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ORIGINAL ARTICLE

Effect of Ethanolic Extract of *Carica papaya* Leaves and their Cytotoxicity and Apoptotic Potential in Human Ovarian Cancer Cell Lines- PA-1

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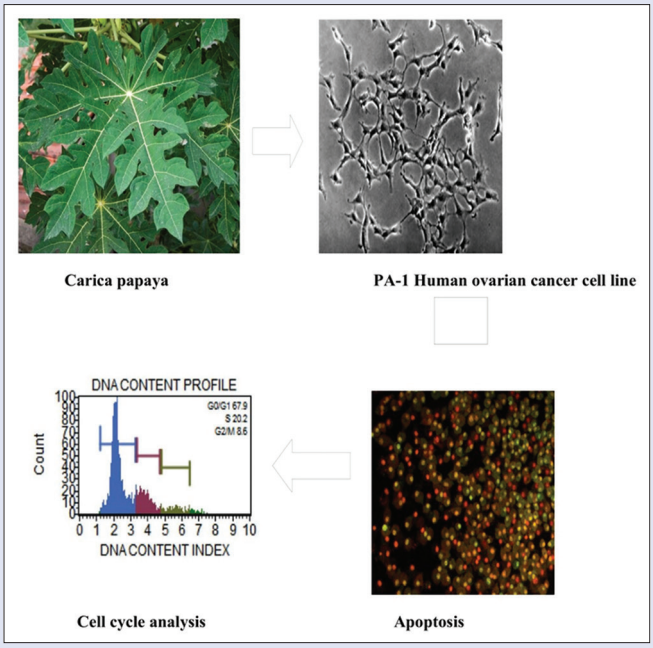
ABSTRACT

Introduction: The aim of the present study is to investigate the efficacy of *Carica papaya* ethanolic extract for antiproliferative, cytotoxicity, apoptotic, cell cycle blockade and wound healing in the ovarian cancer PA-1 cell lines. **Materials and Methods:** PA-1 cells were treated without the sample (act as a control) and with the sample for the above assay as per the described protocol. **Results:** The end result antiproliferative showed that PA-1 cell viability decreases in a concentration dependent manner and the growth inhibitory effect (IC_{50}) values are obtained at a concentration of 100 µg. The increase in number of apoptotic cell was observed and after treating with PA-1 cells the sample with double staining methods. G2/M phase of the cell cycle was significantly blocked by the test sample followed by the S phase in a negligible manner. And *in vitro* cell wound closure or contracture was not significant when compared the sample against control group. **Conclusion:** *C. papaya* ethanolic extract showed to possess excellent cytotoxic effect through inducing apoptosis especially causing arrest at the G2/M phase of the cell cycle.

Key words: Apoptosis, cancer, *Carica papaya*, cytotoxicity, phytochemicals, plant extract

SUMMARY

- In this study, it may be concluded that the ethanolic extract of *Carica papaya* through the presence of several phytochemicals suppressed the subG1 and G_1/G_2 phases of cell cycle progression in PA-1 cell lines (human Ovarian cancer cell lines) by apoptosis mechanism thereby exert its anti proliferative activity. This extract can be developed as a therapeutic candidate for the treatment of Ovarian cancer after, undertaking proper clinical and preclinical evaluation.



Abbreviations used: FBS: Fetal bovine serum; DMEM: Dulbecco's modified eagle medium; PBS: Phosphate-buffered saline; AO: Acridine orange; EB: Ethidium bromide; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide

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INTRODUCTION

Cancer is a major public health burden life-threatening and dreadful disease with unexploring treatment modalities in the conventional system of medicine.[1] Ovarian cancer is the seventh most common cancer and the fourth leading cause of cancer death in women worldwide.[2] The pathogenesis and progression of ovarian cancer is not well understood, which contributes to its poor survival, along with difficulties in early detection among asymptomatic women. In spite of multimodality therapy in terms radiation, chemotherapy, immune

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modulation, and surgery the morbidity and mortality rates are very high claiming an imperative need of new cancer management.[3] The World Health Organization (2001) has estimated that between 80% of the populations are treated through medicines or medicinal formulations obtained from plant sources.[4] Hence, screening for phyto-chemical and pharmacological properties from different traditional plants are necessitated to identify a more efficient chemotherapeutic drug with better efficacy to target different signaling pathway in a precise, specific and sensitive way to control cancer proliferation and induce apoptosis.

