ANTI-CANCER ACTIVITY OF *Pisonia alba* IN TRIPLE NEGATIVE BREAST CANCER CELLS (MDA-MB-231)

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Abstract : Medicinal plants besides therapeutic agents are also a big source of information for a wide variety of chemical constituents which could be developed as drugs with precise selectivity and in line with this the present study was focused on determining the anti-cancer potential of Pisonia alba alcoholic extracts. The extracts were found to have alkaloids, glycosides and tannins as the major secondary metabolites which exhibited significant antioxidant activity when determined by ABTS assay. The extracts show negligible anti-bacterial activity upon agar well diffusion assay. MTT assay on breast cancer cell lines MDA MB has shown a dose dependent inhibition of cell proliferation with an LC 50 value of 15.2ug/ml. Apoptosis was confirmed by fluorescent microscopy and the anti-metastatic activity was determined by clonogenic assay. RT PCR analysis has shown activation of MAPK and ERK which shares significant role in anti-proliferation activity of Pisonia which needs to be explored for utilising its anti-cancer activity.

IndexTerms - **Anticancer*, Poisonia alba*, Triple negative Breast cancer, Phytochemicals and antioxidants.**

I. INTRODUCTION

Many plants derived substances, collectively called phyto nutrients or phytochemicals are becoming increasingly known for their anticancer, anti-diabetic, anti-arthritic activities. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover a new therapeutic agent that lack the toxic side effects associated with current chemotherapeutic agent).(Zucker et al., 2000) Various parts of the plants are collected by local and folk communities all over the world for their use but these are generally collected in low quantities. Plant based products have been effectively proven for their utilization as source for antimicrobial compounds. Plants are important source of potentially useful structures for the development of new chemotherapeutic agents.(Sumathi *et al*.,2011) Programmed cell death (PCD) describes a physiological and pathological process of cell deletion that plays an important role in maintaining tissue homeostasis. It is a highly regulated cellular suicide process essential for growth and survival in all eukaryotes.(Pugazhendy *et. al*., 2015)

Cancer has become a leading cause of death in the world because changed disease spectrum by social and environmental risk factors. Cancer is a series of malignant disease with multiple pathological stages (e.g. cancer initiation, promotion and progression) and involved in multiple factors (genetic, environmental, biological, chemical, physical and psychological factors)(Hamsa *et al.,* 2013). *Pisonia alba* belonging to the family Nyctaginacea, is an evergreen glabrous garden tree with Yong shoots are minutely puberulous. The leaves are ovate- oblong to oblong, 15-25 cm long, 5-7 cm wide usually unequal obtuse at the base and acute apex. (Radha *et al.*,2008) *Pisonia alba* is a large evergreen shrub. It is originally from the beach forest of the Andaman Islands. Leaves are bounty and fresh green in colour. Leaves make good cattle feed to and are mostly used to treat rheumatism or arthritis. In the alternative system of medicine *Pisonia alba* leaves are used as analgesic, anti-inflammatory, diuretic, hypoglycaemic agent and anti-fungal. It is also used in the treatment of ulcer, dysentery and snake bite. The leaves are edible and mostly used to treat wound healing, rheumatism and arthritis (Prabhu *et al.,* 2008.

II. MATERIALS AND METHODS

The leaves of *pisonia alba* were collected from Erode District. The plant specimen was identified and authenticated (**Specimen No: BSI/SRC/5/23/2018/Tech.3365**) by Dr.C.Murugansientist E, Director, Botanical survey of India,

Tamilnadu. 20 gm of *Pisonia alba* powder was immersed in 70% enthanol for 72 hours at 37°c as per the standard procedure. The extract was filtered through two layer muslin cloth and dried in vacuum. The extract was solubilized by using dimethyl sulphoxide (DMSO). Literature says that ethanolic extraction shows best activity. So, with this background the ethanolic extract of *Pisonia alba* was used for further studies.

