 ± 5.75	26.99 ± 1.85	10.53 ± 2.844	4.019 ± 0.709
HEK-293	>500	>500	114.7 ± 17.35	101.6 ± 33.7	99.89 ± 27.59	90.6 ± 18.88	66.83 ± 14.26	60.34 ± 10.66	12.25 ± 3.424	6.039 ± 1.942

Note: Hexane extract (HE), chlorofrom extract (CE), ethyl acetate extract (EAE), methanol extract (ME)**;** paclitaxel = positive control; results are expressed as the mean ± SD (n =3)

DISCUSSION

Different plant extracts exhibit different cytotoxic properties to different cancer cell lines which may be due to the presence of different cytotoxic phytochemicals and their different mode of action to different cancer cell lines [13]. Despite available reports on anti-oxidant, analgesic, anti-tumor, as a growth effector and anti-diarrheal properties [14] of this plant, this is the first comparative study of this plant on cytotoxicities in four different cancer cell lines and two normal cell lines. The strong cytotoxic potential was observed in the methanolic extract to all four cancer cell lines at 24 and 48 h incubations followed by the chloroform and ethyl acetate extracts. The methanol extract showed a higher cytotoxicity to MCF-7 (oestrogen receptor positive) breast cancer cells than to the other two breast cancer cells (MDA-MB-231 (triple negative) and SKBR-3 (Her 2 negative), possibly due to the presence of some phytochemicals which may selectively inhibit the growth of oestrogen receptor positive breast cancer cells. Interestingly, methanol extract was less cytotoxic to human embryonic kidney (HEK-293) cells and normal mammary epithelial cells (MCF-10A) compared to renal adenocarcinoma (ACHN) and human breast cancer cells (MCF-7), respectively. Lupeol, epilupeol, and β-sitosterol which are reported to be cytotoxic compounds to cancer cells [15, 16] have been previously isolated from the hexane and the carbon tetrachloride soluble fraction of the methanol extract of the leaves of *P. paludosa* by other investigators [17].

The comparable results obtained from the two methods of free radical scavenging activity assessment in this study suggest that methanol extract of *P. paludosa* contains significantly high free radical scavenging activity. This observation strongly supports our results as the methanol extract contain the highest amount of polyphenols out of the four organic leaf extracts. There is a strong correlation between polyphenols and free radical scavenging potential of plant species due to the scavenging capability and hydrogen donating ability of hydroxyl groups of polyphenols. Flavonoids also act as scavengers of various “reactive oxygen species (ROS)” and quenchers of singlet oxygen [18]. Our results are fairly in close agreement with the findings of Saha *et al* [19] and Patra *et al* [20] who reported the anti-oxidant activity of ethanol and methanol leaf extracts of this plant, respectively. Results from cytotoxic studies and radical scavenging abilities strongly support that methanolic extract of this mangrove plant can be considered as a source for novel cytotoxic compounds, thus necessitating further studies to isolate active compounds and investigations to ascertain molecular mechanisms of action of the active compounds.

CONCLUSION

Cytotoxic potential of *Phoenix paludosa* Roxb leaves in various cancer cell lines indicate that leaf extracts of this mangrove contain phytochemical constituents which can be developed as anti-cancer agents.

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