Carica papaya is a member of the Caricaceae and is a dicotyledonous, polygamous, and diploid species.[5] It originated from Southern Mexico, Central America, and the northern part of South America. It is now cultivated in many tropical countries such as Bangladesh, India, Indonesia, Sri Lanka, the Philippines, and the West Indies including Malaysia. Malaysia is known to be one of the top 5 papaya exporting countries.[6] The papaya fruit is globally consumed either in its fresh form or the form of juices, jams, and crystallized dry fruit.[7] The ripe fruit is said to be a rich source of Vitamin A, C, and calcium.[8] There are many products derived from the different parts of the *C. papaya* plant, leaves have been used in folk medicine for centuries. Recent studies have shown its beneficial effect as an anti-inflammatory agent for its wound healing properties, antitumor as well as immune-modulatory effects and as an antioxidant.[9] *C. papaya* leaves juice revealed that it was safe for oral consumption.[10] They are also used as anti-inflammatory, antitumor agent and serving as an immune-adjuvant for vaccine therapy[11,12]

However, the leaves are of particular interest in view of anticancer property, but till date, there is limited and incomplete research has been performed on this traditional plant. Moreover, screening of crude extract or phytochemical potential to induce apoptosis has not been properly elucidated in the past as it is the best strategy for developing a novel anticancer drug. Hence, the objective of the present study is to investigate the *C. papaya* leaves potential for their antiproliferative and apoptosis inducing activities on human ovarian carcinoma (PA-1) cell line.

MATERIALS AND METHODS

Chemicals

The chemicals such as fetal bovine serum (FBS), Dulbecco's modified Eagles medium (DMEM), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), Penicillin-Streptomycin, Ethidium bromide were purchased from Sigma-Aldrich, USA, and Trypsin was procured from Gibco, USA. Rest of the chemicals employed in the studies was analytical grade.

Collection of *Carica papaya* leaves and preparation of crude extract

The leaves of *C. papaya* were collected from Chennai. The plant specimen was deposited and authenticated by Dr. S. Sankaranarayanan, Professor and Head, Department of Maruthuva Thavaraiyal, Medicinal Botany and Pharmacognosy Government Siddha Medical College, Arumbakkam, Chennai, India. The herbarium file of the same Centre contains the voucher specimen. The leaves were cleaned in the running tap water and shade dried. The dried material was further subjected to a fine coarse powder using a blender and then the powder was stored in for further use. Crude plant extract was prepared by Soxhlet method. About 150 g of *C. papaya* leaf powder was weighed and macerated in 450 ml of ethanol. The powdered material was kept for occasional shaking for 72 h at room temperature. After 72 h the supernatant was collected by filtration and the solvent was evaporated. The crude extract was stored in 4°C until further use.

Procurement and maintenance of pa-1 (human ovarian cancer) cell lines

The human ovarian cancer (PA-1) cell line was initially procured from National Centre for Cell Sciences, Pune, India, and maintained in DMEM. The cell lines was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100 µg/ml). Cultured cell lines was kept at 37°C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100 µl cell suspension (5×10^4 cells/well) was seeded in 96 well-tissue culture plate and incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

PA-1 cell lines treatment for cytotoxicity assay

The 96 well seeded, 24 h incubated PA-1 cell lines were treated with aliquots of ethanolic extract of *C. papaya*, (EECP) dissolved in 5% dimethyl sulfoxide (DMSO) with the addition of growth medium and incubated further for 72 h to study the anti-proliferative activity. The positive drug Doxorubicin is also used in this study. PA-1 cell lines without any treatment were used as a negative control.

Cytotoxicity assay by direct microscopic observation

The entire plate was observed at the end of 72 h in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) for the detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells which were considered as indicators of cytotoxicity and were recorded as images.

