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EFFECT OF ETHANOLIC EXTRACT OF *COLOCASIA ESCULENTA* LEAVES AND THEIR CYTOTOXICITY AND APOPTOSIS POTENTIAL ON OVARIAN CANCER CELL LINES PA-1

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ABSTRACT

In current years, the application of medicinal plants for the cancer prevention and treatment has gained more interest for the researchers due to the presence of phytoconstituents and lesser side effects. In the current finding, tropical medicinal plant *Colocasia esculenta* leaves was selected for its cytotoxic potential and apoptosis inducing effect against Pa-1 ovarian cancer cell lines. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide] assays were carried out to know the cytotoxic activity and apoptosis effect was examined using flow cytometry of *C. esculenta* leaves of ethanol extract. The cytotoxicity was observed highest, showing the IC_{50} concentration at 93.2 µg/mL of the leaf extract of *C. esculenta* on Pa-1 ovarian cancer cell line and observed dose dependent action against the concentrations. Apoptosis inducing effect of the extract showed 63.63% of late apoptotic cells and 11.22% of early apoptotic cells of Annexin V and PI compared to the standard drug Cisplatin (15uM) showing 41.76% of late apoptotic cells and 46.42% of early apoptotic cells. *C. esculenta* leaves of ethanol extract have potential for development as therapeutic principle for cytotoxicity on Pa-1 ovarian cancer cell line after further investigation.

Keywords: Apoptosis, Cancer cell lines, *Colocasia esculenta,* Cytotoxicity, Medicinal plants.

1. INTRODUCTION

In the modern biological sciecne, medicinal plants have been considered as major biomolecules due to its therapeutic potential to apply in various diseases [1]. *Colocasia esculenta* is an annual herbaceous perennial medicinal plant from tropical regions, belongs to the Araceae family and is a low cost and widely considered as edible plant in the food of human diet [2]. It is commonly called as "Taro" in English, "Arbi" or "Khuyya" in Hindi and "Kesavu" in Kannada. The plant is also known as "elephant ears," due to the broader shape of the leaves, when it is cultivated for decorative, medicinal and nutraceutical purposes [3]. In India, it is most common in coastal area as a wild variety and also cultivated for the use of vegetable, where the leaves, roots and corms can be used as dietary ingredients, but the plant must be cooked. The corms contain calcium oxalate that makes it acrid when eaten raw or partially cooked [4].

This medicinal plant has been reported for various pharmacological activities like antidiabetic, anti-inflammatory, antioxidant, anticancer, analgesic, anti-diarrheal and antimicrobial activities [5]. Main metabolites of the plants are flavonoids, glycosides, sterols, etc., these are exhibited the many biological properties of the *C. esculenta* and becomes important to check its efficacy to explore more therapeutic action [6]. The plant is rich in minerals such as calcium, magnesium, potassium and phosphorus. The starch is abundant in the roots and the tender leaves are rich in Vitamin C. The *in vitro* anticancerous studies on colonic adenocarcinoma cells have showed that the plant possess mechanism of inducing apoptosis in colon cancer cells and also activates lymphocytes to destroy cancerous cells [7]. The anthocyanins in *C. esculenta* have been reported to inhibit human cancer cell growth [8]. The moderate anticancer activity has been reported in the tuber and leaves extract [9].

There is a more attention and exploration of the treasure of hidden molecules of medicinal plants, in that more focus is on indigenous traditional medicinal plants to search safer, promising and easily available as natural products of medicine to treat various diseases. Hence, in the current study, we attempted to identify the efficacy of *C. esculenta* leaves, which was reported for its phytochemicals and to explore cytotoxic and apoptosis inducing effect on Pa-1ovarian cancer cell lines.

2. MATERIAL AND METHODS

2.1. Sample collection and authentication

The selected plant leaves of *C. esculenta* were collected during the period of August-December 2019 from the Hassan district of Karnataka, India, and the plant sample was authenticated by Dr. Shiddamallayya Mathapathi, Research Officer (Botany), at Regional Ayurveda Research Institute, Central Council for Research in Ayurvedic Sciences, Ministry of AYUSH and Government of India.

2.2. Processing of plant material

The healthy leaves were separated and washed thoroughly under tap running water followed by 0.1% NaCl to avoid fungal infections and shade dried at room temperature. Fine powder was prepared from the dried leaves of mesh size 20 mm [10].

