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CARICA PAPAYA: ANTI-CANCER ACTIVITY IN MCF-7 BREAST CANCER CELL LINE

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ABSTRACT: Cancer is the second leading cause of death globally. Among the group of cancer diseases, breast cancer is the most common type of cancer to affect women. The treatment options that are currently available for this include surgery, radiation therapy and therapy through medication or drugs. But, since all these are harmful and have severe side effects, drugs from natural plant compounds are being developed to act against cancer and its metastatic activity. The aim of this article is to perform qualitative and quantitative analysis of alkaloids in leaf extract of *Carica papaya* (CP) and also study the anticancer and anti-metastatic activity of it in MCF-7 breast cancer cell lines. CP, commonly known as papaya or papaw, belonging to the family of Caricaceae has many medicinal uses including dengue treatment, anti-inflammatory and anticancer properties. The leaf extract of CP was prepared by ethyl acetate extraction method. Various tests such as MTT assay, Wound healing assay, DNA fragmentation assay, Caspase 7/9 induction detection assay and Annexin V assay were performed to evaluate the anticancer and anti-metastatic activity of CP leaf extract in MCF-7 cell lines. The results of the tests shows CP has the required properties that acts against breast cancer with increased dose resulting in increased efficiency of CP on the cancer cells.

INTRODUCTION: According to the American Cancer Society, Breast cancer is the second leading cause of cancer death in women. In recent years, incidence rates have increased by 0.3% per year. The chance that a woman will die from breast cancer is about 1 in 38 (about 2.6%). Chemotherapy is the main method of breast cancer treatment. However, since there are side effects of anti-cancer drugs, natural products such as herbs have been used as an alternative therapy [1].

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Carica papaya is commonly called as paw and it belongs to the Caricaceae family is an effective medicinal herb that is being used as food as well as a folk medicine for the treatment of various diseases throughout the world [2].

In traditional medicine, different parts of *C. papaya* including its leaves, barks, roots, latex, fruit, flowers, and seeds have a wide range of reputed medicinal application [3]. Leaves are rich in Alkaloids carpain, pseudocarpain and dehydrocarpaine I and II, choline, carposide, vitamin C and E [4]. Experiments have shown that *C. papaya* possesses anthelmintic, anti-protozoan, anti-bacterial, antifungal, antiviral, anti-inflammatory, anti-hypertensive, hypoglycemic and hypolipidemic, wound healing, antitumor, free-radical scavenging, antisickling, neuroprotective, diuretic, abortifacient, and antifertility activities [3].

Carica papaya can be used for treatment of a numerous diseases like warts, corns, sinuses, eczema, cutaneous tubercles, glandular tumors, blood pressure, dyspepsia, constipation, amenorrhoea, general debility, expel worms and stimulate reproductive organs and many, as a result *Carica papaya* can be regarded as a Neutraceutical [5]. *Carica papaya* leaf decoction, an Australian Aboriginal remedy, has been reported widely for its healing capabilities against cancer, with numerous anecdotal reports. Recently, scientific studies have demonstrated the inhibitory activity of this decoction on the proliferation of both haematopoietic cell lines and solid tumour cell lines [6]. *C. papaya* leaf juice increasing the platelet count for the dengue fever patients [7]. Papaya leaves contain lots of powerful antioxidants and can boost immune system. In particular, papaya leaves produce "Th1," an anti-cancer cytokine. A recent study found that papaya leaf extract could prevent growth of cancer cells including breast cancer. Most commonly, only the cytotoxic activity of the *Carica papaya* have been investigated, hence this study aims at investigating the anti-cancer and anti-metastatic activity of papaya leaf extract on human breast cancer cell line MCF-7.

MATERIALS AND METHODS:

Reagents and Chemicals: PBS, MTT Reagent, DMSO, Annexin Binding buffer, PI working solution, FITC, Digestion buffer, Phenol, chloroform, isoamyl alcohol, sodium acetate, ethyl alcohol, Tris-EDTA buffer, Brafords Reagent, Coomassie blue, Blocking buffer, PBS Tween 20, Primary Antibody against Caspase 7/9 and Secondary Antibody, Chromogen, O Dianisidine and 5N HCl.

