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A study on the anti-oxidant and anti-cancer activity of *Pleiogynium solandri (Benth.) Engl.* against ovarian cancer cells (SK-OV-3)

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ABSTRACT

The Present study was focused on evaluating the Antioxidant and cytotoxic potentials of methanolic extract of different parts of *Pleiogynium solandri (Benth.) Engl*. The GC/MS analysis of the *Pleiogynium solandri* leaves revealed the presence of seven Bioactive compounds proven to have significant therapeutic effects including Antioxidant, Antimicrobial, Anti-inflammatory and anti-tumorigenic effects inducing apoptosis in ovarian and Breast cancer cells. The *Pleiogynium solandri leaf* was found to be more potent than bark and fruit regarding its cytotoxic effectiveness against Ovarian cancer cells (SK-OV-3). The Bark extract was found to have a significant antioxidant effect as compared to Leaf and Fruit. The FACS (Fluorescence-activated cell sorting) studies using a Flow cytometer gave cell populations at different stages of the cell cycle affected by test concentrations with propidium iodide as a dye. The FACS analysis showed cell cycle arrest at the 'S' phase of the cell cycle due to the influence of crude drugs.

Keywords: Pleiogynium solandri (Benth.) Engl, GC-MS Analysis, SKOV3, Flow cytometry, cytotoxic assay

I. INTRODUCTION

Cancer is one of the prominent diseases concerning the world. According to the World Health Organization, 10 million deaths were reported due to cancers in the year 2020, nearly one in six deaths. Each year, approximately 4,00,000 children develop cancer. In a recent analysis stated in the Lancet Journal, the cases of cervical cancer globally estimated that 604 127 cervical cancer cases and 341 831 deaths occurred in 2020[1].

In a normal cell division, cells grow, multiply and undergo apoptosis. But, when this orderly process is interrupted by the influence of harmful substances from the environment such as Ultraviolet rays, High

energy radiations, chemicals such as arsenic, beryllium, vinyl chloride etc. They cause mutations in DNA leading to cancers. Cancers can also be of genetic origin [2].

Despite the fact that there are many existing drugs such as Doxorubicin and cisplatin used in the treatment of cancers presently. They are reported to have massive side effects such as congestive Heart failure, alopecia, anorexia, cardiac toxicity, tinnitus, and myocardial infection [3][4][5].

In perpetual efforts to bring in a reasonable and effective solution to this problem, the scientific community is focused on bringing plant-based methods to bring a cure to this disease.

Pleiogynium solandri (Benth.) Engl. is a semi–deciduous tree belonging to the family Anacardiaceae, also known as the Cashew family. This tree is native to Australia, Malesia and Pacific islands. It is also cultivated in Egypt, for its use as an ornamental plant. In some regions of the world, the fruit is edible and used in preparations of Jams and jellies [6]. the plant possesses a wide range of biological activities including antioxidant, anti-inflammatory, anti-microbial, and hypolipidemic activities. Phytoconstituents like phenols, flavonoids, and anthraquinones are believed to be responsible for these activities. Some of the reported compounds across different parts of Pleiogynium solandri were Apigenin, catechin, gallic acid, Coumaric acid, taxifolin, and chlorogenic acid [7][8][9][10].

This study was focused on evaluating the Antioxidant and cytotoxic potential of different parts of *Pleiogynium solandri (Benth.) Engl.*

II. METHODS AND MATERIAL

A. Collection of Sample

The leaves, bark and fruit of *Pleiogynium solandri* (Benth.) were collected from Lal Bagh Botanical Garden, Bengaluru. The plant sample was identified and authenticated by Dr V. Rama Rao, Research Officer (Botany), Central Institute of Ayurveda Research Institute, Bengaluru. The samples were shade-dried for 3-5 days.

B. Extraction of Phytochemicals

The samples were ground into fine powder and extracted using methanol. 25g of the samples were weighed and subjected to steam-based extraction along with methanol as a solvent. The obtained extracts were filtered using Whatman No1 filter paper and used for analysis.

C. Qualitative Analysis

The preliminary qualitative screening for Phytochemical constituents was done by following protocols [11].

