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Anticancer Studies of Aqueous Extract of Roots and Leaves of *Pandanus Odoratissimus* f. *ferreus* (Y. Kimura) Hatus: An *In Vitro* Approach

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ABSTRACT

A number of medicinal plant extracts are being used against various diseases in different systems of medicine such as Ayurveda, Unani, and Siddha, but only a few of them have been scientifically explored. The objective of the present study was to explore the dose-dependent *in vitro* anticancer effects of the extracts of *Pandanus odoratissimus* whose scientific documentation as an anticancer agent is lacking despite being used traditionally. The dried parts of roots and leaves were extracted with methanol (MEPO) and water (AEPO). The extracts were then subjected to *in vitro* cytotoxic and antimitotic screening by brine shrimp lethality assay and onion root tip method, respectively. Further, the behavior of the extracts on calu-6 (non-small cell lung cancer cell lines), PBMC (peripheral blood mononuclear cells) and WI (lung fibroblast cell lines) was studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay followed by flow cytometric analysis on calu-6 cell lines. AEPO showed significant cytotoxic and antimitotic activities. It showed 100% lethality of brine shrimps at 80 µg/ml and an LC_{50} of 33.33 µg/ml, which was eightfold higher than that of synthetic standard podophyllotoxin (4.16 µg/ml). AEPO at 10 mg/ml concentration showed significant antimitotic activity by showing 3% mitotic index. which was more than that of standard cyclophosphamide with 4% mitotic index in comparison to control. There was a significant reduction in cell proliferation of calu-6 cells, ranging from 56 to 35%, after 24-48 h of treatment with 200 µg/ml ($P < 0.001$) of AEPO, while AEPO remained unaffected on PBMC and WI-38 cel lines. Cell cycle analysis revealed that AEPO at 50 µg/ml and 100 µg/ml significantly increased the number of cells in sub G0–G1 phase, indicating the cells entering in to apoptotic phase. These results suggest that aqueous extract of *P. odoratissimus* possesses better anticancer activity. The plant has the potential to be used in anticancer therapy, and this study scientifically validated the folklore use of this plant.

Key words: Anticancer, Antiproliferation, Cytotoxic, *Pandanus odoratissimus*

INTRODUCTION

There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional systems of medicine. *Pandanus odoratissimus* f. *ferreus* (Pandanaceae) is one such plant distributed commonly throughout India. In Ayurveda, Unani, and Siddha systems of medicine, the leaves are used for treating backache, rheumatic diseases, epilepsy, wound healing, nervous

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disorders, loss of appetite, indigestion, constipation, diabetes, infertility, skin diseases, urinary disorders, and fever.[1] The plant is known to possess a broad spectrum of medicinal, pharmacologic, and therapeutic properties. Tribals believe that this herb is an effective remedy for a wide range of illnesses.[2] Leaves of *Pandanus* plants contain alkaloids such as pandanamine and pandamerilactones with pyrroline-derived structures as the major chemical constituent, and were found to possess antioxidant, anti-inflammatory, and antidiabetic activities.[3-5] Further, the ethanolic extract from *Pandanus amaryllifolius* showed selective cytotoxicity against different human colon, cervical, hepatocellular, and breast cancer cell lines.[6] In Ayurveda, a paste of *P. odoratissimus* with sugar is used for treating cancers.[7] The pharmacognostic and phytochemical investigation was carried out in the leaves of *P. odoratis simus*.[8] The phytochemical constituents present in the *P. odoratissimus* methanol extract were reported to be steroids, saponins, terpenoids, glycosides, tannins, and flavonoids.[9] Active principles of the extract of whole plant are 3-(4-(dimethylamino) cinnamoyyl)-4-hydroxycomarin, 3,3′-methylenebis (4-hydroxycomarin), erythro-9,10-dihydroxyoctadecanoic acid, octadecanedioic acid, and dihydroagathic acid.[10] Scientific evidence in support of the antitumor activity of *P. odoratissimus* f. *ferreus* is lacking despite its use as a potential antitumor agent in traditional system. Hence, it was decided to illustrate the ethnobotanical uses of the plant, and this study aimed to evaluate the dose-dependent cytotoxic, antimitotic, antiproliferative, and apoptotic effects of the methanol and aqueous extracts of *P. odoratissimus* f. *ferreus* (MEPO and AEPO, respectively).