Cell Culture: MCF-7 cell lines were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with FBS, penicillin and streptomycin. The cells were subcultured after trypsinisation with 0.25% in 0.5mM EDTA and were cultured under 5% CO_2at 37degree Celsius.

Sample Preparation: The *Carica papaya* plant leaf was dried and powdered. The dried powder was weighed and found to be of 120g. 200ml of 70% ethanol was added to it and mixed thoroughly in rotatory shaker for 48 h. Then the extract is obtained by squeezing the mixture using muslin cloth. Ethanol is evaporated by drying the extract. The dried extract (9.45g) was transferred to the centrifuge tube to which 30ml of distilled water was added. It was homogenized by vortex shaker. Fractionation was done with a separating funnel. To the funnel, the homogenized mixture and 100ml of 90% ethyl acetate were added. After thorough shaking, it was left to settle down. The supernatant was transferred to a petri dish and left for drying. To the organic phase, 30 ml of 90% ethyl acetate was added and left for separation. The final dried extract of *Carica papaya* was scrapped off and transferred to an eppendorf tube. Then it was weighed and found to be 922 mg.

MTT Assay: Cells were seeded into a 96 well plate washed with PBS. To 1mg of sample, 0.1% of DMSO was added and mixed thoroughly. The sample was then added to the well plates containing cells, in the concentration of 6.25, 12.5, 25, 50 and 100µg/ml. The cells treated with the sample were left for 24 h incubation. After the incubation period, the cells were studied under microscope. Media was removed and 10µL of MTT reagent was added to each well. Then it is incubated for 2 h. 50µL of DMSO was added to each well. The contents were transferred into another well plate and kept for incubation in dark for 10 minutes and readings were obtained using ELISA reader at 492nm.

Caspase 7/9 Induction Detection Assay: IC_{50} concentration of the sample was added to the cells and kept for overnight incubation. The cells were transferred to tubes and washed twice with PBS. 200µL of blocking buffer (0.2% gelatine in 0.05% Tween 20 in PBS) was added to the tubes and incubated for 1 hour at room temperature. Then the tubes were washed twice with PBS Tween 20. Primary antibodies for caspase 7/9 were added and left for 2 h incubation at room temperature. Primary antibodies were then removed from the tubes and washed twice with PBS Tween 20. Secondary antibodies were added and kept for 1 hour incubation at room temperature. Then washed twice with PBS Tween 20.Chromogen O-Dianisidine (1mg/100ml methanol, 21ml citrate buffer, 60ml H_2O_2) of concentration 200µL was added and left for 30 min dark incubation. Stop the reaction by adding 50µL of 5N HCl. OD is read at 415nm in ELISA reader. 10µL of sample and 200µL of Brafords reagent were mixed and OD is read at 595nm.

DNA Fragmentation Assay: Cells treated with sample of IC_{50} concentration were incubated for 36 hours. The cells were trypsinised and centrifuged for 10 min at 6000 rpm. The cells were washed with PBS. The pellet was resuspended in 1 ml Digestion Buffer (100mM NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS; pH-8) and 0.1 mg/ml proteinase K was added. The sample was incubated at 50 degree celsius for 12 h. Phenol/Chloroform/ Isoamyl alcohol (IAA) in the ratio 25:24:1 was prepared and 600µL of this reagent was added to each tube. The tubes were centrifuged at 12500 rpm for 15 min. The aqueous layer was collected in separate tubes and 20µL of 7.5M sodium acetate and ethanol were added to the tubes. The tubes were kept in freezer for 12 h incubation. Then the samples were centrifuged at 12500 rpm for 10 min. The DNA obtained were electrophoresed to visualise the bands.

Wound Healing Assay: The cells were seeded into well plates and kept for 24 h incubation. Scratch wounds were made with a sterile pipette tip through a premarked line. The wells were rinsed three times with PBS followed by incubation with sample at IC_{50} concentration for 0 h, 24 h, 48 h and 72 h. Images of the wound areas were taken to study the effect of the sample on wound closure.