In vitro anticancer and wound healing assay

Cytotoxicity assay by direct microscopic observation

Entire plate was observed at an interval of each 24 h; up to 72 h in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as shrinking of cells or rounding, granulation, and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay

The antiproliferative effect of ethanolic extract of *C. papaya* was assessed by the method of Laura B. Talarico assay was against in PA-1 cell line. 15 mg of MTT was reconstituted in 3 ml phosphate-buffered saline (PBS) until completely dissolved and sterilized by filter sterilization. After 24 h of incubation period, the sample content in wells was removed. 30 µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well and then incubated at 37°C in a humidified 5% CO_2 incubator for 4 h. After incubation period, the supernatant was removed and 100 µl of MTT solubilization solution (DMSO) 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 using microplate reader at a wavelength of 570 nm. Ethanolic extract efficiency for cytotoxicity was measured in terms of growth inhibition percentage for different concentration using the below formula.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of growth inhibition} = \left[\frac{\text{Mean OD samples}}{\text{Mean OD of control group}}\right] \times 100$$

Ethanolic plant extract compound stock and test concentration for apoptosis and cell cycle analysis

1 mg of plant compound was added to 1 ml of DMSO and dissolved completely by cyclomixer. Different concentration of the test sample was prepared in serial dilution were (25 µg/ml, 50 µg/ml, and 100 µg/ml) of 5% DMEM. In short after 24 h, the growth medium was removed, freshly prepared each plant extracts in 5% DMEM were five times serially diluted by two fold dilution (25 µg/ml, 50 µg/ml and 100 µg/ml 5% DMEM and each concentration of 100 µl were added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO_2 incubator for 24 h.

Assessment of apoptosis by double staining acridine orange/ethidium bromide

The type of cell death which occurs due the treatment of the extract was determined by acridine orange/ethidium bromide (AO/EB) staining according to the method of Zhang *et al*. After incubation the cells were washed in cold PBS and then stained with a mixture of AO (100 µg/ml) and EtBr (100 µg/ml) at room temperature for 10 min. The stained cells after washing twice with 1X PBS is viewed under fluorescence microscope (blue filter-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). The control and the positive control cells were also treated in the same manner.

Apoptosis by annexin V-FITC/PI assay

The quantitative assessment of apoptosis was performed by method of annexin *V-FITC/PI* assay. Here, the treated cells were trypsinized and centrifuged for 5 min at 1200 rpm. Cell pellets were washed and resuspended in PBS and to produce the concentration of 1×10^6 cells per ml after centrifugation. By using annexin*5-FITC* apoptosis detection kit and following the manufactures instructions, stained apoptotic cells were analyzed in the flow cytometry.

Cell cycle analysis

The treated cells were washed in PBS and using 70% ice cold ethanol and stored for 12 h at 4°C. The samples were centrifuged at for 5 min at 3000 rpm supernatant was removed and pellet was dissolved in 250 µl PBS and once again centrifuged. The pellet was dissolved in 250 µl of MUSE cell cycle reagent and centrifuged. The supernatant was discarded and the resultant solution was incubated for 30 min at dark and flow cytometric analysis was performed. DNA content as well as percentage of cells in phases of the cell cycle was determined. Gating was performed comparable with untreated control cells and samples.

Scratch wound healing assay

Exponentially growing cells were trypsinized and seeded at a density of 200,000 cells per well into 12-well plate for 24 h incubation (~90% confluence). The scratch wounds were made by a sterile 1 mL pipette tip through a pre-marked line. After removal of the resulting debris from five lineal scratches, the PA-1 cell monolayer was subsequently rinsed three times with PBS followed by incubation with sample for 24 h. The wound areas were displayed by taking images just above the interchanges between scratched wound areas and premarked lines and the effect of sample on wound closure was determined microscopically (×4 magnification, Olympus CKX41) after 24 h of incubation. The effect of sample on wound closure was captured using MRI-Image software.

Statistical analysis

All the experimental samples were done in triplicate to validate the test results. Statistical analyses were performed using latest SPSS version. The efficiency of the test sample was compared between treated and untreated PA-1 Human Ovarian cancer cells by employing independent sample '*t*-'test and paired sample *t*-test. A $P < 0.05$ was considered as a significant value.

RESULTS

Measurement of cell viability

There were no detectable changes in the morphology of the cells, such as rounding or shrinking of cells were observed. Apart from that, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity. The experimental cells which showed the viability above 95% was carried out for further experiments. Increased cytotoxic activity is noticed in the cells treated with 25 mg/ml and 50 mg/ml of EECP extracts [Figure 1].