MATERIALS AND METHODS

Chemicals

Cyclophosphamide, podophyllotoxin, doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reagent, Ficoll, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), propidium iodide (PI), fetal bovine serum (FBS) Media, Dulbecco's modified Eagle's medium (DMEM), and trypsin were obtained from Sigma Aldrich (Bangalore, India) and Himedia Ltd (Mumbai, (India)). All other chemicals and solvents were obtained from Biochem Pharmaceuticals (Ahmedabad, India) and SD Fine Chemicals (Mumbai, India) and were of analytical grade with highest purity.

Collection, authentication, and extract preparation of *P. odoratissimus* f. *ferreus* (Y. Kimura) Hatus

The plant *P. odoratissimus* f. *ferreus* used for the present study was collected from the forest near Punalur at Kollam district, Kerala during midwinter season of 2012. The plant was identified, confirmed, and authenticated by Dr. M. D. Rajanna, Professor and Head, Department of Botany (No. 3/proj/B-Garden), University of Agricultural Sciences, GKVK, Bangalore, Karnataka, India. A voucher specimen was deposited in the department for future reference.

The roots and leaves of the plant were shade dried, chopped into small pieces, and powdered by a mechanical mixer. Five hundred grams of the coarse material was extracted with two different solvents, i.e., methanol (2.5 L) and distilled water (2.5 L), separately using Soxhlet extraction apparatus. The solvents were evaporated using a rotary vacuum evaporator (YamatoRE 300, Japan) at 50ºC and dried in desiccators.[11]

Phytochemical analysis

The qualitative and the quantitative analyses of the plant's constituents were carried out by the methods described by Trease and Evans.[12]

Cytotoxicity assay

Brine shrimp lethality bioassay was performed as a cytotoxicity assay using the method of Meyer *et al*.[13] In this bioassay, the eggs of the brine shrimps, *Artemia salina* Leach, were collected and placed to hatch in a hatching chamber. After 48 h, the larvae (nauplli) from the hatched eggs were observed, collected, and transferred into test tu bes of 10 ml capacity each, containing different concentrations of the extracts. After 24 h of drug exposure, the numbers of dead shrimps were counted. Podophyllotoxin was used as reference standard.

Antimitotic assay

Antimitotic activity was performed by onion root tip method as described previously by Aprem *et al*.[14] The root tips of *Allium cepa* are generally used for studying the actions of various compounds on cell division or on chromosomes. Onions of good quality were rooted in water, and the roots were treated with different concentrations of the extracts and standard drug cyclophosphamide for 24 h. The root tips of around 2–3 mm were cut and fixed in acetic acid: Alcohol (1:3) and stained with a mixture of acetocarmine and 1 N HCl (9:1). Further, the root tips were squashed and observed under a microscope (45×) to calculate the mitotic index. Mitotic index was calculated using the following formula:

Mitotic index = number of dividing cells/number of non-dividing cells.

Antiproliferation assay

Tumor cell lines and their maintenance

The non-small cell lung cancer lines (calu-6) and normal lung fibroblast cell lines (WI-38) were obtained from National Centre for Cell Science (NCCS), Pune, and were processed and maintained in the Cellular and Molecular Biology Section of Department of Biotechnology, Gautham College of Pharmacy, Bangalore. The cell lines were grown and maintained in a humidified incubator at 37ºC in an atmosphere of 5% CO_2. DMEM supplemented with 10% FBS, penicillin, and streptomycin was used as the culture medium for these adherent cells. The medium was changed every 2 or 3 days during the experimental period.