Annexin V-FITC Assay: Cells were cultured as per the standard procedure and treated with sample in its IC_{50} concentration. It was kept at incubation for 24 h. Cells were trypsinized and 100ul of cells in the suspension were transferred to separate tubes. Centrifugation at 6000 rpm for 10 min was done and the supernatant was removed. The cells were washed with PBS.

And then 50µL of 1x binding buffer, 2.5µL of FITC and 0.5µL of PI working solution were added to each tube. Then the cells were incubated in dark for 15 min. After incubation, 200µL of 1x binding buffer was added. The cells were analysed in flow cytometer using flow cytometer software. Cells were gated against untreated control cells and analysed for apoptosis using Muse FCS 30 software.

RESULTS:

MTT Assay: The percentage of the cells that were viable, were determined by calculating the optical density of the cells treated with the extracts against blank as control containing only the cells, shown in **Fig. 1** at 540nm.Then, the inhibitory concentration, IC_{50} values for CP was found to be 237.752µg/ml respectively, using ED_{50} plus v1.0.

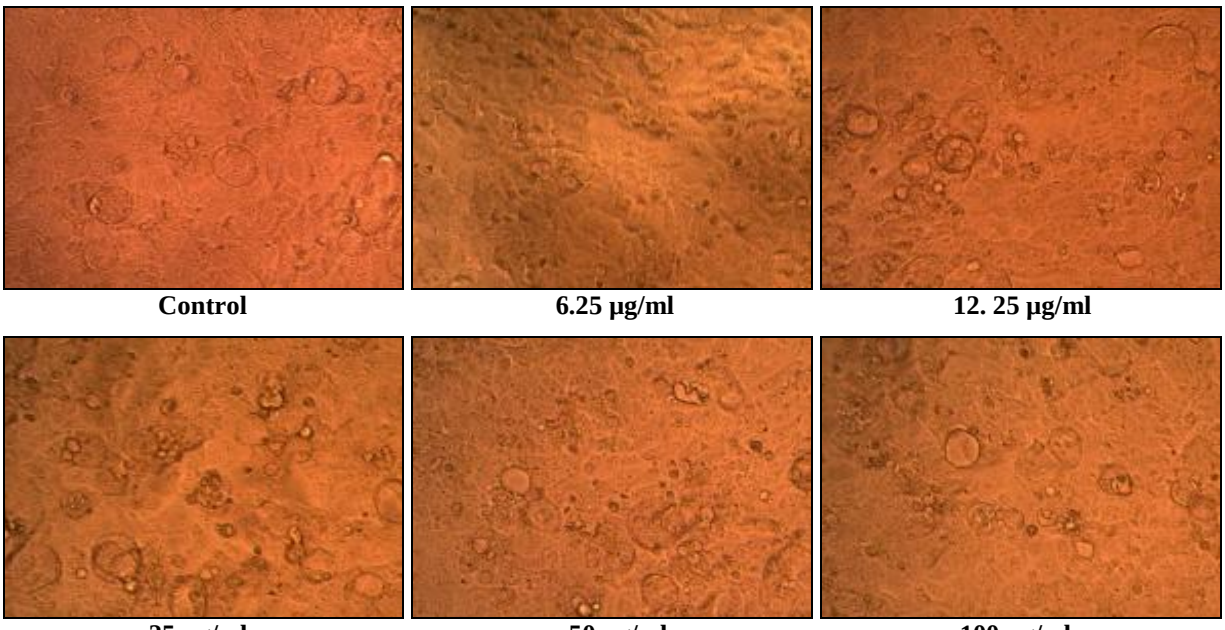

FIG. 1: MTT ASSAY OF CELLS TREATED WITH *CARICA PAPAYA* EXTRACT

DNA Fragmentation Assay: DNA fragmentation assay is performed to study the apoptotic activity in the cell groups treated with CP extract. As a result of this assay, a DNA laddering pattern was observed after running in agarose gel electrophoresis.

