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IN-VITRO EVALUATION OF CYTOTOXIC AND APOPTOGENIC PROPERTIES OF MUCUNA PRURIENS ON MCF7 CELL LINES

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ABSTRACT

Since last two decades researchers have come up with various synthetic chemotherapeutic agents to treat breast cancer. Ironically, these synthetic agents either cause various side effects or breast cancer cells develop resistance towards them. The present study is aimed to evaluate the cytotoxic and apoptogenic properties of extract from *Mucuna pruriens* seeds on breast adenocarcinoma MCF7 cell lines. MCF7 cells treated with the different concentrations of ethanol extract of the plant sample was subjected to cytotoxic assay, hemolysis assay, G2M phase cell cycle studies and apoptotic studies. *Mucuna pruriens* seeds extract in concentration of 320µg/ml was found to induce apoptosis in 52.3% of MCF7 cells and was non-hemolytic because of which it manifest to be potential prospect to fight against breast cancer.

KEYWORDS: Adenocarcinoma, anti-neoplastic, typtamines, *Mucuna pruriens*.

INTRODUCTION

Cancer is the uncontrolled and unwanted growth of cells. From the past two decades it is major concern as it is the leading cause of highest rate of mortality. Breast cancer, a type of adenocarcinoma is not only affecting 1 in 8 US women but also1 in 1000 US men are also at risk of contracting it (Breastcancer.org, 2017). Various therapeutic strategies to combat breast cancer tumor cells are mastectomy, radiotherapy, application of chemo-therapeutic agents like cytotoxic drugs and immunotherapeutic agents. Discouragingly, these therapeutic

approaches exhibit various adverse effects on the patient. Also, prolonged usage of these drugs can induce resistance in the cancer cells.

The use of natural products as remedy to breast cancer can be promising without manifesting any side effects. Secondary metabolites from plants like poly-phenols, alkaloids, taxols, flavanoids possess cytotoxic properties. Plant extracts from *Ricinus communis* which is rich in oils, glycerides and phenolic compounds and *Vernonia amygdalina* which is rich in alkaloids and tannins have shown antiproliferative activity (Scarpa & Guerci, 1982; Yedjou, 2008). Bark and root Pfaffia paniculata which is also known as Brazilian ginseng is rich in pfaffic acid and saponins which are able to restrict the growth of breast cancer cells (Nagamine *et al.*, 2009). Today there are various phytochemical drugs that can effectively serve as an active ingredient to combat cancer.

Mucuna pruriens is a herbaceous, leguminous plant which is well known for its anti-parkinson (Katzenschlager *et al.*, 2004), anti-depressant(Oudhia, 2002) and aphrodisiac properties(Amin *et al.*, 1996). The plant commonly called as Magic Bean is twining shrub about 15 m long and possess white or purple coloured flowers with black or white coloured seeds encased in pod. Besides nutritional components, its seeds are rich in alkaloids like mucunine, mucunadine, prurienine and tryptamines like serotonin, nicotine, bufotenine(Duke, 1992). Furthermore, studies have shown that seed powder of Mucuna has capability to cure pulmonary tuberculosis, scorpion sting(Guerranti *et al.*, 2004), snake bite(Roberto *et al.*, 2008) and possess anti microbial (Sathiyanarayanan & Arulmozhi, 2007), anti-epileptic(Gupta *et al.*, 1997), anti- neoplastic(Gupta *et al.*, 1997) and uterine stimulant properties(Sridhar & Bhat, 2007).

Although this plant has been widely studied, biochemical studies of their anti-carcinogenic effects on MCF7 breast cancer cell line have not been reported. Many chemotherapeutic drugs eliminate cancer cells by inducing, a genetically programmed form of cell death. It is therefore important to establish the chemopreventive efficacy of the plant by evaluating cytotoxicity and apoptosis induction in cancer cell lines before whole animal studies or clinical trials begin. We therefore decided to screen the seed extract of *M.pruriens* for its anti-cancer activity against the human breast carcinoma MCF-7.

MATERIALS AND METHODS

Plant Material

Based on the literature survey, *Mucuna pruriens* was selected for the evaluation of cytotoxic and apoptogenic activity on MCF7 cell lines. The seeds of *Mucuna pruriens* were collected from Amruth Kesari, Bangalore.

Preparation of Plant Extract

Plant material collected was shade dried for almost 5 days and was then milled into fine powder. 10 g of each powdered material was dissolved in 50 ml of ethanol and kept on hot water bath at 50º C for 4 hours. The extract then obtained was filtered through Whatman No.1 filter paper and the filtrate was kept in water bath at 80 ºC for few hours until it get into semisolid form. 320 mg of semi-solid crude extract was dissolved in 1 ml of DMSO in an eppendorf tube to make 320 mg/ml stock solution and was kept on hot water bath at 60 ºC for 1 hour for proper dissolution of the pellet. Working concentration of the test samples i.e. 0, 10, 20, 40, 80, 160 and 320 µg/ml was prepared from the stock solution.