2.3. Preparation of extracts

Leaf powder was extracted with ethanol using Soxhlet extractor for 3-4 h. Three hundred grams of leaf powder were subjected to Soxhlet extraction with 900 ml ethanol in the ratio of 1:3. The extract was collected and the solvent was evaporated under vacuum. The dried sample was stored at 4°C for the further experimental purpose [11].

2.4. Cell lines procurement and maintenance

Cell lines were obtained from National Centre for Cell Sciences, Pune, and Maharashtra, India, corresponding to ovarian (Pa-1) cancer cell lines. The cancer cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose supplemented with 10% fetal bovine serum. Incubation was carried out at 37°C with an atmosphere of 5% CO_2 maintained the cultures until they reach to 70% confluence growth and avoided the cross contamination.

2.5. MTT (3-(4,5-dimethylthiazol-2yl)-2,5-Diphenyltetrazolium Bromide) cytotoxicity assay

The assay was carried out on ovarian (Pa-1) cancer cell lines were determined by the MTT. Cell density (20,000 cell/well) was plated in 0.02 ml of medium/well in 96-well plates. The cell lines were culture in required medium and to allow them to grow for 24 hours. Appropriate concentrations of ethanolic crude extract (31.25, 62.5, 125, 250, and 500 µg/ml) dissolved in DMEM media high glucose (Cat No. AL111, HiMedia) were added and incubated for 24 h at 37°C with an atmosphere of 5% CO_2. Cisplatin with the concentration of 15 µM was used as a positive control for the study. After the incubation period, discarded the used media and 100 µl of MTT reagent (Cat No: 4060, HiMedia) was added followed by incubation for 3 h at 37°C. After incubation period, the formed formazan crystals were dissolved with 100 µl of DMSO (Cat No. 1309, Sigma) and the absorbance readings were taken by ELISA Reader (ELX 800, Biotek) at 570 nm and the inhibitory concentration (IC_{50}) value is calculated using linear regression equation, that is, Y =Mx + C derived from the cell viability graph.

The cells viability was determined by the following formula:

% of viability = (OD of test compound treated cells/OD of untreated cells) x 100

2.6. Apoptosis inducing effect by flow cytometry

Pa-1 cells were cultured in 6-well plates (5x105 cells/ well) and treated with 0.25 mg/mL, 1 mg/mL, 2 mg/mL, and 4 mg/ mL concentrations for *C. esculanta* leaves for 24 hrs. After treatment, cells were washed with Phosphate buffer solution (PBS), trypsinized, washed, and resuspended in binding buffer. Five microliters of propodium iodine (PI) and 5 µL Annexin V-FITC were added. After incubation for 15 min in the dark, 500 µL of binding buffer was added and ten thousand cells per group were analyzed using flow cytometry (BD FACSCanto A, BD Biosciences, USA). To detect PI and Annexin V-FITC, green solid state 488 laser was used for excitation. Filter configurations for PI and FITC were 556/LP and 585/ 40, and 502/LP and 530/30, respectively.

2.7. Statistical analysis

All the experiments were carried out in triplicates and were expressed as mean±standard error of the mean. The data were statistically analysed using Microsoft Office Excel 2007.

3. RESULTS AND DISCUSSION

The cytotoxic effect and apoptosis inducing effect of the ethanolic extract of *C. esculenta* leaves was evaluated on Pa-1 ovarian cancer cell lines. Research interest has been tremendously increasing into bioactive potential of medicinal plants to prevent and remedy for types of cancer due to lower toxicity, no side effects, easily available natural source, cost effective and stability.

3.1. Cytotoxic assay

Ability to reduce the cell viability capacity of cancer cell lines was assessed using the MTT assay. The ethanolic