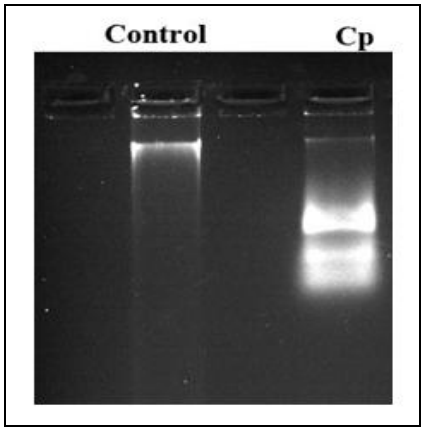

FIG. 2: LADDERING PATTERN OF THE DNA IN CP EXTRACT TREATED CELLS

On comparison with the control, the laddering pattern of the DNA in CP extract treated cells shows induction of apoptotic cell death, shown in **Fig. 2**.

Caspase 7/9 Induction Detection Assay: In this study, the effect of *Carica papaya* on caspase 7/9 activation was analysed. The Caspase 7 induction in control is 0.1356 activity unit/mg of protein, in the cells with sample *C. papaya* is 0.2513 activity unit/mg of protein. The Caspase 9 induction in the control is 0.1273 activity unit/mg of protein, in the cells treated with *C. papaya* is 0.1889 activity unit/mg of protein. The result shows that caspase activity was enhanced while the MCF-7 cells were treated with *C. papaya* extract.

Wound Healing Assay: The effect of *Carica papaya* on the migration of MCF-7 cells was determined by wound healing assay. The inhibition to cell migration in the presence of samples was observed at 24 h, 48 h and 72 h incubation after treating the cells with *Carica papaya.* The wound closure at every interval was compared with the initial wound width (at 0 h). The cells treated with *Carica papaya* assessed for percentage wound area and the results were 62.08% in 24 h, 47% in 48 h and 47.55% in 72 h. In the control, the wound closure was almost complete, after 48 h incubation, as shown in **Fig. 2**. The wound closure was minimum in the cell groups treated with the sample which shows that there is inhibition to the cell migration in a time-dependent manner.

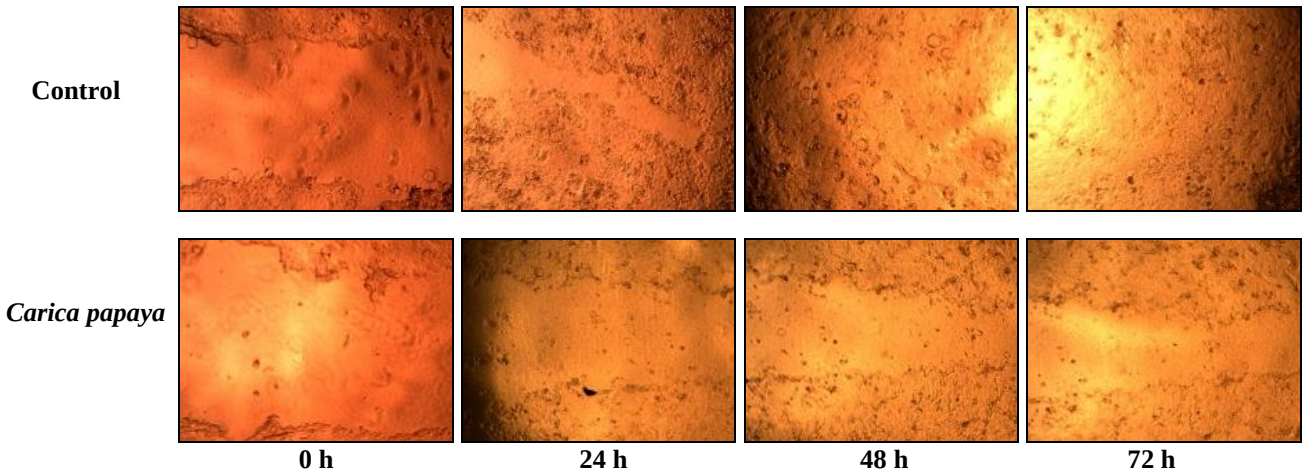

FIG. 3: RESULTS OF VIABLE CELLS AFTER TREATMENT WITH THE CP EXTRACT

The percentage of viable cells after treatment with the extracts of *Carica papaya* is 96.34%, 94.08%, 90.30%, 86.67% and 77.23% for the different concentrations 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml respectively.