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assay

Anti-proliferative potential in terms of anticancer assessment was done on *C. papaya* determines the viability of the cells against PA-1 cells at varying concentration ranges. The end result showed that PA-1 cell viability decreases in a concentration dependent manner and the growth inhibitory effect (IC_{50}) values are obtained at a concentration of 100 µg/ml. This concentration was used in cell cycle analysis by flow cytometry analysis [Figure 1].

Apoptosis assessment

The DNA-binding dyes AO and EB were used to identify the apoptosis and necrosis of the PA-1 cells due to EECP treatment. In the present study due to the plant extract treatment there was a considerable cell death in the PA-1 cells when comparable to control untreated cells [Figure 3]. The cells treated with 50 µg/ml of EECP extract showed both showing early apoptotic cells with bright green colored nuclei with chromatin condensation and late apoptotic cells with bright orange colored nuclear areas with condensed chromatin. However, necrotic cells are also visualized with bright orange colored uniform nucleus with intact membrane. The PA-1 cells treated with 100 µg/ml of EECP extracts showed an increased late apoptosis and necrosis when comparable to the group treated with 50 µg/ml of EECP extracts. It was also observed the number of apoptotic cells were more than the necrotic cells as evidenced by the presence of irregular shaped and shrinkage of cell membrane a characteristic feature of apoptotic cells than necrotic cells which exhibits intact cell membrane.

Apoptosis by annexin V-FITC/PI assay

In order confirm the apoptosis process exhibited by the EECP, the annexin *V-FITC/PI* assay was performed. It is found that EECP at lower concentration of 50 µg/ml significantly induced the apoptosis when comparable ($P < 0.01$) to higher concentration of 100 µg/ml which showed less apoptotic potential in the preset study [Figure 5 and Table 1].

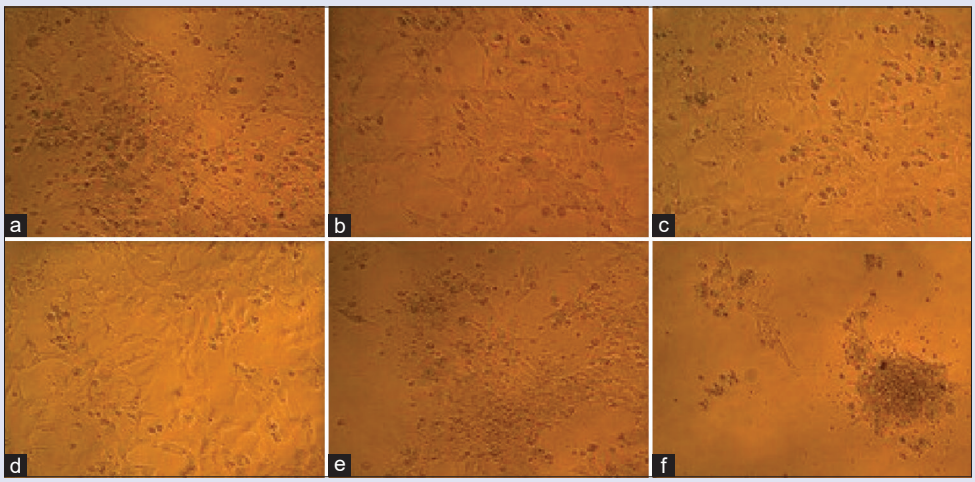

Figure 1: Morphological changes of PA-1 cells after 72 h of treatment (a) untreated (control) treated with (b) 6.25 mg/ml (c) 12.50 mg/ml (d) 25 mg/ml (e) 50 mg/ml of *Carica papaya* leaf extract. (f) Doxorubicin treated cells viewed under an inverted light microscope (×200)