extract has shown cytotoxic potential against the Pa-1 ovarian cell lines in cytotoxic MTT assay. A concentration dependent activity was exhibited by ethanolic extract of *C. esculenta* leaves in decreasing the number of viable cells. By increasing the concentration of the extract, the cell viability decreased gradually. The cytotoxicity effect of the extract was very high against the human ovarian cancer cell lines (Pa-1) by exhibiting the IC_{50} concentration at 93.2µg/mL and it has been considered as best concentration (Table 1 & Fig. 1). Similar studies on cell viability found at IC_{50} concentration at 130 and 30 mg/mL with *Pergularia daemia leaves* on ovarian cancer OAW-42 and Pa-1 cell lines respectively [12]. The plant extract having the ability to trigger, exhibit and execute cytotoxicity and apoptosis in cancer cell lines is clear and the MTT assay may be the involvement of mitochondria [13]. Reports of Machana et al exhibited the cytotoxic and apoptotic effects of six herbal plants against the human hepatocarcinoma (HepG2) cell line [14]. It is also came to know that, herbal drugs interplay to enhance immune system to detoxifying body cells and act as anticancer agents by inhibition of angiogenesis during cell differentiation [15].

Table 1: IC_{50} concentration of the ethanolic extract of *Colocasia esculenta* leaves on cell lines.

Sl. No	Treatment	Cell line	IC_{50}
1	Ethanol extract	Pa-1	93.2***µg/mL***

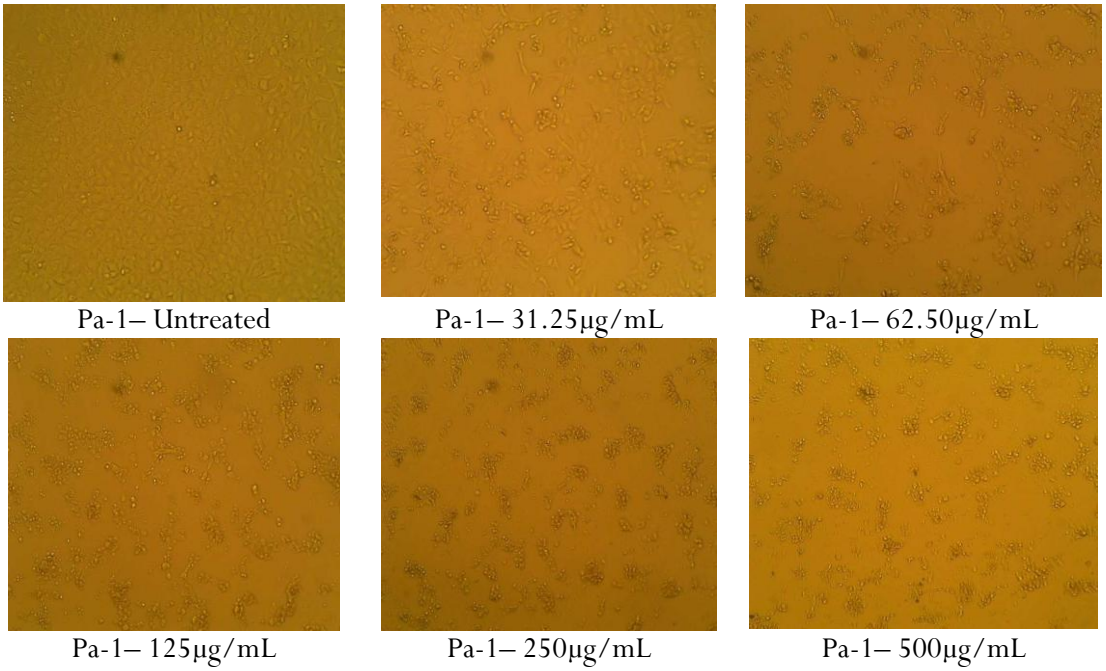


Fig. 1: Effect of ethanloic extract of *Colocasia esculenta* leaves on Pa-1 cell line at graded concentrations

3.2. Apoptosis assay by flow cytometry

In apoptotic cells, the membrane phospholipid phosphatedylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa $Ca2^{+}$ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including fluorescein isothiocyanate (FITC). Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation (Tables 2, 3 & Fig.2-6). The apoptosis was continued by evaluating the quantification of DNA, it is resulted as DNA fragmentation and exhibit the final step of apoptosis [16] The apoptotic inducing effect of the ethanolic extract of *C. esulenta* leaves was explored by staining the cells with APOPercentage dye and evaluation by flow cytometry. The results showed that the induction of cytotoxicity observed occurs through the mechanisms associated with apoptosis. The extract induced apoptosis in a concentration-dependent manner. Apoptosis is a tightly controlled and programmed cell death with abrupt expression of characters; morphological features are distinct in nature, like nuclear condensation, shrinkage

of cells, blebbing membrane, DNA fragmentation and apoptotic cell bodies were exhibit cell breakdown significantly [16].