Isolation of peripheral blood mononuclear cells

Isolation and preservation of PBMCs were carried according to the procedure described by Mallone *et al*.[15] The isolated and maintained PBMCs were subsequently used for testing the AEPO and MEPO by MTT assay.

MTT assay

Antiproliferation assay was carried out by the modified method of Lau.[16] Cell proliferation was determined using MTT assay, which reflects the normal function of mitochondria and cell viability. Briefly, calu-6, PBMC, and WI-38 cells were seeded onto flat-bottomed 96-well culture plate at a density of 5×10^4 cells/well in DMEM. After 24 h, the cells were washed and placed in a culture medium with different concentrations of MEPO and AEPO, ranging from 25 to 200 µg/ml, for 48 h and 72 h. Next, non-FBS culture medium containing 10% MTT was added to each well of a microtitre plate and the samples were then incubated for 4 h at 37ºC. After removing the culture medium, DMSO was added to each well. The absorbance was then read at 570 nm using ELISA reader. The absorbance of control cells (treated with DMSO) was considered as 100%. The percentage cell viability was calculated using the following formula:

Mean value of [(OD in control group – OD in treated group)/ OD in control group] ×100%.

Apoptotic assay

A modified method described by Lee and Zhou[17,18] was used for the evaluation of apoptotic activity. The calu-6 cells were adjusted to a density of 5×10^5 cells/ml using DMEM and added to six-well plate and incubated at 37ºC for 24 h. The cells were rinsed with phosphate-buffered saline (PBS) and cultured in the medium along with MEPO and AEPO in a concentration ranging from 25 to 100 µg/ml. After 24 h, the cells were collected by routine enzyme digestion and rinsed with PBS (pH 7.4) twice. The fixed cells were suspended in 70% ethanol at 4ºC overnight, centrifuged (1500 rpm, 5 min), and washed with PBS twice. Cells were placed in the dark with PI and RNase A in PBS at 25ºC for 30 min. Stained cells were analyzed by flow cytometry at 488 nm laser and 15 mW work rate. The percentage of apoptotic cells was determined using CellQuest and the cell cycle was analyzed by MultiCycle software program. Doxorubicin active against non-small cell lung cancer was used as the positive control.

Statistical analysis

The values were expressed as Mean ± standard error of mean (SEM). The data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism software. $P < 0.05$ were considered as statistically significant.

RESULTS

The results of phytochemical analysis showed the presence of alkaloids (1.2%), flavonoids (4.6%), glycosides (2.6%), and phenolic content (3.1%) in AEPO and alkaloids (1.7%), flavonoids (1.3%), and carbohydrates (2.8%) in MEPO.

Cytotoxicity assay

The results of cytotoxicity assay, when carried out in triplicates, revealed that AEPO and MEPO caused an increase in percentage death of shrimps in a dose-dependent manner. The LC_{50} of AEPO was found to be 33.33 µg/ml, which was eightfold higher than that of synthetic standard podophyllotoxin, i.e., 4.16 µg/ml, while MEPO showed lesser response (95.23 µg/ml) in comparison to AEPO [Figure 1].

Antimitotic activity

AEPO showed significant reduction in the mitotic index of squashed onion root tips with increase in concentration [Table 1]. Antimitotic activity was found to be significant at 10 mg/ml of AEPO (0.03 ± 0.02; $P < 0.05$) when compared with the control mitotic index (0.43 ± 0.02), and was also more than that of standard cyclophosphamide (0.04 ± 0.01; $P < 0.05$).