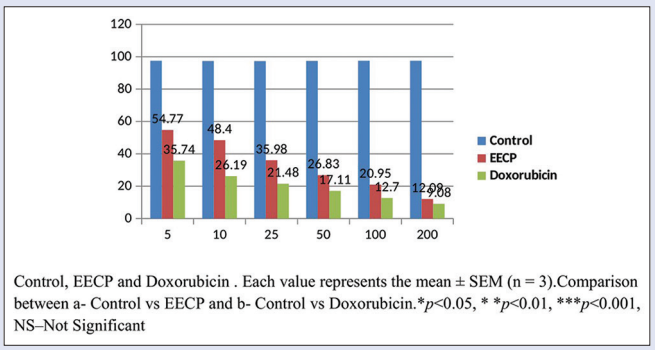

Figure 2: Cell viability of PA-1 cell lines (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide assay) of control, EECP, and doxorubicin. Each value represents the mean ± standard error of mean (n = 3). Comparison between (a) Control versus EECP and (b) Control versus doxorubicin. *P

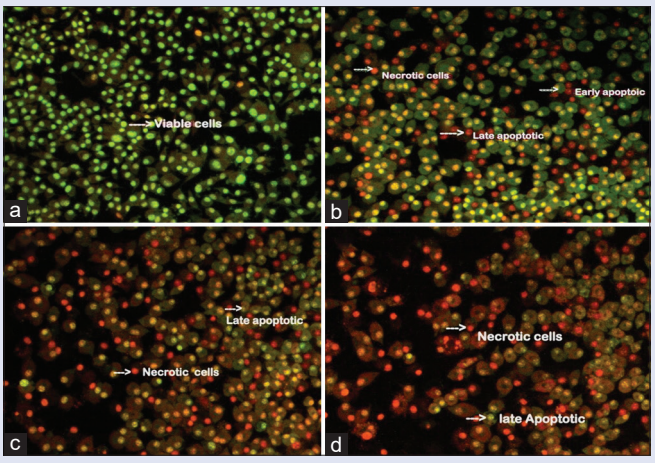

Figure 3: Apoptosis assessment in PA-1 cells by double staining acridine orange/ethidium bromide staining of PA-1 cells treated with ethanolic extract Excoecaria agallocha for 72 h and viewed under the fluorescence microscope (×200). (a) Control untreated viable cells with normal green nucleus (b) 60.5 µg/ml extract treated cells showing early and late apoptotic cells and necrotic cells (c) 100 µg/ml extract treated cells showing late apoptotic cells and necrotic cells (d) doxorubicin-treated cells showing the late apoptotic cells and necrotic cells

Table 1: Apoptosis-Annexin *V-FITC/PI* assay of PA-1 cell lines (of Control, EECP and Doxorubicin

PA-1 Cell Treatment	% Apoptosis
Control (Untreated)	5.35 ± 0.12
EECP Treated (50µg/ml)	25.34 ± 1.08a**
EECP Treated (100 µg/ml)	12.31 ± 0.30b**
Doxorubicin Treated (10µg/ml)	40.35 ± 0.69c**

Each value represents the mean ± SEM (n = 3).Comparison between a-Control Vs EECP 25mg/ml treated, b- Control Vs EECP 50mg/ml treated and c-Control Vs Doxrubicin. *P<0.05, **P<0.01, ***P<0.001, NS–Not Significant

Flow cytometry analysis

The efficiency of the ethanolic leaf extract on the cell cycle progression through apoptotic induction was measured by flow cytometry after staining with propidium iodide [Figure 4]. Result shows in the untreated group that 67.9% of cells were in G0/G1 phase, 20.2% cells were found in "S" phase and 8.6% cells were seen in the G2/M phase. In the case of the EECP leaf extract treated PA-1 cells, only 58.5% of cells were in G0/G1 phase, 25.8% cells were found in "S" phase and 11.4% cells were seen in the G2/M phase. From these results, it is evident that the EECP induces cell death in PA-1 cells in sub G1 phase and its accumulation when comparable to the control cells. Although there was increase in the gated cells in "S" phase and G2/M phase of treated cells, the significant decrease in the G0/G1 phase indicates the antiproliferative potential of our extract. This result indicates that the major block in cell cycle progression is induced at the G2/M phase followed by S phase by the ethanolic extract of the leaf.

Wound scratch healing assay

PA-1 cell line was tested through the wound scratch healing assay to determine to identify capacity of these cells to migrate under phytoextract stimulus, the inhibitory potential of plant extracts toward the cells responsible for the wound healing and closure [Figure 5]. In the present investigation comparable to the untreated control cells EECP-treated cells showed a slower migration and wound closure of the scratch indicating the impaired multiplication of cells which is a good indicator for any extract with antiproliferative activity.