Cell Culture

Michigan Cancer Foundation-7 (MCF7) cell line obtained from the American Tissue Culture Collection (ATCC) was cultured in the Dulbecco's Modified Eagle's Medium (DMEM) media (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1000 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified incubator.

Thawing and Revival

Cryo-vials containing the frozen cells from liquid nitrogen storage were quickly thawed (< 1 minute) by gently swirling the vial in the 37°C water bath. Thawed cells were transferred to a sterile tube containing required amount of medium corresponding to the cell lines and inverted for uniform distribution. The cell suspension was centrifuged at 1200g for 5 minutes. Clear supernatant was checked for visibility of the complete pellet, re suspended in complete growth medium and transferred to T-25 flask under the recommended culture environment (5% CO2 at 37o C). Growth was monitored and cells were trypsinized and sub cultured once they reached a confluence of 70-80%.

Anti carcinogenic Activity of Plant Extract

MTT Assay

MCF7 cells (5.0 X 10^4) were plated in 96 well plates with different concentrations of each plant extracts made in complete DMEM media (0, 10, 20, 40, 80, 160 and 320 µg/ml) and incubated for 24 hours at 37˚C in a 5% CO_2 incubator. Next day the media was removed and 100 µl of MTT reagent was added to each well and incubated again for 4 hours. MTT reagent was removed and 100 µL DMSO was added to each well for solubilizing the formazan product. The plate was shaken well and absorbance reading was taken at 570nm using microplate reader. The percentage inhibition was determined using a formula [% Inhibition = 100-(optical density of sample/optical density of control) × 100]. IC50 values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell.

Hemolysis Assay

5ml blood was collected from the healthy person in a vial containing 5.4 mg of EDTA. Erythrocytes were collected by centrifuging the blood at 1000 rpm for 10 mins at 4°C. Plasma and white buffy layer was discarded and erythrocytes were washed with 1×PBS thrice, pH 7.4. In 50µl of the 10 times diluted RBCs, 100 µl of 160 and 320 µg/ml dilution of each plant extract was added in separate eppendrof tubes. For positive and negative control, 1% SDS solution and 1×PBS was added respectively. They were then incubated for 1 hour in incubated shaker at 37°C. After incubation, the volumes of reaction mixture were adjusted to 1 ml using 1XPBS. Finally, centrifuged at 3000 rpm for 3 min and the resulting haemoglobin in supernatant was measured at 540 nm by Tecan micro plate reader and determined the concentration of haemoglobin using Magellan- data analysis software. The haemolysis caused by 100 µl of 1% SDS was taken as 100 % haemolysis and the percentage haemolysis was calculated [% Haemolysis = [(control – sample)/ control] * 100].

G2M phase cell cycle study

MCF7 cells (1×10^6) were seeded in 6 well plate with 160 µg/ml and 320 µg/ml concentration of each plant extract and one well as control with 1% DMSO. The plate was incubated for 24 hours at 37˚C in a 5% CO^2 incubator. Next day cells were collected in individual vial by trpsinization and centrifuged to get cell pellet at 1500 rpm for 5 minutes. After washing the cell pellet with 1×PBS, 1 ml of 70% ethanol was added slowly while continuous stirring and another 1 ml was added directly to it. The cells were kept for fixing at 4°C overnight. Next day pellet was collected by centrifugation and washed twice by cold 1× PBS. To cell pellet

500µl of propidium iodide solution (0.05 mg/ml PI and 0.05 mg/ml RNase A in PBS) was added and cell at various stages were recorded using FACS Caliber (BD Biosciences, San Jose, CA).

Apoptotic study

After seeding about 1×10^6 cells in 6 well plate, next day cells were treated with 160 and 320µg/ml concentration of each plant extract and incubated at 37°C in CO2 incubator overnight. Cells were harvested by trypsinization and cells was washed twice by cold 1×PBS. Cell pellet was resuspended in 1× PBS at a concentration of ~1 x 10^6 cells/mL. To the 100 µL of cells (~1 x 10^5 cells) transferred to a 5-mL FACS tube, 5 µL Annexin V and 5 µL PI was added, gently mixed and incubated for 15 minutes at RT in the dark. 400 µL of 1X Binding Buffer to each tube and analyze by using FACS Caliber (BD Biosciences, San Jose, CA).