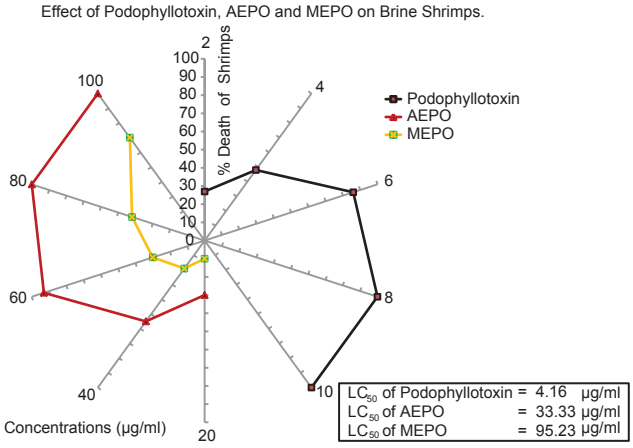

Figure 1. The LC50 of AEPO was found to be 33.33 µg/ml, which was eightfold higher than that of synthetic standard podophyllotoxin (4.16 µg/ml). Increase in percentage lethality of shrimps in a dose-dependent manner reveals that AEPO was cytotoxic to shrimps. The numbers on line tips in the figure indicate different concentrations and the scale on the lines indicates percentage death of shrimps

Table 1. Effect of cyclophosphamide, AEPO, and MEPO on the mitotic index of onion root tips

Treatment	Concentration (mg/ml)	Interphase	Cells in dividing stage (per 500 cells)				Total	Mitotic index	% Mitotic index
			Prophase	Metaphase	Anaphase	Telophase			
Normal control	-	350	75	45	20	10	150	0.43±0.02	43.00
Cyclophosphamide	5	460	20	10	5	5	40	0.08±0.03*	8.00
	10	480	9	5	2	4	20	0.04±0.01*	4.00‡
AEPO	5	450	23	14	9	4	50	0.11±0.04*	11.00
	10	485	8	2	4	1	15	0.03±0.02*	3.00‡
MEPO	5	390	50	35	10	15	110	0.28±0.07ns	28.00
	10	410	40	30	10	10	90	0.22±0.06ns	22.00

AEPO: Aqueous extract of *P. odoratissimus f. ferreus*; MEPO: Methanolic extract of *P. odoratissimus f. ferreus*

Antiproliferation assay

Antiproliferative effect of AEPO on calu-6 revealed significant reduction in cell proliferation at concentrations of 25, 50, 100, and 200 µg/ml ($P < 0.001$) after 24 and 48 h of treatment. AEPO inhibited the proliferation of calu-6 cell lines up to 44% after 24 h of treatment and up to 65% after 48 h of treatment at the highest concentration of 200 µg/ml [Figure 2]. IC_{50} of AEPO was found to be 153.84 µg/ml, while that of MEPO was found to be >200 µg/ml. Data obtained from MTT assay clearly indicate that AEPO was cytotoxic to human lung cancer cells in a dose- and time-dependent manner, and increasing the incubation time from 24 to 48 h resulted in increased toxicity. The growth rate of WI-38 cell lines was relatively high, indicating no cytotoxicity to normal lung fibroblast cell lines. Studies with PBMCs isolated from whole blood revealed that the critical components in the immune system, such as T cells, B cells and natural killer (NK) cells of lymphocytes and monocytes, remained unaffected by AEPO and MEPO after 48 h of exposure.

Apoptotic activity

Flow cytometric analysis of DNA cell cycle for calu-6 cell lines treated with AEPO showed sub G_0–G_1 phase arrest of calu-6 cell lines and the apoptosis was found to be marginal at 50 µg/ml and wide at 100 µg/ml [Figure 3f and g], thereby influencing the cells entering G_1 phase, whereas the methanol extract did not show any changes on the cell cycle. These results clearly indicate that AEPO arrested the growth of calu-6 cells and the effect increased with increase in concentration of the drug. On the other hand, doxorubicin, a chemotherapeutic anthracycline antibiotic active against S phase cells, exerted greater effects on exponentially growing cells than on resting cells of non-small cell lung cancer *in vitro* [Figure 3h].