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) to allow the investigator to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, where as the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative.

Table 2: Details of drug concentrations used for the study

Sl. No	Test Compounds	Cell Line	Concentration treated to cells
1	Cell Control	PA-1	No treatment
2	Standard Control (Cisplatin)	PA-1	15uM
3	Ethanol extract of the *C. esculenta* leaves (Samples)	PA-1	93.2uG/mL

Table 3: Table showing the % of cells of gated in different quadrants of Apoptosis in the untreated, standard and ethanolic extract of *C. esculenta* leaves treated Pa-1 cells and overlay of the results plotted in bar graph as below

Quadrant	% of Necrotic Cells	% Late Apoptotic Cells	% Viable Cells	% of Early apoptotic cells
Label	UL	UR	LL	LR
Untreated Cells	0	0	99.89	0.11
STD (Cisplatin)	0.49	41.76	11.33	46.42
Ethanolic extract of *C. esculenta* leaves	0.02	63.63	25.13	11.22

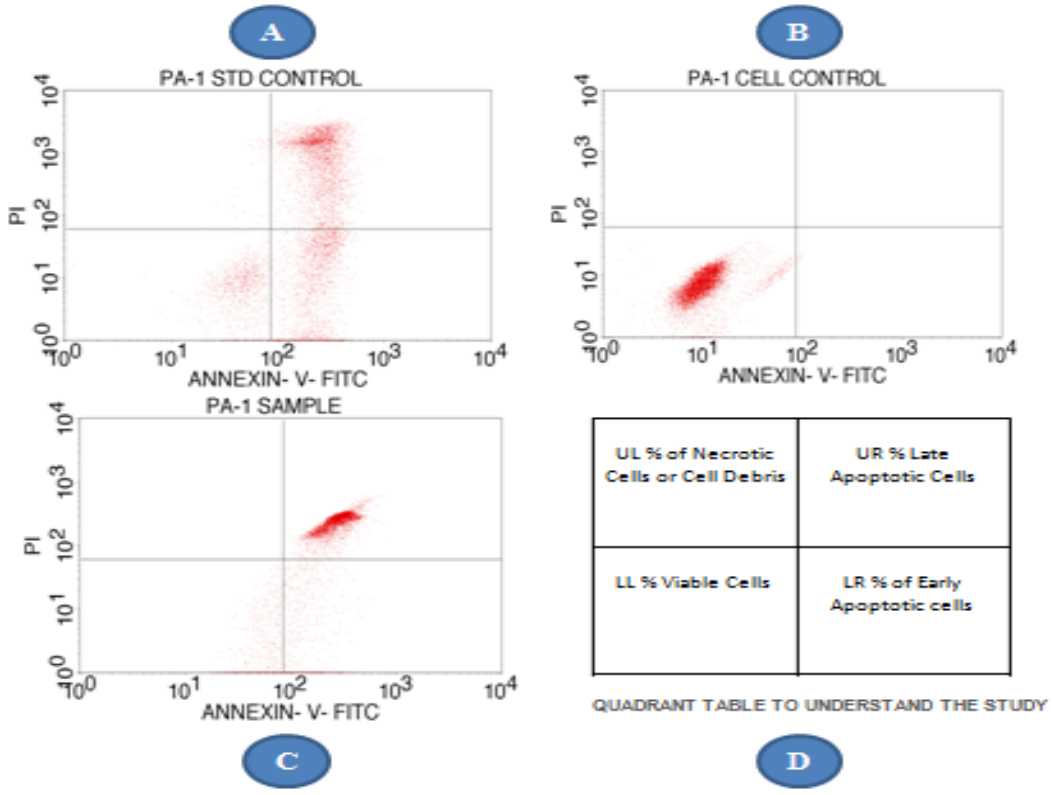
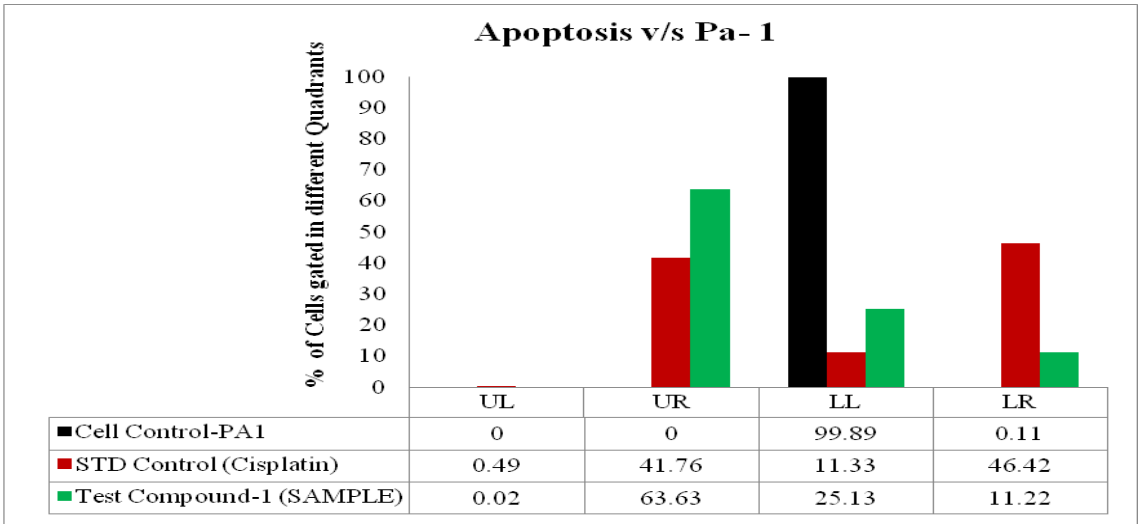
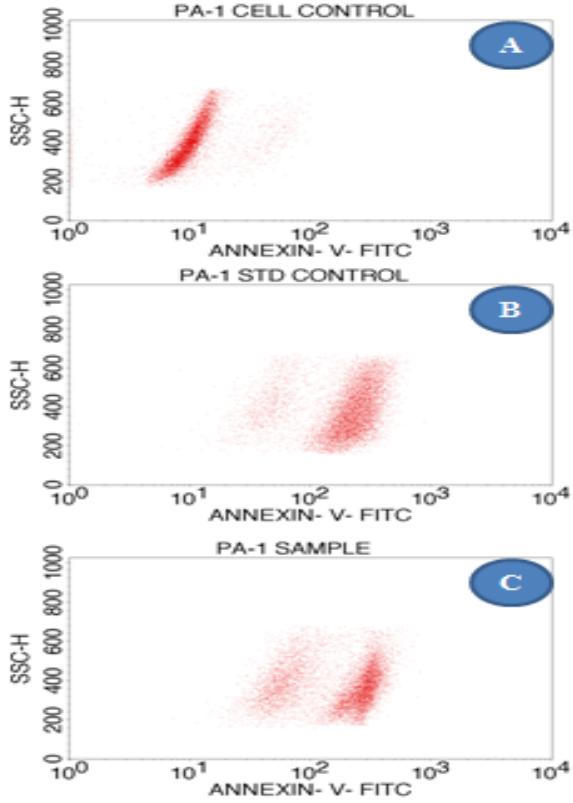