1.PRELIMINARY PHYTOCHEMICAL SCREENING OF *PISONIA ALBA*

1.1 TEST FOR ALKALOIDS

A fraction of *Pisonia alba* was treated with 1.27 g of iodine and 2 g of potassium iodide in 100 mL water and observed for the formation of reddish brown colour precipitate. There was a formation of reddish brown colour confirming the presence of alkaloids.

1.2 TEST FOR FLAVONOIDS

To 0.5 ml of alcoholic extract, 5- 10 drops of dilute HCL and small piece of Zinc chloride or Magnesium chloride were added and solution was boiled for few minutes. Presence of reddish pink or dirty brown color indicates positive flavanoid reaction

1.3 **TEST FOR TANNIS**

To 1 ml of the extract, 2 ml of 5% FeCl3 was added. A dark blue or green black indicates the presence of tannis.

1.4 TEST FOR PHENOL (Ferric Chloride Test)

The sample is treated with aqueous ferric Chloride blue or green color occur (Anubratapaul *et al*., 2006).

1.5 TEST FOR STEROIDS (Salkowski Test)**:** To 2ml of chloroform extract 1ml of Sulfuric acid is added and observed

1.6 TEST FOR GLYCOSIDES: The *Pisonia alba* was dissolved in sodium hydroxide solution .

2. **ANTIOXIDANT ASSAY:** The antioxidant activity of ethanolic extraction of *Pisonia alba* identified using ABTS assay. The performed medical motivation of 2,2 azonobol 3ethyl benzothioozoline 6 sulfonic acid in generated by oxidation of ABTS with potassium per sulfate (a blue chromogen) and is reduced in the presence of such hydrogen donating antioxidant.

3.ANTIMICROBIAL ACTIVITY

Antibacterial activity was measured using the standard method of diffusion disc plate on agar (

4. MTT CELL VIABILITY ASSAY: To determine cytotoxic effects of the sample *Pisonia alba* in MDA MB 231cell line different concentrations of samples 6.25, 12.5, 25, 50, and 100µg/ml were added to the MDA-MB-231 cells and incubated for 24hrs. The medium was prepared as per standard procedure described above and the cells after 72hrs growth were harvested and used for further studies. The cytotoxicity induced was determined by MTT assay and Lc50 value calculated.

5. DETERMINATION OF APOPTOSIS: Apoptosis was determined morphologically after staining with acridine orange and ethidium bromide by fluorescent microscopy. The MDA-MB-231 cells were subculture kept for 3 days incubation. After 80% confluent, 50 µl of acridine orange and ethidium bromide solution was added in the ratio 1:1. Then kept it for 10 minutes incubation and washed to remove the stain using 1ml PBS. Then the cellular morphology was evaluated by Olympus (CKX41) fluorescent microscope.

6. CLONOGENIC ASSAY (M J Marcus 2008): Clonogenic assay or colony formation assay is an in vitro cell survival assay based on the ability of single cell to grow into a colony (M J Marcus).

7.mRNA GENE EXPRESSION OF MDA-MB-231 CELL LINE: mRNA gene expression is done by analysis of the gene expression of *Pisonia alba*.

III. RESULTS AND DISCUSSION

1. PHYTOCHEMICAL ANALYSIS OF *PISONIA ALBA*

All the plants were showed the presence of phytochemicals. The phytochemicals present in the plants can inhibit the growth of many phytopathogenic fungi. Inhibition of pathogen may be due to the presence of these phytochemicals. The result of phytochemicals are tabulated in the table.The table I showed the result of phytochemicals analysis of *Pisonia alba*

The table showed the presence of phytochemical in *Pisonia alba*. In this more alkaloid are present in ethanolic extract of *Pisonia alba.* A trace amount of glycosidase, tannins, phenols and there was an absence of flavanoids and steroids.

2. ANTI-OXIDANT ASSAY OF ETHANOLIC EXTRACT OF *Pisonia alba*

Then the sample was subjected in to ABTS assay to determine the percentage of inhibition. ABTS assay measures the relative ability of antioxidant. Antioxidants play an important role in inhibiting & scavenging radicals, thus providing protection to humans against infection and degenerative diseases.