Test for Alkaloids

Dragendoff's test: 0.2ml of sample along with 0.2ml of HCl was added. To this 2-3 drops of Dragendoff's reagent were added and the appearance of orange or red precipitate and turbid solution indicates the presence of alkaloids.

Test for Anthraquinones

Bontrager's test: 0.2 ml of extract was taken and 0.2 ml of 5% concentrated sulphuric acid was added. The mixture was boiled in a water bath and then filtered. Filtrate was then shaken with an equal volume of chloroform and kept standing for 5 min. the bottom layer was separated and mixed with half of its volume of dilute Ammonia. The formation of rose pink to red colour at the ammoniacal layer indicates the presence of anthraquinones.

Test for Coumarins

Lacton test: 2ml of the test sample was treated with 3ml of 10% NaOH. The appearance of yellow colour indicated positive results.

Test for Carbohydrates:

Molisch's test: 0.2 ml of sample was mixed with a few drops of Molisch's reagent. 0.2 ml of sulphuric acid was added along the sides of the test tube and observed for the appearance of a purple colour ring for the positive test.

Test for Flavonoids:

Alkaline reagent test: 0.2 ml of plant extract was taken in a test tube and mixed with dilute NaOH solution. To this diluted HCL was added. Observation of a yellow solution that turns colourless later would indicate the presence of flavonoids.

Test for Glycosides:

0.2 ml of sample along with 0.2 ml of chloroform was mixed with 0.2 ml of acetic acid and the mixture was cooled on ice. Sulphuric acid was added carefully and the colour change from violet to blue to green indicates the presence of a steroidal nucleus

Test for phenols

To 0.2ml of extract 0.4ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. The formation of blue or green colour indicated the presence of flavonoids.

Test for Saponins:

Foam test: To 0.2ml of the extract was added 0.6ml of water in a test tube. The contents were shaken vigorously and observed for the formation of persistent foam indicating the presence of saponins.

Test for Steroids:

Lieberman Burchard tests: 0.2 ml of sample was mixed with 0.2 ml of chloroform. To this 0.2ml of concentrated sulphuric acid was added. The appearance of a red colour in the lower layer of chloroform indicates the presence of steroids.

Test for Tannins:

Braymer's test: 0.2 ml of plant extract was mixed with 2 ml water and heated on water bath for 10 minutes. The mixture was filtered and ferric chloride was added to the filtrate and observed for dark green solution which indicates the presence of tannins.

Test for Terpenoids:

Salkowski's test: 2ml chloroform was mixed 5mL plant extract, the contents were evaporated on water bath. After evaporation 3mL conc. H2SO4 was added and boiled on water bath. The solution turns grey colour indicating the presence of Terpenoids.

D. GC/MS analysis

The Clarus 680 GC was used in the analysis employed a fused silica column, packed with 5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250μm df) and the helium gas was used as a carrier to separate the at a constant flow of 1 ml/min. The injector's temperature was maintained at 260°C during chromatographic run. 1μL of sample extract was introduced into the instrument, the oven temperature was maintained at 60 °C (2 min); followed by 300 °C at the rate of 10 °C min–1; and 300 °C, where it was held for 6 min. The mass detector conditions were as follows; transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were analyzed by comparing with the database of spectrum of known components recorded in the GC-MS NIST library.

E. Antioxidant Assay using DPPH method

DPPH assay was carried out as per the method of Rajakumar., 1994 [12]. The test samples i.e., leaves, Bark and fruit were prepared to 1mg/ml using

methanol and diluted to different concentrations. The different concentrations of test samples were made up using HPLC grade methanol and treated with DPPH. The reaction mixture was mixed and incubated at 25°C for 15 minutes. The absorbance was measured at 510nm using spectrophotometry. A control reaction was carried out in absence of the test sample. (Standard: Quercetin)

F. Cytotoxicity assay using MTT

Cell culture: Skov3 cell line was procured from ATCC, stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100µg/mL) in a humidified atmosphere of 5% CO2 at 37oC until confluent. The cells were trypsinized and centrifuged at 1000rpm for 5 minutes. The cell pellet was gently resuspended using 2mL DMEM complete media. The viability of the cells was checked and a single cell suspension of $3.0x10^4$ cells/mL was prepared.