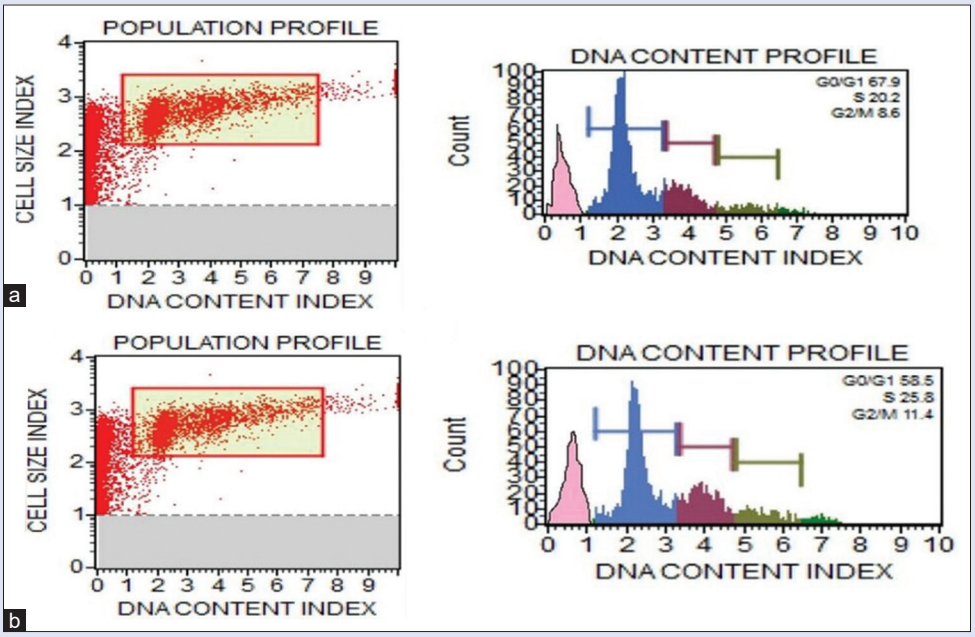

Figure 4: Cell cycle analysis of PA-1 (a) Gated cells of untreated group, (b) Gated cells of ethanolic extract of *Carica papaya* treated group