Fig. 2: Annexin V-PI expression study of ethanolic extract of *C. esculenta* leaves on Pa-1 cells using BD FACScalibur, Cell Quest Pro Software (Version: 6.0).



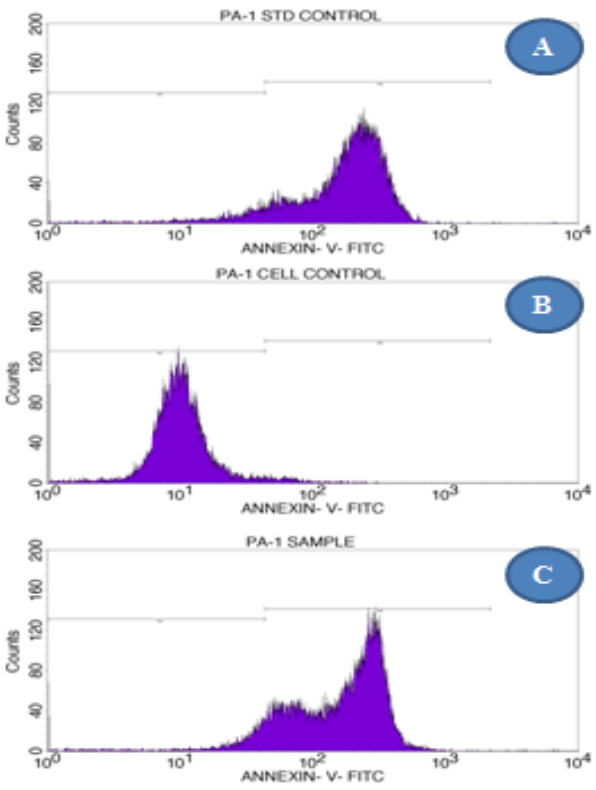
	UL	UR	LL	LR
Cell Control-PA1	0	0	99.89	0.11
STD Control (Cisplatin)	0.49	41.76	11.33	46.42
Test Compound-1 (SAMPLE)	0.02	63.63	25.13	11.22