Annexin V FITC Assay: This assay was used to determine the percentage of apoptotic cells after treatment with the CP extract. It can be observed that after staining the cells with FITC annexin V and PI, the apoptotic cells showed green fluorescence, dead cells showed red and green fluorescence and live cells showed little or no fluorescence in the control and both cell groups treated with samples. These populations were distinguished using a flow cytometer with the 488 nm line of an argon-ion laser for excitation in the control and CQ treated cells.

Using Muse FCS 3.0 software, as shown in **Fig. 4** the percent of apoptotic cells in the control was found to be 8.18% and in cells treated with CP was found to be 30.85%.

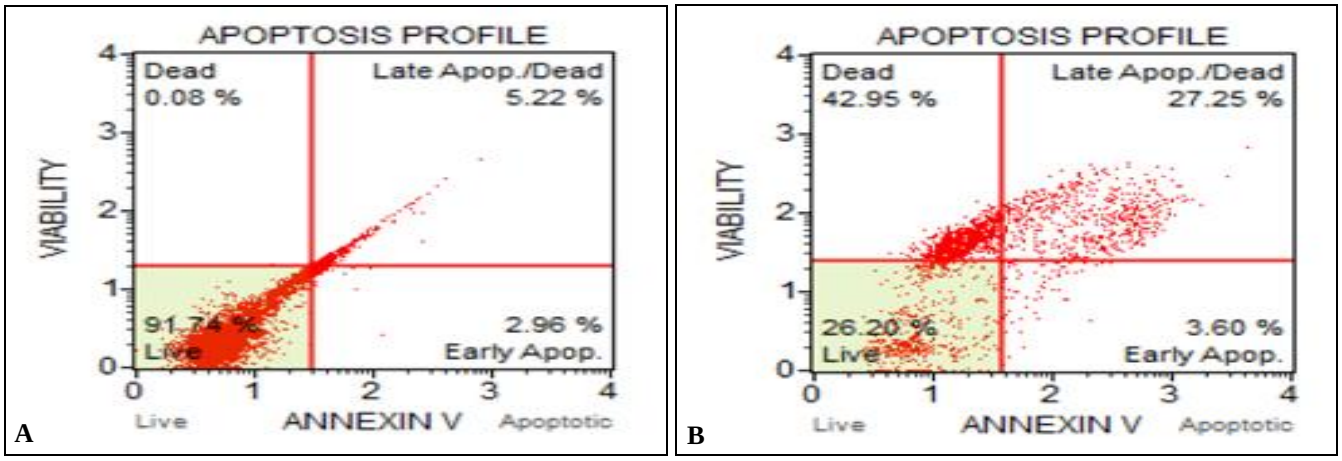

FIG. 4: APOPTOSIS PROFILE OF UNTREATED CONTROL CELLS- MCF-7 (A) AND CELLS TREATED WITH CP EXTRACT

DISCUSSION: This study is to assess *Carica papaya* for potential anticancer activity against human breast cancer cell line, MCF-7. Regulation of growth is lost in tumor cells, they survive beyond their normal lifespan and proliferate abnormally. Anticancer agents derived from plants can block proliferation, resulting in cell cycle arrest and apoptosis [8]. MTT assay is a standard colorimetric assay that measures the cell respiration and the amount of formazan produced to give the number of viable cells in the culture treated with the desired compound. The more the number of viable cells in the culture, the more formazan crystal formation occurs, from which the cell proliferation and thus the cytotoxicity of the sample are determined. IC_{50} is the concentration of sample that causes 50% cell death and it shows the amount of cytotoxicity [9]. In this study, the ethyl acetate extract of the sample CP treated with the breast cancer cell line shows the IC_{50} value of 237.752 µg/mL at a concentration of (6.25, 12.5, 25, 50 and 100 µg/mL). Thus, IC_{50} value of CP shows that it inhibits the cell proliferation and has a potential anti-cancer activity.

