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(RESEARCH ARTICLE)

EVALUATION OF ANTICANCER ACTIVITY OF JUSTICIA CARNEA

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ABSTRACT

Cancer is one of the leading causes of mortality all over the world, prompting continuous search for safer and effective anticancer agents globally. Herbal medicine has gained wide popularity as medicines with less or minimal adverse effects. In this study, we evaluated the anticancer activity of a herbal medicine *Justicia carnea,* commonly known as Brazilian plume flower or blood leaf, belonging to Acanthaceae family, which has been widely used in folk medicine. We have evaluated *Justicia carnea* leaf extract against MCF-7 human breast cancer cell line. The cytotoxic effect of the extract was studied using MTT assay, while double staining with Acridine orange and Ethidium bromide (AO/EtBr) was performed to observe morphological changes associated with apoptosis. The extract exhibited concentration-dependent cytotoxic activity against MCF-7 cells, with an IC_{50} value of 237.13 µL/ml. The treated cells showed morphological alterations, including cell shrinkage and nuclear changes, suggesting the possible induction of apoptosis. Overall, the findings suggest that *Justicia carnea* leaf extract possesses preliminary in vitro cytotoxic activity against MCF-7 breast cancer cells and may induce apoptosis associated morphological changes. Further studies are required to elucidate its mechanism of action, establish its selectivity for cancer cells and assess its potential for future anticancer drug development.

Keywords: MTT assay, Cytotoxicity, Apoptosis, Acridine orange, Herbal formulation.

1 INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality worldwide and is a major global public health challenge. It is a complex and heterogeneous disease characterized by uncontrolled proliferation of abnormal cells resulting from genetic and epigenetic alterations that evade normal cellular regulatory mechanisms. Carcinogenesis is initiated when cellular regulatory process becomes dysregulated as a result of accumulated genetic mutations, epigenetic alterations and aberrant signaling pathways. Continuous proliferation leads to tumourigenesis. Based on their biological behaviour tumours are classified into malignant and benign tumours [1,2]

Herbal medicine has been used to treat various ailments for centuries, and cancer is no exception. Due to a large number of bioactive compounds present in plants, herbal medicine has been extensively studied as potential anticancer drugs.

Vinca alkaloids such as vinblastine and vincristine extracted from Madagascar periwinkle plant (*Catharanthus roseus*) are one of the earliest examples of herbal anticancer agents. These compounds which are widely used in various cancers such as breast cancer, Hodgkin's lymphoma, acute lymphoblastic leukemia, non-Hodgkin's lymphoma, neuroblastoma, rhabdomyosarcoma and wilms tumor were introduced since 1960s.Paclitaxel from pacific yew(*Taxus brevifolia*) used for breast , ovarian and lung cancers, camptothecin extracted from *Camptotheca acuminata* which led to the development of Topotecan and Irinotecan used to treat ovarian, lung and colorectal cancer are some of the earliest examples. Inspired by these early successes, more research is focusing on plant- based anticancer agents. [3,4,5]

Justicia carnea also known as Brazilian plume flower, Jacobinia is a plant commonly used as ornamental across the globe. It is a member of Acanthaceae family and genus Justicia, which has many plants used in herbal and traditional medicinal practices. *Justicia carnea* is known as "ogwuobara" in Nigeria meaning blood tonic and has been used in treating gastrointestinal disorders, inflammation, anemia, diabetes mellitus, malaria and respiratory disorders. Several studies conducted on phytoconstituents of *Justicia carnea* showed the presence of flavonoids, alkaloids, tannins, glycosides etc. which may contribute to the plant's antioxidant and anticancer properties. [6,7]

Although previous studies have investigated different pharmacological properties of *Justicia carnea*, the anticancer properties of its leaf extract are comparatively less explored. Therefore, further investigation is required to determine whether the plant extract can inhibit cancer cell proliferation.

Our current study is designed to evaluate the invitro anticancer activity of methanolic leaf extract of *Justicia carnea* using MTT cell viability assay and Ethidium bromide staining against MCF-7 cell line.

2 MATERIALS AND METHODS

2.1 Plant material

The plant Justicia *carnea* was obtained from "Floranza", a plant nursery located in Purakkalam, Kuthuparambu, Kerala, India. The plant was authenticated by a qualified botanist at Nirmalagiri College, Kuthuparambu, Kerala. A specimen of the plant was preserved in the institutional herbarium for future reference.