S. No.	Concentration of enthanolic extraction of *pisonia alba* (µg/ml)	Percentage of inhibition
1	12.5	73.49
2	25	80.634
3	50	89.12
4	100	91.84
5	200	99.43

S.No	Phytochemicals Present in *Pisonia alba*	Ethanolic extract of *Pisonia alba*
1	Alkaloids	++
2	Glycosidase	+
3	Flavanoids	_
4	Tannis	+
5	Steroids	_
6	Phenol	+

Figure 1

3. ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT OF *Pisonia alba*

In order to access the antimicrobial activity of isolated plant extract act against different microorganisms by standard agar gel well diffusion method. The antimicrobial activity of *Pisonia alba* against different pathogen like *E.coli*, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Klebsiella pneumoniae*. The purified sample showed positive result on *Klebsiella pneumoniae.*

S.No	Microorganisms	AB	100	50	25
1	*E.coli*	1.5cm	Nil	Nil	Nil
2	*Klebsiellapneumoniae*	0.8cm	0.1cm	Nil	Nil
3	*Staphylococcusaureus*	1.2	Nil	Nil	Nil

4. MTT CELL VIABILITY ASSAY OF ETHANOLIC EXTRACT OF *Pisonia alba*

This is a colorimetric assay that measure the reduction of yellow 3-(4,5dimethyl thiozal 2-yl)-2,5diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cell and passes in to the mitochondria where it is reduced to an insoluble,coloured,(dark purple) formazan product. The cells are then solubilized with an organic solvent (DMSO) and released solubilized formazan is measured spectrophotometrically. Since the MTT can only occur in metabolically active cells, the level of activity is measure of the viability of the cells.

S. No	*Pisonia alba* in µg/ml	Optical density	Test	Percentage of viability
1	6.25	0.3062	0.1732	56.56%
2	12.5	0.3062	0.1605	52.41%
3	25	0.3062	0.1481	48.36%
4	50	0.3062	0.1461	46.24%
5	100	0.3062	0.1211	39.49%

5. MORPHOLOGICAL STUDIES OF APOPTOSIS IN ETHANOLIC EXTRACT OF *Pisonia alba*

5.1. Ethidium Bromide and Acridine Orange

Ethidium bromide (EtBr) is a molecules that intercalated into nuclic acids and can be used to visualize the nuclear changes in apoptotic cell.The cells were subjected in to pisonia alba were stained with ethidium bromide and the nuclear changes were observed.