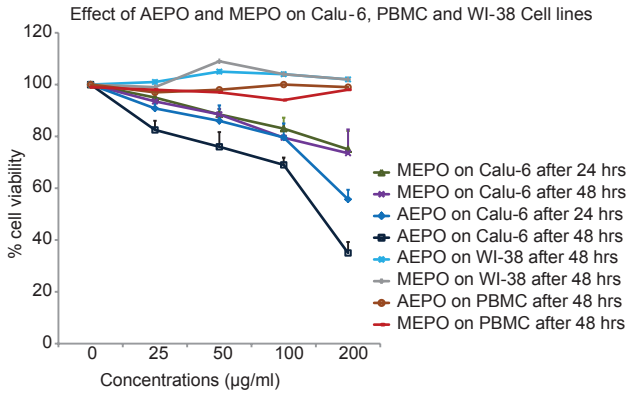

Figure 2. Antiproliferative effect of AEPO on calu-6 reveals significant reduction in cell proliferation at 25, 50, 100, and 200 µg/ml ($P < 0.001$) after 24 and 48 h of treatment, whereas the growth rate of WI-38 cell lines was relatively high, indicating no cytotoxicity to normal lung fibroblast cell lines. Studies on PBMCs isolated from whole blood reveal that the critical components of the immune system, such as T cells, B cells, and natural killer (NK) cells of lymphocytes and monocytes, remained unaffected by AEPO and MEPO after 48 h of exposure. AEPO inhibited the proliferation of calu-6 cell lines up to 65% at the highest concentration of 200 µg/ml. IC50 of AEPO was found to be 153.84 µg/ml, while that of MEPO was found to be >200 µg/ml. Data obtained from MTT assay clearly indicate that AEPO was cytotoxic to human lung cancer cells in a dose- and time-dependent manner. Values are Mean ± SEM (of triplicates from two independent experiments) analyzed by one-way ANOVA followed by Dunnett's test

DISCUSSION

The discoveries of plant-based anticancer agents have led to significant interest in evaluating various other natural products for their efficacy. Phytochemicals like flavonoids and polyphenols have been reported to act as anticancer agents by regulation of signal transduction pathways of cell growth and proliferation, suppression of oncogenes and tumor formation, induction of apoptosis, modulation of enzyme activity related to detoxification, oxidation, and reduction, stimulation of the immune system and DNA repair, and regulation of hormone metabolism.[19] Polyphenols have a protective role in carcinogenesis, inflammation, atherosclerosis, and thrombosis, and have high antioxidant capacity.[20] The data obtained in this study show that AEPO contains rich flavonoids and phenols which might contribute to its anticancer effects. AEPO inhibited proliferation of calu-6 cell lines without affecting normal lung fibroblast cells (WI-38) and PBMCs, proving its cytotoxicity to only human lung cancer cells in a dose- and time-dependent manner. Effect of AEPO and MEPO on PBMCs remained unaffected, indicating their safety on immune cells like T cells, B cells, and NK cells. The cytotoxicity may be due to the release of cytochrome *c* from mitochondria, leading to apoptosis.[21] Control of the cell cycle is accomplished by the coordinated interaction of cyclins with their respective cyclin-dependent kinases (CDKs) to form active complexes and drive cells into the next phase at the appropriate time. Any disorder in the cell cycle may result in genomic instability and apoptosis. Bcl-2 family proteins are important regulators of apoptosis. The family comprises both anti-apoptotic (e.g.,, Bcl-2) and pro-apoptotic (e.g., Bax) proteins with opposing biological functions.[22] Apoptosis in cells might occur through complex mechanisms.[23] It has been suggested that apoptosis may occur by disruption of mitochondrial function and induces lysosomal damage as the fi rst target which leads to other cellular events including reactive oxygen species (ROS) production andoxidative damage,[24] lysosomal damage, lipid peroxidation, DNA strand breaks, gene expression, chromosomal aberrations, inhibition of DNA repair processes.[25] Apoptosis of calu-6 cell lines by AEPO might be due to any of the reported reasons. Onion root tip cells are in the active stage of division and can be used to study the effects of various compounds on cell division or chromosomes by the number of dividing and non-dividing cells which gives the mitotic index. Reduction in mitotic index caused by AEPO indicates inhibition of actively dividing cells. Brine shrimp lethality assay is proposed as a simple bioassay for natural product research and this procedure determines the LC_{50} values of active compounds in brine medium. Effect of AEPO on brine shrimps reveals its cytotoxic potential.