2.2 Preparation of methanolic extract

Fresh leaves of *Justicia carnea* were collected and washed with pure water and dried under shade avoiding direct exposure to the sun up to one month. The dried plant leaves are powdered using a mixer grinder and stored in air tight container until extraction. The weighed quantity of dried plant powder is mixed with methanol in the ratio of 1:10 in a conical flask and is allowed to macerate for 72 hours in dark with occasional shaking. The macerated extract is filtered and excess solvent is removed using a rotary evaporator under reduced pressure. The concentrated extract is used for further investigation. [8]

2.3 Cell line and cell culture

2.3.1 Procedure

MCF-7 (Human Breast Cancer) cell line was initially procured from National Centre for Cell Sciences (NCCS) Pune, India and maintained in Dulbecco's modified Eagle medium, DMEM (Sigma Aldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

2.4 Cells seeding in 96 well plate:

Two-day old confluent monolayer of cells was trypsinised and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO2 incubator.

2.5 Preparation of compound stock:

The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

2.6 Anticancer Evaluation:

After 24 hours the growth medium was removed, freshly prepared compounds were added at concentrations of 100 µL/ml, 50 µL/ml, 25 µL/ml, 12.5 µL/ml, and 6.25 µL/ml of DMEM. Each concentration was added in triplicate to the respective wells and incubated at 37ºC in a humidified 5% CO2 incubator. Untreated control cells were also maintained. [9]

2.7 MTT assay

2.7.1 PRINCIPLE

The MTT assay is a colorimetric method used to assess cell viability and metabolic activity. It is a preliminary screening method to analyze the cytotoxic potential of anticancer drugs. Viable cells convert the yellow tetrazolium salt (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) into purple formazan crystals through cellular reducing activity. Viable cells contain NAD(P)H dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with deep purple colour.

The amount of formazan produced is measured spectrophotometrically and is used as an indicator of cell viability relative to the untreated control. The formazan crystals are dissolved using a solubilizing solution and absorbance is measured at 500 to 600 nm using a plate reader. The darker the solution, the greater the number of viable, metabolically active cells.[10]

2.7.2 PROCEDURE

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004).[11]

The percentage of growth inhibition was calculated using the formula:

$$\%\ of\ viability = \frac{\text{Mean OD of Samples}}{\text{Mean OD of control group}} \text{x } 100$$

2.8 Determination of apoptosis by acridine orange (Ao) and ethidium bromide (ETBR) double staining

2.8.1 Principle

DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells (Zhang et al, 1998). Acridine orange is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). Ethidium bromide is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA.[12]

2.8.2 Procedure

MCF-7 (Human Breast Cancer) cell line was cultured as per standard procedures described earlier and treated with concentration of sample – 237.13 µL/ml and incubated for 24 hours at 37ºC in a humidified 5% CO 2 incubator. Untreated control cells were also maintained in this condition.

After incubation, the cells were washed by cold PBS and then stained with a mixture of AO (100µg/ml) and EtBr (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).[13]

3 RESULT AND DISCUSSION

Table 1: MTT assay results of *Justicia carnea* leaf extract against MCF-7 cell line at different concentrations.

Sample Concentration (µL/mL)	OD I	OD II	OD III	Average Absorbance at 540nm	Percentage Viability
Control	0.6341	0.6221	0.6418	0.6327	100.00
Sample code- SAMPLE					
6.25	0.6158	0.6205	0.6197	0.6187	97.78
12.5	0.5995	0.6015	0.5874	0.5961	94.22
25	0.5646	0.5819	0.5704	0.5723	90.45
50	0.5301	0.5418	0.5501	0.5407	85.45
100	0.4951	0.4851	0.5026	0.4943	78.12

3.1.1 IC_{50} VALUE(HYPOTHETICAL)

IC_{50} Value of SAMPLE:237.13 µL/mL (Calculated using ED50 PLUS V1.0 Software)

3.2 STATISTICAL ANALYSIS

Table 2: Statistical analysis of MTT assay of *Justicia carnea* leaf extract against MCF-7 cell line

sample	Concentration(µL/ml)	OD I	OD II	OD III	Percentage viability1	Percentage viability2	Percentage viability 3	Average	Std Dev	Std Error
control	0	0.6341	0.6221	0.6418	100	100	100	100	0	0
Justicia carnea extract	6.25	0.6158	0.6205	0.6197	97.11402	99.7428066	96.55656	97.8045	1.70164	0.98244
	12.5	0.5995	0.6015	0.5874	94.543447	96.6886353	91.523839	94.252	2.59471	1.49805
	25	0.5646	0.5819	0.5704	89.039584	93.5380164	88.875039	90.4842	2.64595	1.52764
	50	0.5301	0.5418	0.5501	83.598801	87.0921074	85.71206	85.4677	1.75943	1.01581
	100	0.4951	0.4851	0.5026	78.079167	77.9778171	78.311	78.1227	0.1708	0.09861