Treatment: To each well of the pre-labelled 96-well microtiter plate, 100 µL of the prepared cell suspension (30,000 cells/well) was added and incubated at 37°C with 5% CO2. After 48h of incubation, the supernatant was removed and the monolayer was rinsed with plain media. To each pre-designated well, 100µL of test drugs (32mg/ml) at various concentrations were added and incubated for 24 hours. After incubation, the test solutions in the wells were discarded and 100 µL of MTT reagent (5 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37° C in 5% CO2. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to solubilize the formazan crystals. The absorbance was measured at 590nm using a multimode plate reader. The percentage growth inhibition and IC50 values were calculated using GraphPad Prism 5.0 software. (Standard: Doxorubicin) [13].

G. Cell cycle studies using FACS

1 $x10^6$ cells were seeded and cultured for 24 hours in a 6-well plate containing serum-free media. Cells were then treated with desired concentrations of the samples prepared in complete media and incubated for another 24 hours. Cells were then harvested and centrifuged at 2000 rpm for 5 minutes at RT. Cell pellet was washed by resuspending in 2mL of 1XPBS twice. The supernatant was discarded retaining the pellet. Cells were fixed by resuspending in 300 µl of Sheath fluid followed by the addition of 1mL of chilled 70% Ethanol drop by drop with continuous gentle shaking and another 1mL of chilled 70% Ethanol added slowly at once. The cells were then stored at 4 °C for 30 minutes or overnight. Post-fixing, the cells were centrifuged at 2000 rpm for 5 minutes. The cell pellet was rinsed twice with 2 ml of chilled 1XPBS. The cell pellet was then resuspended in 500µl of sheath fluid containing 0.05 mg/ml PI and 0.05 mg/ml RNaseA and incubated for 20 minutes in the dark. The percentage of cells in various stages of the cell cycle in compounds treated and untreated populations were determined using FACS Caliber (BD Biosciences, San Jose, CA) [14].

III.RESULTS AND DISCUSSION

A. Extraction of Phytochemicals:

The % yield of leaf, Bark and fruit extract was found to be 12.09%, 0.72%, 12.12%. The % yield was calculated using the below formula.

$$\% \text{ yield} = \frac{\text{Weight of extract}}{\text{weight of sample}} \times 100$$

B. Phytochemical Analysis

The Qualitative Analysis of *Pleiogynium solandri* found the presence of secondary metabolites such as Flavonoids, Phenols, and tannins in all three extracts. The presence of other secondary metabolites such as Alkaloids, Anthraquinones, Coumarins and terpenoids was also found. The results are shown in **Table-1**

Table 1 – Phytochemical screening of *Pleiogynium solandri*

Name of the compound	Phytochemical test	*Pleiogynium solandri*		
		Leaf sample	Bark sample	Fruit sample
Alkaloids	Dragendroff test	-	+	+
Anthraquinones	Bontrager's test	+	-	+
Carbohydrates	Molisch test	-	+	+
Coumarins	Lacton test	+	-	+
Flavonoids	Alkaline reagent test	+	+	+
Glycosides	General test	-	-	-
Phenols	$FeCl_3$ test	+	+	+
Steroids	Liebermann-Burchard test	-	-	-
Saponins	Frothing test	+	+	-
Tannins	Ferric chloride test	+	+	+
Terpenoids	Salkowski test	-	+	+

Note: (+) Present; (-) Absent

C. GC/MS Analysis

The Gas Chromatography /Mass Spectroscopy results for Pleiogynium solandri are shown in **Table 2**. The GC/MS Analysis revealed the presence of seven bioactive compounds including n-hexadecanoic acid, cis-vaccenic acid and octadecanoic acid present in major concentrations followed by oxalic acid - ethyl neopentyl ester, Myo-inositol, 4-c-methyl-, isophytol and geranyl acetate, 2,3-epoxy-.