Fig. 3: Annexin V-PI expression study of the ethanolic extract of *C. esculenta* leaves against Pa-1 Cell line



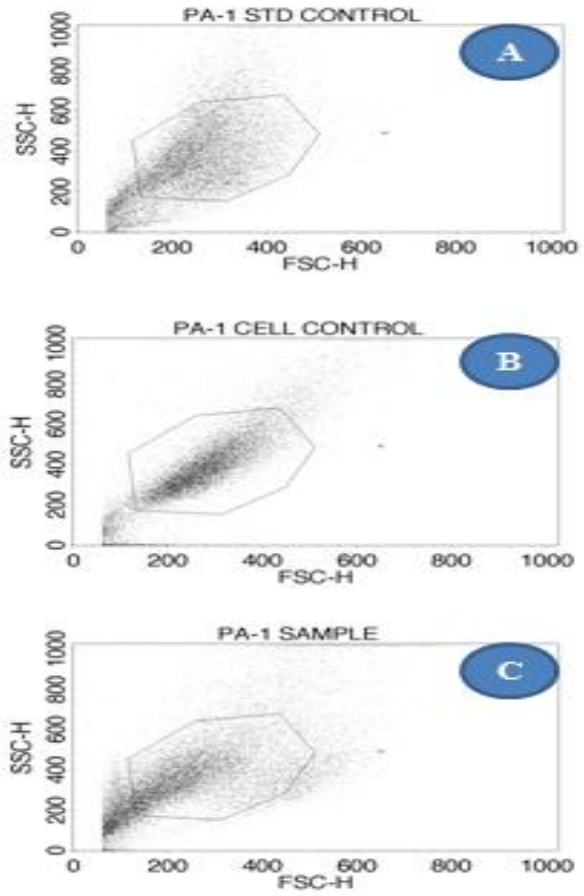
Flow cytometry analysis for Annexin V-ANNEXIN-V-FITC for forward scatter height (FSC-H) AND side scatter height (SSC-H) plot gating can be used to identify the distinct cell types in sample. A. showing the expression of given untreated PA-1 Cells, B. showing standard drug (Cisplatin-15uM) treated PA-1cells and C. showing ethanolic extract of C. esculenta leaves (PA-1 Sample) treated PA-1 cells against the Annexin V-FITC and Propidium Iodide Stain

Fig. 4: Evaluation of apoptosis induced by ethanolic extract of *C. esculenta* leaves treatment



Flow cytometry analysis for Annexin V-ANNEXIN-V-FITC staining. A. showing the expression of given untreated PA-1 cells, B. showing standard drug (Cisplatin-15uM) treated PA-1cells and C. showing ethanolic extract of C. esculenta leaves (PA-1 Sample) treated PA-1 cells against the Annexin V-FITC and Propidium Iodide stain.

Fig. 5: Evaluation of apoptosis induced by ethanolic extract of *C. esculenta* leaves treatment



Flow cytometry analysis for forward scatter height (FSC-H) AND side scatter height (SSC-H) plot gating can be used to identify the distinct cell types in sample. A. showing the expression of given untreated PA-1 Cells, B. showing standard drug (Cisplatin-15uM) treated PA-1cells and C. showing ethanolic extract of C. esculenta leaves (PA-1 Sample) treated PA-1 cells against the Annexin V-FITC and Propidium Iodide Stain

Fig. 6: Evaluation of apoptosis induced by ethanolic extract of *C. esculenta* leaves treatments.