RESULTS AND DISCUSSION

The viability of the MCF7 cells on treatment with the M. pruriens plant extract was evaluated by MTT (3-[4, 5-dimethylthiazol2-yl]-2, 5 diphenyl tetrazolium bromide) assay.

The cytotoxic effect of the plant extract at different concentration is shown in Table 1.

A dose dependent curve response was obtained between the concentration of extract and % inhibition. Based on which, inhibitory concentration at which half of the cells die was calculated as 118.33µg/ml (IC50).

Table 1: Cell Viability on MCF7 cell lines.

Conc. (µg/mL)	Dilution	OD	% Inhibition
0	-	1.293	0
10	1:32	1.213	6.13
20	1:16	1.132	12.46
40	1:8	0.986	23.69
80	1:4	0.725	43.93
160	1:2	0.561	56.60
320	Neat	0.200	84.50

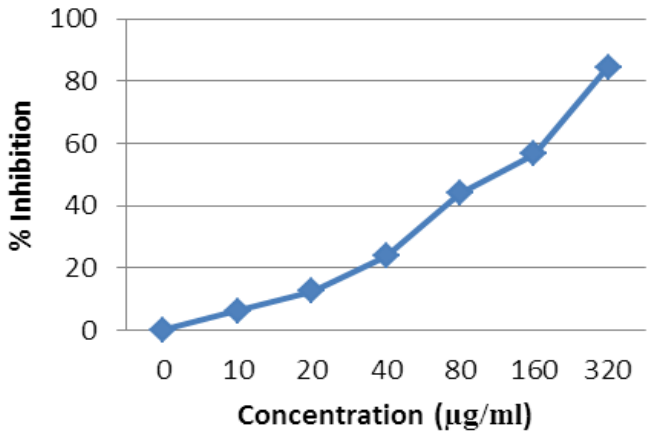


Figure 1: % inhibition of seed extract on MCF7 cell lines.

After analyzing the results of MTT assay, the concentration of the plant extract to be used for further analysis was set as 160 and 320 µg/ml. hemolysis assay was done to determine the lytic activity of plant extract in erythrocytes. Interestingly, ethanol extract of plants have not shown hemolysis on human erythrocytes at 160 and 320 µg/ml with the 1% SDS as positive control as shown in table The present breakthrough in this study, thereby gives enough scope to use these plant extracts for further investigation at molecular level to identify the compound accountable for anticolorectal cancer activity..

Table 2: Hemolysis Assay OD.

Conc. (µg/mL)	Absorbance	% Hemolysis
PBS	-	0
1% SDS	1:32	6.13
160	1:16	12.46
320	1:8	23.69

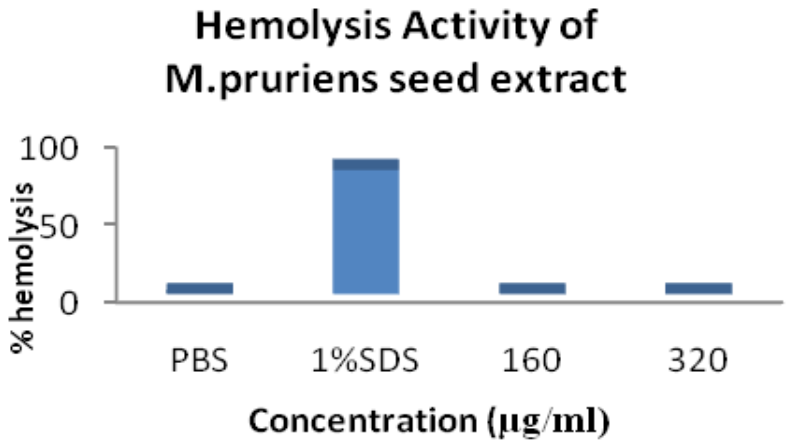
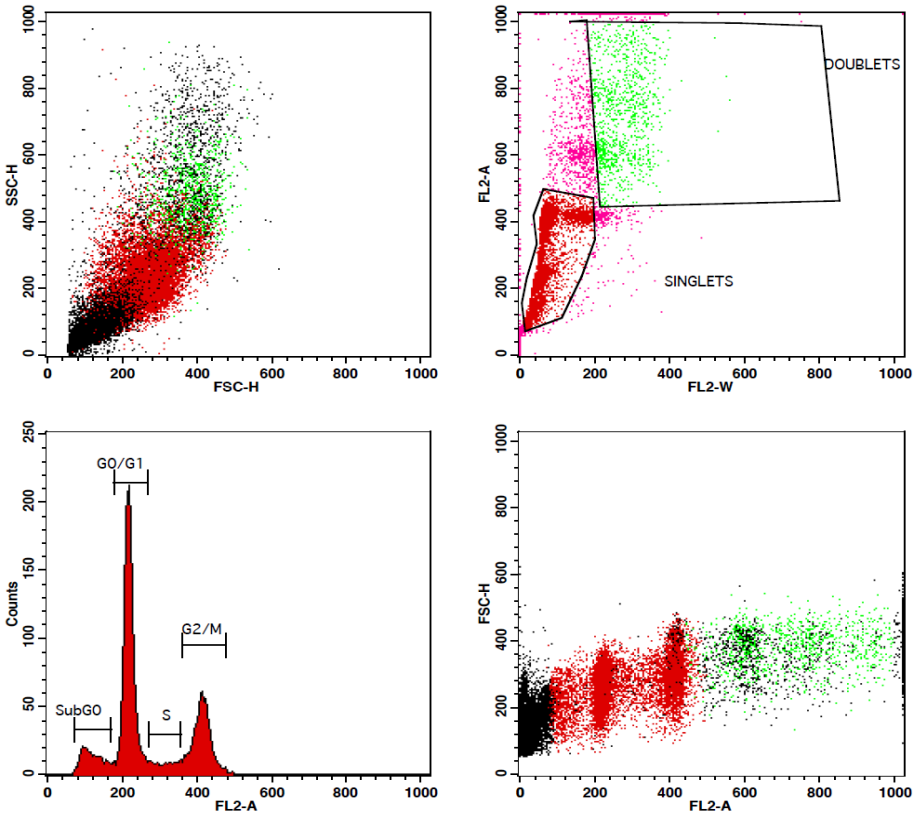