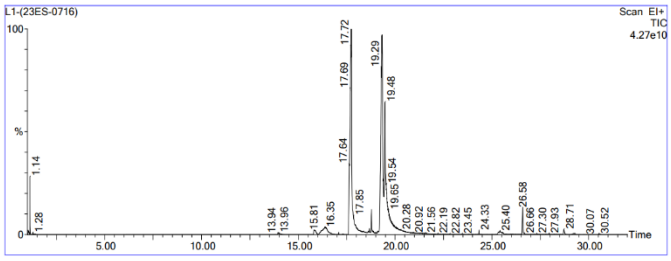

Fig 1: Chromatogram of GC/MS analysis of *Pleiogynium solandri* – Leaf

Table 2: GC/MS Analysis of *Pleiogynium solandri* – *Leaf*

SL NO	Compound name	MW (g/mol)	Formula	RT (min)	Area %	Activities	Reference
1.	oxalic acid, ethyl neopentyl ester	188.22	C9H16O4	15.809	0.962	Anti Adipogenic activity	[15]
2.	myo-inositol, 4-c-methyl-	194	C7H14O6	16.395	3.893	Antioxidant, anti-inflammatory, anti-cancer	[16] [17]
3.	n-hexadecanoic acid	256	C16H32O2	17.735	36.680	Antitumorigenic, promote tumour cell apoptosis, Influence cell cycle ovarian cancer	[18] [19]
4.	isophytol	296	C20H40O	18.775	1.191	Antimicrobial	[20][21]
5.	cis-vaccenic acid	282	C18H34O2	19.326	35.550	Anti-microbial, Anti-oxidant, Anti-cancer, hypolipidemic effects in rats	[22][23]
6.	octadecanoic acid	284	C18H36O2	19.476	19.976	Antioxidant, Induces Apoptosis in Human Breast Cancer Cells	[24][25]
7.	geranyl acetate, 2,3-epoxy-	212	C12H20O3	25.393	1.749	Anti-microbial, Anti-inflammatory, Anti-cancer	[26] [27][28]

D. Antioxidant by DPPH method

DPPH radical scavenging activity of leaf, bark and fruit extract was determined by calculating the percentage inhibition of the free radical. Half maximal Inhibitory concentration (IC50) was calculated using Graph pad Prism 5.0 software. The IC 50 values of Leaf, bark and fruit were found to be 16.85 µg/ml, 7.53 µg/ml, and 20.94 µg/ml respectively. The IC 50 value of the standard (Quercetin) was found to be 2.289 µg/ml.

The percentage inhibition was calculated using the below formula:

$$\% \text{ inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

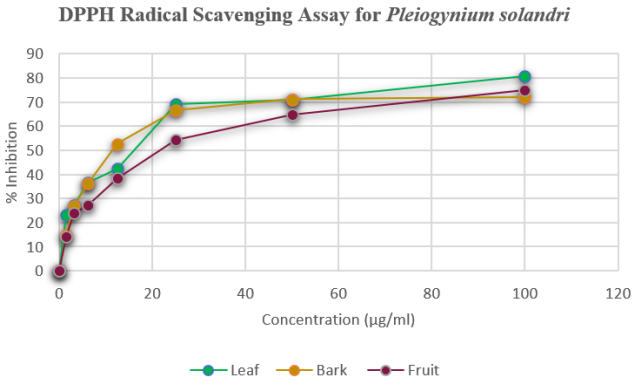

Fig 2 : DPPH radical scavenging Assay for *Pleiogynium solandri*

E. Cytotoxicity assay using MTT

The leaf, Bark, and fruit extracts of *Pleiogynium solandri* were tested for cytotoxicity against Human Ovarian cancer cell lines (SKOV3) by MTT assay. The IC_{50} values of Leaf, Bark, and Fruit were found to be 68.79 µg/ml, 140.5 µg/ml and 146.2 µg/ml respectively. (Doxorubicin - IC_{50}: 21.4 µg/ml).