APOPTOSIS ASSESSMENT BY ACRIDINE ORANGE AND ETIDIUM BROMIDE STAINING

Control (DMSO+MDA-MB-231 Cell line) *Pisonia alba*+ DMSO+ MDA-MB-123

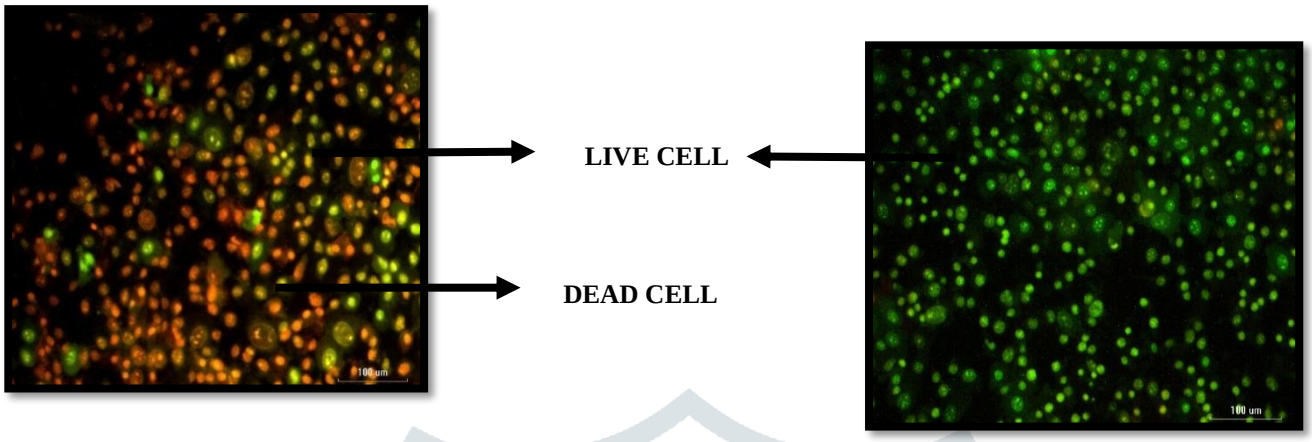

Figure 2 Acridine Orange and ETBR Staining

6. CLONOGENIC ASSAY

Clonogenic assay or colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. The treatment of *Pisonia alba*has reduced the number of colonies revived and from the results it can be observed that the metastatic property i.e., efficiency of a single cell to grow as a colony is decreased by treatment with *Pisonia alba.*

Control Ethanolic extract of *Pisonia alba*

Figure 3 Colonogenic assay

7. mRNA GENE EXPRESSION OF MDA-MB-231 CELL LINE

This method is used to determine mRNA gene expression of MDA-MB-231 cell lines for the decision of anti-cancer activity of *Pisonia alba* in ethanolic extract. In this method two genes are used MAPK and ERK.