Metastasis is the spread of tumor from its primary site to distant organ, generally called secondary site, in a sequential manner. Any disturbance to one of the sequential steps will completely arrest the tumor metastasis. Secondary sites of breast tumor are bone, lung, liver and brain. Over 90% of breast cancer mortality (more than 0.5 million a year) is of metastatic. Metastasis initially begins with the local invasion of tumor into the surrounding host tissue. Tumor cells migrate from their confined primary site, singly or co-ordinately for facilitating the invasion [10]. Thus inhibition of migration will arrest metastasis. Effect of CP on cellular motility and migration is studied by wound healing assay, since they are the primary need of tumor invasion, intravasation, diffusion to distant site, extravasation and colonization at secondary site [11]. The result of wound healing assay indicates that there is significant inhibition of migration when the cells are treated with CP at 24, 48 and 72 h. The wound closure was minimum in the cell groups treated with the samples. Hence, there is inhibition to the cell migration in a time-dependent manner.

Compared with other genes involved in apoptosis (*e.g., p53* and the *Bcl-2* family), relatively little work has been carried out on caspase expression in breast cancer. The aim of this study was therefore to investigate caspase 7/9 expression and its relationship to apoptosis in breast cancer. Currently at least 14 different caspases are known to exist, of which two-thirds play a role in apoptosis. Caspases belongs to the family of conserved cysteine proteases which are central components of the machinery responsible for apoptosis. C9 is an initiator caspase and C7 is a downstream effector caspase. C7 is a member of C8, C9 and C10 group of initiator caspases that degrade multiple cell proteins and are responsible for the morphological changes in apoptosis. In this study, caspase 7 and caspase 9 induction in the cells treated with sample CP is studied, in order to reduce the number of cancer cells.

The caspase 7 activity increased to 0.351442 activity units/mg of protein when the MCF-7 cell line treated with the sample CP. The untreated sample has a caspase 7 activity of about 0.135651 activity units/mg of protein.

The caspase 9 activity was increased to 0.222429 & 0.264279 activity units/mg of protein when the MCF-7 cell line was treated with the sample CQ & CP. The untreated sample has a caspase 9 activity of about 0.127397 activity units/mg of protein. Thus, here CP induced more caspase activity of both C7 and C9. Caspase pathways are considered as suitable candidate targets for anticancer drug discovery. It has been reported that some commonly used plant-derived anticancer drugs such as tamoxifen, doxorubicin, etoposide and cisplatin promote apoptosis in tumor cells through caspase pathways.

One of the hallmarks of cancer is very less apoptotic activity, which therefore, results in the uncontrolled growth of malignant cells and eventually, their metastasis. Hence, it plays a pivotal role, as the increasing treatment strategies for cancer involve the induction of apoptosis in the cancer cells [12]. In this study, the induction of apoptosis by the CP ethyl acetate extracts in the MCF-7 cell lines was confirmed using Annexin V assay with flow cytometric analysis. The flow cytometric analysis clearly shows a significant increase in the percentage of apoptotic cells on treatment with CP extracts in MCF-7 cell lines as opposed to the untreated cells.

Apoptosis is characterised by chromatin condensation and fragmentation, overall cell shrinkage/rounding and formation of apoptotic cell bodies and any disturbance to the regulation of apoptosis plays a major role in cancer growth. Thus, any compound which regulates apoptosis is considered anti-cancerous. DNA fragmentation is the hallmark of apoptosis and DNA ladder observation is one of the methods to assess it [13]. DNA laddering pattern in agarose gel electrophoresis confirms DNA cleavage in the cells as result of apoptosis induction. In this study, CP induces apoptosis and hence expected DNA laddering pattern was observed.

CONCLUSION: It can be concluded that *Carica papaya* has potential anti-cancer compared to other medicinal plants. The phyotochemical properties were identified by the anti-cancerous activity through MTT assay, DNA fragmentation assay, caspase 7/9 induction detection assay and Annexin-V FITC assay and through wound healing assay it is understood that it also has significant anti-metastatic activity.

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CONFLICTS OF INTEREST: The authors confirm no conflict of interest pertaining to this work.

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