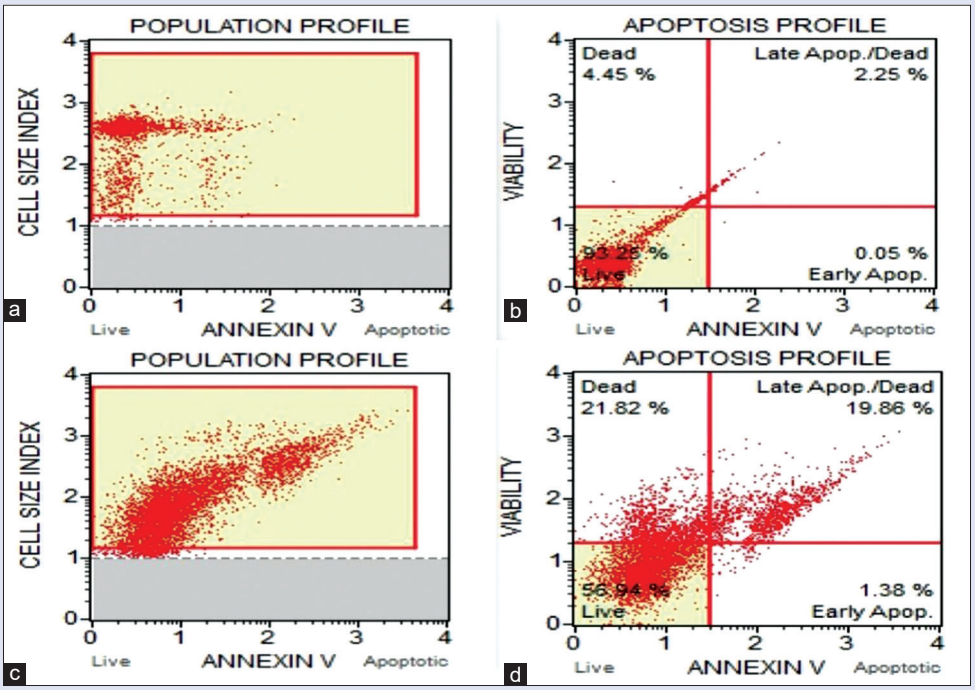

Figure 5: Apoptosis- annexin V-FITC/PI assay of PA-1 cell lines

DISCUSSION

India is enriched with flora and fauna and plenty of healthful plants have the lore claim of treating dreadful diseases as well as cancer. The leaves of papaya is of particular interest seeable of metastatic tumor property, since until date there are restricted and incomplete analysis are performed on this ancient plant at the moment it's understood that the phyto-pharmacologic activity exhibited by any ancient medicine are thanks to their additive phyto constituents instead of the only compound.[13] These complicated mixture of the bimolecules thanks to their synergistic additionally as agonistic character exert the specified pharmacological activity.[14] Keeping these facts in mind we tend to create an endeavor to review antiproliferative activity of genus *C. papaya* ethanolic leaf extract in PA-1 cell lines that is employed to review the female internal reproductive organ cancer.

Apoptosis, programmed death and a well-structured physiological method to eliminate unregulated/abnormal or broken cells stay as golden screening terminus for locating anti-cancer medicine.[15] they are the

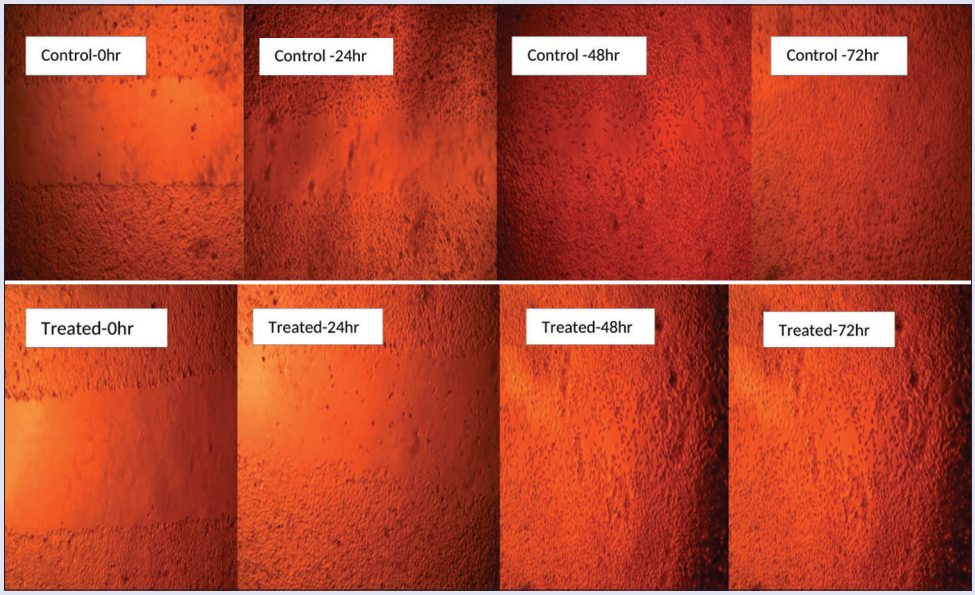

Figure 6: Effect of wound closure in the control and ethanolic extract of *Carica papaya* treated PA-1 cells at different time intervals

common targets for presently clinically used and promising metastatic tumor agents. There is a lot of rational for human to use screening methodology through necrobiosis, the agents that induce broad toxicity are often excluded. Broad vary of natural substance that has the potential to induce necrobiosis for various neoplasm cell of origin has been documented. These natural substances of clinical values are typically gift in plant sources and consumed by humans in lesser quantities. Hence, it becomes essential to searched for the inducers of necrobiosis from such plant resources.[16]

Antioxidant on the market from nature possesses a broad vary of organic chemistry functions as well as inhibiting reactive O species, scavenging free radicals and living thing oxidation-reduction potential modifications. Recently, Chaudhary and Ali[17] have reviewed papaya supported its pharmacognostic and ethnomedicinal properties and finished that additional studies (pre-clinical and clinical) have to be compelled to be performed to elucidate the most potential of this plant.