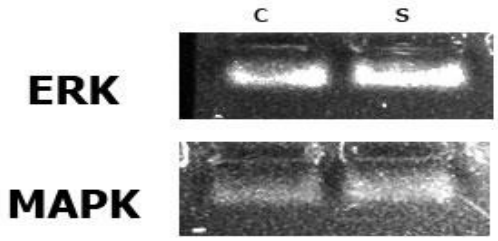

Figure 4 Gene expression

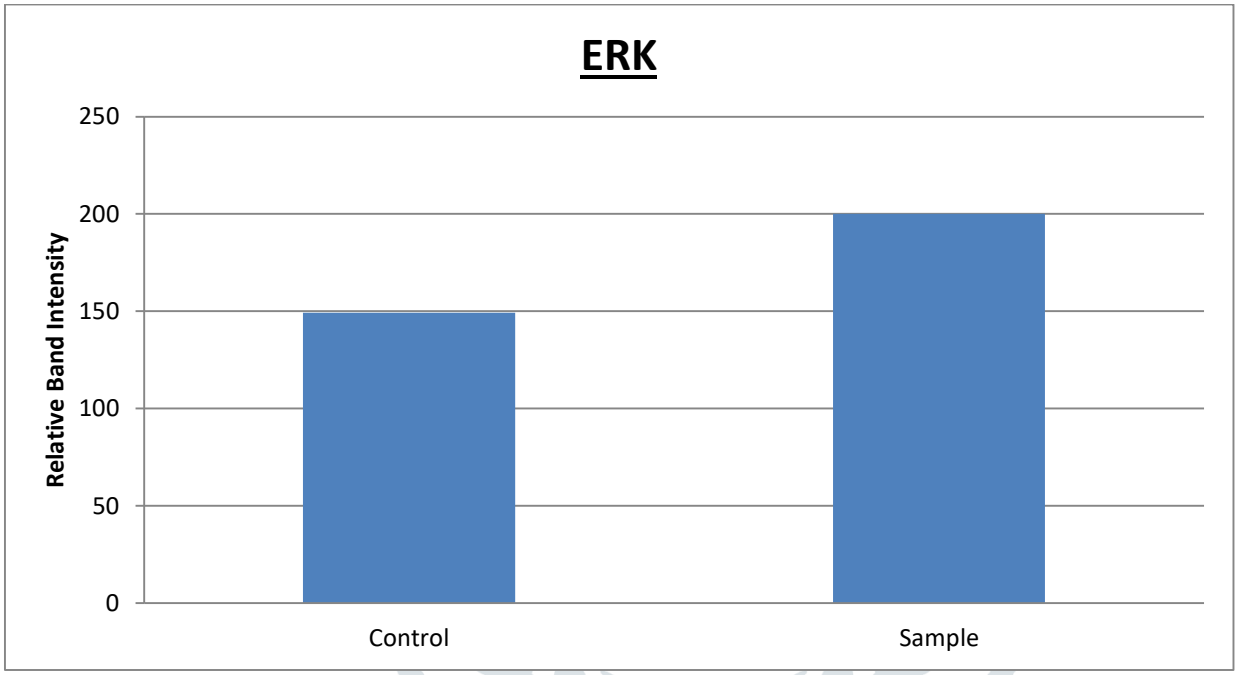

Figure 5

Along X axis – sample names
(i) Untreated cells kept as control
(ii) Cells treated with Sample
Along Y axis- Relative Band intensity in arbitrary units
Raw density measured using image J analysis software
Mean intensity is measured using ImageJ analysis

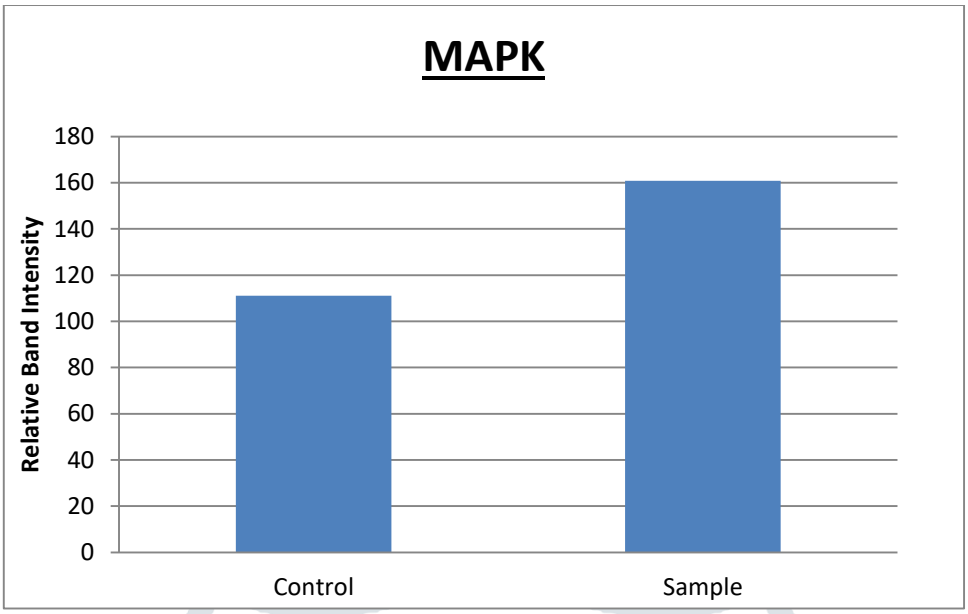

Figure 6 RELATIVE EXPRESSION OF MAPK

Along X axis – sample names
(i) Untreated cells kept as control
(ii) Cells treated with Sample
Along Y axis- Relative Band intensity in arbitrary units
Raw density measured using image J analysis software,
Mean intensity is measured using ImageJ analysis

IV. ACKNOWLEDGEMENT

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V. SUMMARY AND CONCLUSION

Medicinal plants besides therapeutic agents are also a big source of information for a wide variety of chemical constituents which could be developed as drugs with precise selectivity and in line with this the present study was focused on determining the anti-cancer potential of Pisonia alba alcoholic extracts. The extracts were found to have alkaloids, glycosides and tannins as the major secondary metabolites which exhibited significant antioxidant activity when determined by ABTS assay. The extracts show negligible anti-bacterial activity upon agar well diffusion assay. MTT assay on breast cancer cell lines MDA MB has shown a dose dependent inhibition of cell proliferation with an LC 50 value of 15.2ug/ml. Apoptosis was confirmed by fluorescent microscopy and the anti-metastatic activity was determined by clonogenic assay. RT PCR analysis has shown activation of MAPK and ERK which shares significant role in anti-proliferation activity of Pisonia which needs to be explored for utilising its anti-cancer activity.

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