3.3 GRAPH

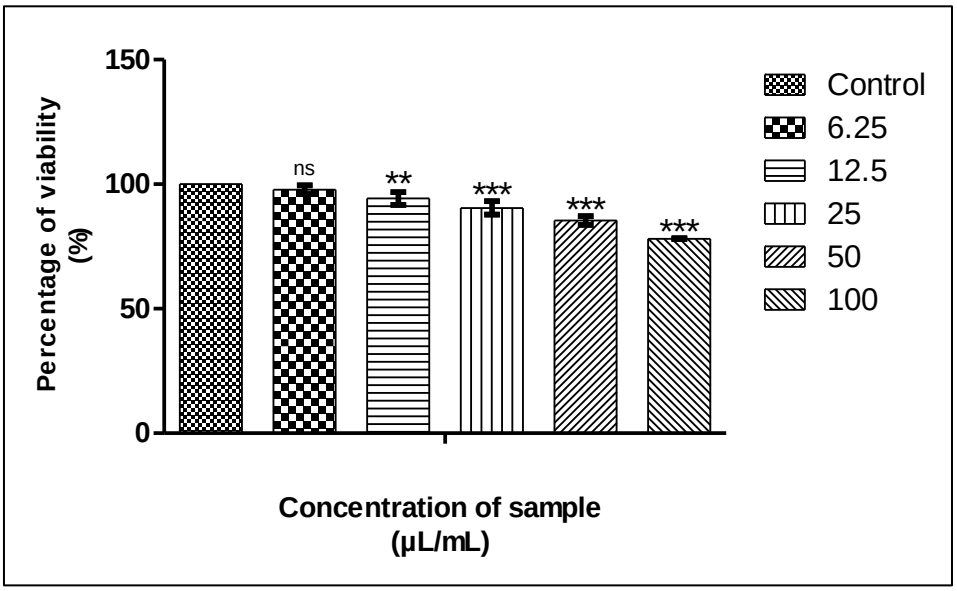


Figure 1: Graphical representation depicting the anticancer effect of sample by MTT assay- Along Y-axis Percentage viability, Along X-axis varied concentration of sample. All experiments were done in triplicates and results represented as Mean+/- SD. One-way ANOVA and Dunnett's test were performed to analyse data. ***p< 0.0001 compared to control group, **p< 0.001 compared to control group, ns-non significant compared to control group.

Concentration-dependent reduction in cell viability was observed in MCF-7 cancer cell line administered at different concentration of *Justicia carnea* extract. Maximum cytotoxicity was observed with 100µl/ml of the sample (78.122% viability). Thus, it can be concluded that *Justicia carnea* extract exhibits concentration-dependent cytotoxic activity and its activity increases with increase in concentration.

3.4 DOUBLE STAINING METHOD USING ACRIDINE ORANGE AND ETHIDIUM BROMIDE

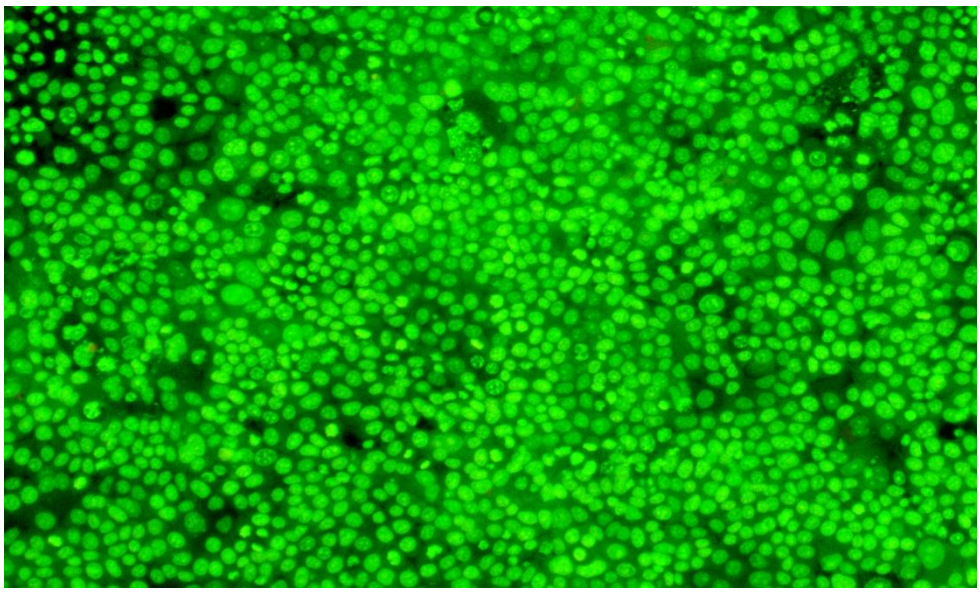


Figure 2(A): Control MCF-7 cells showing uniform green fluorescence(viable)