Figure 2: % hemolysis of RBCs on treatment with seed extract.

To investigate whether the extract affects cell cycle regulation, flow cytometry was performed. Fig 1 shows incubation of Mucuna extract with MCF-7 for 24 h significantly arrest 26.60% of cells at G2M phase of cell cycle. 56.1% of cells were in G0/G1 phase, with 78.10% of cells in the same phase in the respective control. Fig 2 shows the annexin V based apoptotic studies of the MCF7 cells treated with M. pruriens plant extract. At concentration of 160 and 320 µg/ml, M. pruriens has induced significant apoptosis in 15.0% and 52.38% of the MCF7 cells respectively as compared to untreated MCF7 control cells of only 0.24% cells.



Histogram Statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	33.37	265.86	37.34
SubG0	71, 172	1070	10.70	3.57	119.67	21.25
G0/G1	179, 268	5576	55.76	18.61	218.46	6.38
S	273, 357	576	5.76	1.92	315.88	8.17
G2/M	362, 478	2660	26.60	8.88	411.94	5.34

Figure 3: Flow cytometry plots of MCf7 treated with 320 µg/ml of M.pruriens seed extract.

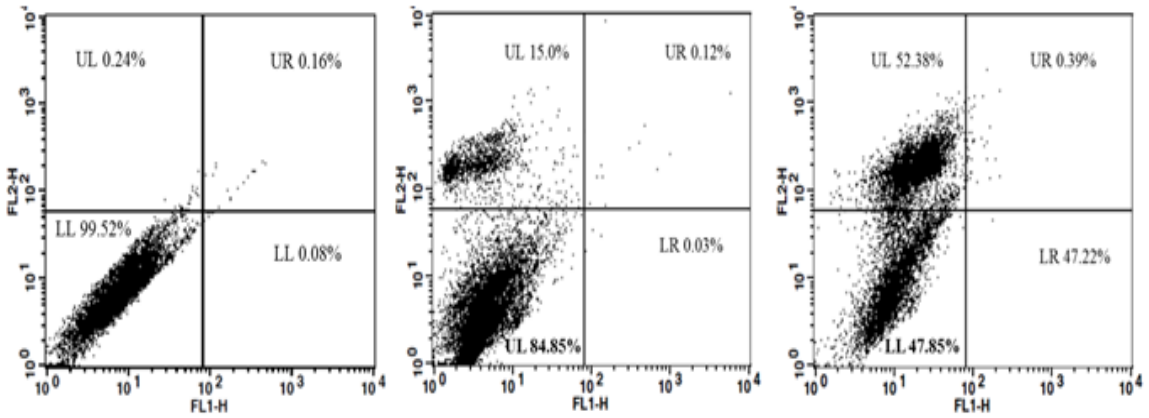


Figure 4: Apoptoic studies. Control, MCF7 cells treated with 160 and 320 µg/ml of Mucuna pruriens seed extract respectively.

CONCLUSION

Hemolytic, Cytotoxic and apoptogenic studies of MCF7 cells when treated with M.pruriens seed extract shows that it is able to induce cell death in the breast cancer cells and without affecting erythrocytes. Hence, it can be a promising chemotherapeutic agent to treat breast cancer.

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