CONCLUSION

The above results suggest that the aqueous extract of roots and leaves of *P. odoratissimus* f. *ferreus* possesses cytotoxic, antimitotic, antiproliferative, and apoptotic effects. The plant has the

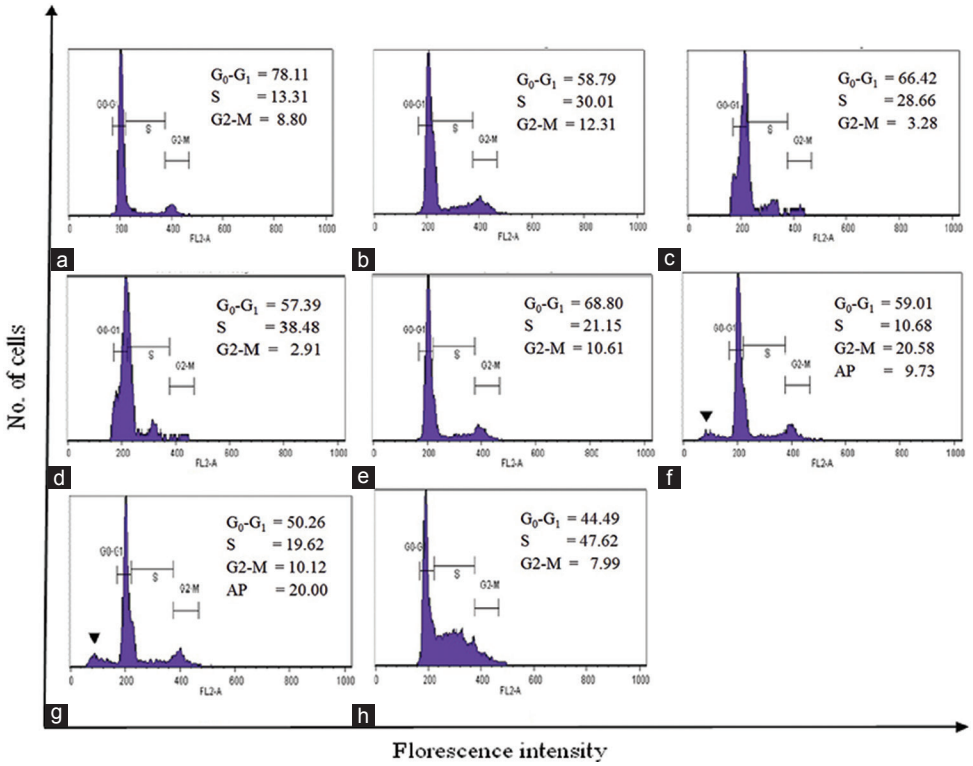

Figure 3. Flow cytometric analysis of the DNA cell cycle for calu-6 cell lines treated with AEPO showed sub G0–G1 phase arrest of calu-6 cell lines, which was found to have marginal apoptosis at 50 µg/ml and a wide range of apoptosis at 100 µg/ml (as shown in Figure 2f and g), thereby influencing the cells entering G1 phase. But the methanol extract did not show any changes in the cell cycle. These results clearly indicate that AEPO arrested calu-6 cells, which might increase its potential to arrest with increase in concentration of the drug. (a) Untreated, (b) 25 µg/ml MEPO, (c) 50 µg/ml MEPO, (d) 100 µg/ml MEPO, (e) 25 µg/ml AEPO, (f) 50 µg/ml AEPO, (g) 100 µg/ml AEPO, (h) 25 µg/ml doxorubicin

potential to be used in cancer therapy, and this study scientifically validated the traditional use of this plant.

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