The direct assessment of cell viability by confocal research confirms the composition necrobiosis in terms of rounding error or shrinking of cells, granulation and status within the living substance, etc., the photographs obtained from EECP extract treated cluster showed a attenuate variety of cells with shrinkage as admire the management untreated cluster shows that the phyto constituents gift within the extract possesses a possible to induce the death within the treated PA-1 cells. The MTT assay may be a colorimetrical assay to assess the cellular metabolic activity. It is a wide used methodology for screening the cytotoxic activity of crude extracts, isolated compounds, and artificial medicine projected for metastatic tumor activity in several cell lines.[15] The research worker Bruno Walter Last, in his findings have according that the papaya leaf tea or extract possesses the property of neoplasm destruction.[16] The growth restrictive activity of the EECP extract on PA-1 cell lines, the female internal reproductive organ neoplastic cell line confirms the power of the extract in neoplasm destruction.

The vital characteristic feature of any metastatic tumor drug is to inhibit the cell proliferation by causing apoptosis.[17] The antiproliferative activity of the plant extract on PA-1 cells in terms of necrobiosis and mortification was confirmed AO/EB staining ways within the gift study. but within the discovery of metastatic tumor medicine the agents that induces necrobiosis or programmed death is taken into account as a beneficiary physiological method that scavenges the unregulated or abnormal cells, instead of mortification Associate in Nursing inflammatory response that invariably destroys the conventional cells the encompassing microenvironment.[18] to verify the apoptotic mode of death thanks to the EECP administration, Annexin/PI flow cytometric analysis was performed that evaluates the phosphatidylserine amino acid flipping. Annexin/PI assay may be a well-accepted biomarker for the necrobiosis method.[19] Cancer cells have associate in Nursing inherent property to resist necrobiosis to continue their uncontrolled cell proliferation and thence any compound that modulates necrobiosis could act as potential therapy agent for cancer medical care.[20] within the gift investigation a degree dependent apoptotic potential was discovered for the EECP extract as proven by shrinkage of cells, chromatin condensation that most likely could turn out the apoptotic bodies were engulfed by macrophages to shield the neighboring traditional cells.

In order to verify the metastatic tumor activity of the EECP extract the cell cycle analysis was performed. The results of the cell cycle analysis reveals that the plant extract considerably arrests the progression of cell cycle within the sub-G1 and additionally G0/G1 part as proven by the attenuate variety of cells therein part. There are many neoplasm suppressor proteins such as p53 and MDM2 play important role for the progression of the cell cycle at G0/G1.[21] This is attributed to the actual fact that the phyto chemicals gift within the extract plays a very important role in distressing these proteins thereby have an effect on the cell cycle progression. Parts such as Alkaloidscarpain, pseudoscorpion, and dehydrocarpaine I and II, choline, carposide, Vitamin C and E are according to[22] within the leaves of papaya could answerable for stunning the cell cycle progression. It is accepted that neoplasm development toward metastasis depends on the cell migration and invasion in any kind of neoplasm.[23] The wound scratch healing assay was performed to spot the extent of migration of the PA-1 cell lines at completely different time intervals treated with the EECP. Although the plant extract did not fully inhibit the migration of cells a discount within the multiplication and migration is discovered in comparison to the untreated cells, indicating the papaya leaves could also be developed into a promising drug for the treatment of female internal reproductive organ cancer [Figure 6].[24-26]

CONCLUSION

This study demonstrates that treatment with *C. papaya* ethanolic extract alone induce growth inhibition effect on the PA-1 cell lines in a dose dependent manner. Our study concludes that apoptosis plays role in *C. papaya* induced on ovarian cancer PA-1 cell lines exert Cytotoxicity activity by apoptosis mechanism through its phytochemical constituents. Moreover, the extract also suppressed the cell cycle progression at subG1 and G1/G2 phases indicating the agonistic action of the *C. papaya* bio molecules on the apoptotic regulatory proteins. Added to these inhibitions of cell migration were also observed in the present study. Based on the above findings, *C. papaya* leaf extract may be developed into a potential candidate for the treatment of ovarian cancer with proper preclinical and clinical evaluation level following a molecular investigation to explain the bioactive components and its associated mechanism that could trigger growth inhibition through this herbal extract.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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