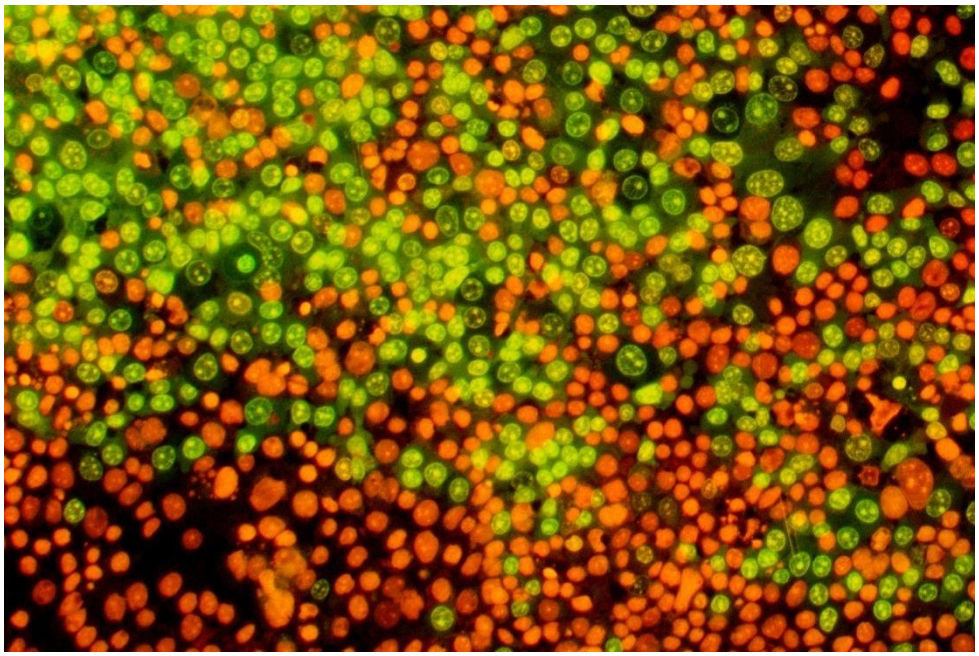


Figure 2(B): Cells treated with IC_{50} showing bright green (early apoptosis) and orange nuclei (late apoptosis).

At IC_{50} concentration (237.13µL/ml) AO/EtBr staining revealed induction of apoptosis. Control cell showed uniform green fluorescence (viable), treated cells showed bright green condensed nuclei (early apoptosis), orange fragmented nuclei (late apoptosis) and uniform orange nuclei(necrosis) confirming cell death via apoptotic pathway.

4 CONCLUSION

Breast cancer is one of the leading cancers in women around the world.MCF-7 is a standard human breast cancer cell line model. Current chemotherapy often causes anaemia and other toxic reactions hence cancer drugs which may mitigate these side effects are better candidates for treatment. *Justicia carnea* commonly known as blood leaf has been used in folk medicine in Africa and many parts of the world to ameliorate anaemia due to its rich constituents.

The selection of *Justicia carnea* plant was based on reported anticancer activities of other species of genus Justicia such as *Justicia adhatoda* moreover several studies have proven the antioxidant activity of *Justicia carnea*

The present study was conducted to evaluate the in vitro anticancer potential of *Justicia carnea* leaf extract against the MCF-7 breast cancer cell line using the MTT assay and Acridine orange/Ethidium bromide (AO/EtBr) double staining.

MTT assay demonstrated concentration-dependent reduction in viability of MCF-7 cells. IC_{50} was found to be 237.13µL/ml indicating moderate cytotoxic effect compared to standard doxorubicin.

AO/EtBr staining provided additional evidence of cell death mechanism where control showed uniform bright green nuclei indicating live cells while treated cells showed orange nuclei suggesting apoptosis induction.

From the experimental results we can suggest a moderate anticancer activity for *Justicia carnea* along with its traditional anti-anaemic use the plant may warrant further investigation as a potential adjuvant, but further studies are necessary to evaluate the haematinic activity, experimentally identify active constituents, investigate drug interaction and assess safety in preclinical models.

In conclusion, *Justicia carnea* seems to be a promising candidate for further research as an anticancer drug because of its traditional use as an anti-anaemic agent. We are hopeful that it can be developed to a complementary adjuvant to reduce this side effect if relevant studies in this area are carried out with high grade laboratory precision.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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