In this study, ethanolic extract of the *C. esculenta* leaves with the IC_{50} concentration is used to check the Annexin V - PI expression study on the 1 cell line namely, Pa-1. The used concentrations of the extract, cell control and standard showing 63.63% of late apoptotic cells (UR) and 11.22% of early apoptotic cells (LR) of Annexin V and PI compared to the standard drug Cisplatin (15uM) showing 41.76% of late apoptotic cells (UR) and 46.42% of early apoptotic cells (LR).

Apoptotic inducing effect of Pa-1 cell lines at the 93.2uG/mL concentration of ethanolic extract of the *C. esculenta* leaves exhibited cells which are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because in either case, the dead cells will stain with both FITC Annexin V and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V and PI negative (viable, or no measurable apoptosis), to FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death). The movement of cells through these three stages suggests apoptosis. In contrast, a single observation indicating that cells are both FITC Annexin V and PI positive, in of itself, reveals less information about the process by which the cells underwent their death. The reports of Tayarani-Najaran et al. [17] got maximum activity in antiproliferative and apoptosis inducing effect on HeLa and MCF-7 cells by hydroalcoholic and n-hexane extracts of *Lavandula angustifolia* with the induction of essential oils for that the rate of apoptosis was 27.4% on 400 µg/mL after 48 h of treatment. In the cell lines of prostate found effective treatment with 0.05% (v/v) for 48 h showed an apoptotic rate (early and late) of 74.76% for PC3 cells and 10.64% for DU145 cells [**18**]. In each quadrants showing the expression of given untreated Pa-1 Cells (A) and standard drug (Cisplatin-15uM) treated Pa-1cells (B) and ethanolic extract of *C. esculenta* leaves (Pa-1 Sample) (C) treated Pa-1 cells against the Annexin V-FITC and Propidium Iodide Stain with their apoptosis study understanding table (D).

Table 3 shows the % of apoptotic cells gated in the different samples like untreated, standard drug and test compound namely ethanolic extract of *C. esculenta* leaves treated cells. Lower left quadrant population represents the viable cells. 99.89%, 11.33%, 25.13% of cells were found in untreated, standard and ethanolic extract of *C. esculenta* leaves respectively in lower left quadrant. Upper left quadrant represents cells debris or necrotic cells. 0%, 0.49% and 0.02% of cells were found in untreated, standard and ethanolic extract of *C. esculenta* leaves respectively in upper left quadrant. Upper right quadrant represents late apoptotic cells. 0%, 41.76%, 63.63% of cells were found in untreated, standard and ethanolic extract of *C. esculenta* leaves respectively in upper right quadrant. Lower right quadrant represents early apoptotic cells. 0.11%, 46.42% and 11.22% of

cells were found in untreated, standard and ethanolic extract of *C. esculenta* leaves respectively in lower right quadrant. The phytochemicals present in the crude extract of plants might be alkylate the DNA, causing breakage of DNA strand and leads to damage for the cell death [14]. The findings revealed that crude extract showed an induced apoptosis effect in the ovarian cell line Pa-1 cells.

Given ethanolic extract of *C. esculenta* leaves showing 63.63% of late apoptotic cells (UR) and 11.22% of early apoptotic cells (LR) of Annexin V and PI compared to the standard drug Cisplatin (15uM) showing 41.76% of late apoptotic cells (UR) and 46.42% of early apoptotic cells (LR). The observations suggest us that the test compound may have possible therapeutic potential against human ovarian cancer (Pa-1) derived diseases.

Hence present experimental approach proves the potential use of ethanolic extract of *Colocasia esculenta* as a source of anticancer agent.

4. CONCLUSION

The *in vitro* experimental findings on cytotoxic and induced apoptotic effect of the ethanolic extract against Pa-1 ovarian cancer lines with controls was investigated by the MTT assay compared with Cisplatin as a reference standard drug. Remarkable findings observed on cytotoxic activity of the extract were high against Pa-1 cell lines and it is evidenced with the findings of induced apoptosis studies by inhibition of growth, apoptosis, by arresting cell cycle and inducing of reactive oxygen species level in cancer cells. Thus the crude extract of plant which exhibited significant anticancerous activity further planned for future investigation on the isolation of novel anticancer bioactive molecules and their characterization.

5. ACKNOWLEDGEMENTS

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***Conflicts of interest**: none*

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