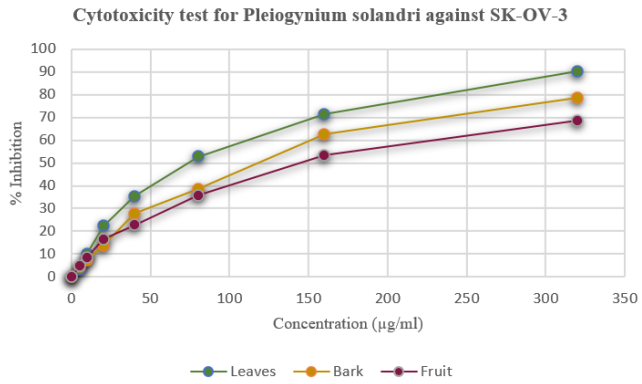

Fig 3: Cytotoxicity Test for *Pleiogynium solandri*

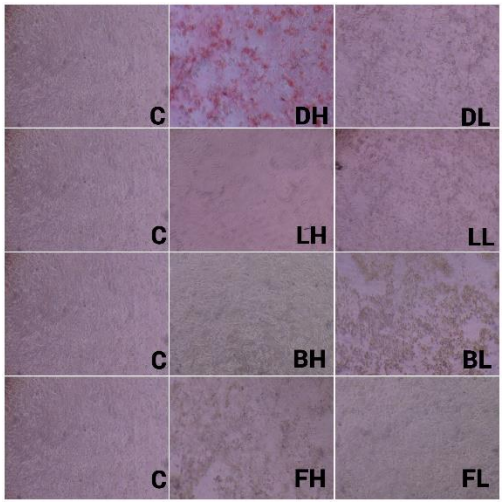

Fig 3 : Cytotoxic effects of *Pleiogynium solandri (Benth.) Engl* on Ovarian cancer cells (C – Control; **DH** – Doxorubicin Higher concentration; **DC** – Doxorubicin Lower concentration; **LH** – Leaf Higher concentration; **LL** – Leaf Lower concentration; **BH** – Bark Higher concentration; **BL** – Bark Lower concentration; **FH** – Fruit Higher concentration; **FL** – Fruit Lower concentration).

F. Cell cycle studies using FACS

The leaf sample showed a good IC50 value of 68.78µg/ml as compared to Bark and fruit in the cytotoxic assay. Hence, the Leaf sample was selected for cell cycle studies using the flow cytometric method. The leaf sample of 40µg/ml and 80µg/ml concentrations for cell cycle arrest has shown 14.32% and 25.41% of cells gated in the S phase compared to the control cells having shown 11.68% of cells gated in the same phase.

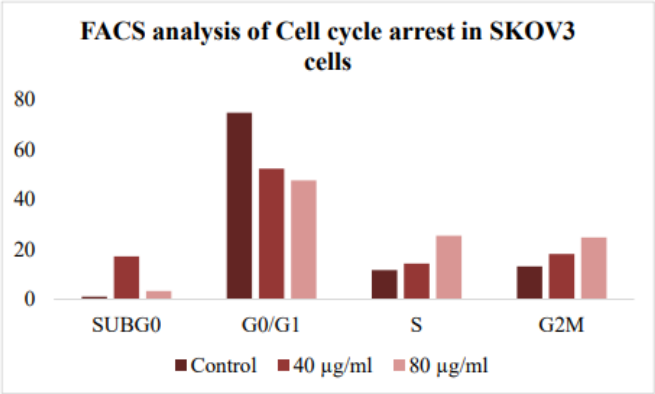

Fig 4: FACS analysis of *Pleiogynium solandri* against SKOV3 cells

IV. CONCLUSION

This study aimed to evaluate the Antioxidant and cytotoxic potentials of different parts of *Pleiogynium solandri (Benth.) Engl.* The leaf extract was found to have profound cytotoxic potential as compared to Bark and fruit with IC50 of 68.79 µg/ml while IC50 of compared Standard (Doxorubicin) remained to be 21.4 µg/ml. On the other hand, while determining the Antioxidant potential of *Pleiogynium solandri (Benth.) Engl. Bark* showed significant antioxidant potential. The GC/MS Analysis of the leaf of *Pleiogynium solandri* revealed the presence of seven Bioactive compounds that may be responsible for the potential cytotoxic effects of leaf of *Pleiogynium solandri*. Out of the seven Bioactive compounds detected, -n-hexadecanoic acid, cis-vaccenic acid and octadecanoic acid were found to be in major concentration. This analysis was further progressed for Cell cycle studies

by fluorescence-associated cell sorting method using flow cytometry. The cell populations at different stages of the cell cycle treated with different concentrations of leaf extract determined 80µg/ml of the drug to be effective in arresting the cell cycle at 'S' phase. Though *Pleiogynium solandri* is cultivated in Australia, Malesia the Pacific islands and Egypt for its various uses by locals, this plant has not received much attention from the scientific community. Hence, this can serve as source for bringing Novel plant-based drugs for many therapeutics especially in cancer research.

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