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Anticancerous efficacy of *Mansoa alliacea* leaf extracts on human epidermoid carcinoma A431 cells

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Abstract
Mansoa alliacea (Lam.) A. H. Gentry is a woody vine belonging to Bignoniaceae. The present study evaluated the antincancerous efficacy of *M. alliacea* leaf extracts against Human epidermoid carcinoma cell line A-431. Leaf extracts were prepared using different solvents *viz*., petroleum ether, chloroform, ethyl acetate, ethanol and water. Cell viability was tested with MTT assay. Ethyl acetate and ethanol extracts showed better IC 50 values *i.e*., 71.31µg/ml and 73.32 µg/ml. Further Annexin V and PI staining was used to check apoptosis and CASPASE-3 expression was evaluated using FITC Caspase 3 antibody with camptothecin as positive control. Ethyl acetate and ethanol extract showed 90.42% and 79.82% of early apoptotic cells compared to Standard drug Camptothecin showing 74.53% of early apoptotic cells. These results suggest that the Ethyl acetate and ethanol leaf extracts of *M. alliacea* may have possible therapeutic potential against Human epidermoid carcinoma cell line A-431.

Keywords: *M. alliacea*, A-431, caspase-3, apoptosis, bignoniaceae

Introduction
Cancer is the second leading cause of death worldwide after cardiovascular diseases. It remains as one of the leading causes of morbidity and mortality globally. It is responsible for one in eight deaths worldwide more than AIDS, tuberculosis, and malaria together. In the United States, one in four deaths is attributed to cancer. Many attempts were made to fight against this deadly disease through surgery, radiotherapy, and chemotherapy either alone or in combination (Shewach and Kuchta, 2009) [1]. Most cancers of the anus, cervix, head, neck and vaginal areas are epidermoid carcinoma, also called squamous cell carcinoma [2]. Human papillomavirus (HPV) may be involved in the multistep process of skin carcinogenesis as a co-factor with UV radiation. It is an emerging concern worldwide and is the second most common cutaneous malignancy with a higher mortality rate after basal cell carcinoma (Combalia and Carrera, 2020) [3]. For centuries researchers have been working on the anticancerous activity of the plants. Medicinal plants have been in use from time immemorial, and their utility has been increasing day by day in the present day scenario. Naturally obtained compounds are considered safer and readily biodegradable than chemotherapeutic drugs. Because chemotherapeutic medicines are more toxic and create many complications. And the problem of drug resistance observed in synthetic drugs is also reduced. Plants contain many phytochemical compounds and secondary metabolites used to treat several human ailments. According to an NCI report, approximately out of 35,000 medicinally important plants, 3,000 plants have shown effective anticancer activity (Desai *et al*., 2008) [4]. *Mansoa alliacea*, commonly called "garlic vine", is an important medicinal plant that belongs to the family Bignoniaceae. It has a specific garlic-like odour which makes it a unique plant having analgesic, anti-inflammatory, and anti-rheumatic properties and widely used for arthritis, rheumatism, body aches, muscle aches, injuries and pain [5]. Itokawa *et al*., (1991) [6] isolated and purified two important compounds such as 9-methoxy-α-lapachone and 4-hydroxy-9-methoxy- α-lapachone having cytotoxic ability against V-79 cells. Hence the present study is prompted us to investigate the anticancerous ability of *M. alliacea* leaf extracts to inhibit the growth of A431 skin cancer cells and to induce apoptosis.

Material and Methods
Plant Collection and Identification
M. alliacea plant was collected from Basavanagudi, Bengaluru, Karnataka, and was authenticated by the Botanical Survey of India (BSI), Pune [Accession/voucher No.:136264]. The Herbarium is kept in the Department of Botany, Bangalore University, Bengaluru.

Preparation of leaf extract
Fresh leaves of *M. alliacea* were collected and brought to the laboratory and immediately washed and dried in the shade for about three weeks. The dried leaves were powdered and extracted (Soxhlet extraction method) with five different solvents *viz*., Petroleum ether, Chloroform, Ethyl acetate, Ethanol and Water and concentrated by using Rotary evaporator. The crude extracts were stored at 4˚C for further use.

Cell culture
The A431 – Human Skin Cancer cell line cultures were Procured from National Centre for Cell Science, Pune and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium containing 10% (v/v) FBS until they reached confluency. Then they were trypsinised and washed with PBS for cell counting and seeding.

In vitro cytotoxicity MTT assay (Mosmann, 1983) [7]
Mosmann (1983) [7] developed a colourimetric method to evaluate the *in vitro* cytotoxicity based on the tetrazolium salt MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). Living cells reduce MTT to dark blue

formazan, which is measured using a spectrophotometer. MTT reagent was prepared by dissolving in PBS at 5mg/ml. 200 µl cell suspension was seeded into each well of 96-well plate with required cell density (20,000 cells per well). The cells were allowed to grow for about 12 hours. Later, they were treated with different concentrations (20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml, 320 µg/ml) of petroleum ether (MPE), chloroform (MC), ethyl acetate (MEA), ethanol (ME), aqueous (MW) leaf extracts and camptothecin (as standard) and incubated for 24 hours in 5% CO_2 incubator at 37ºC. Then the samples were removed, and the cells were treated with MTT reagent and incubated for another 3 hours. After the removal of MTT reagent, 100 µl DMSO was added to solubilize the formazan. The absorbance of the test samples was recorded with an ELISA reader at 570nm. The IC_{50} value was determined by using a linear regression equation, i.e., Y =Mx+C using MS Excel 2010. Where, Y = 50, M and C values were obtained from the viability graph.

Flow cytometry analysis

Preparation of cells

Ethyl acetate and ethanol leaf extracts showed IC 50 values of 71.31 and 73.32, respectively against the A431 cells. Hence, these extracts were used to study the mechanism of apoptosis by cell cycle analysis, Annexin V and PI staining and Caspase 3 activity using flow cytometry. A431 cells were cultured using DMEM media in three separate 6-well plates at a density of 3 x 10^5 cells/2 ml each and incubated in a CO_2 incubator at 37°C for 24 hours. The medium was removed and cells were washed with 1ml of 1X PBS. Later, the cells were treated with 71.31 µg/ml of ethyl acetate, 73.32 µg/ml of ethanolic leaf extracts, camptothecin (as positive control) and an untreated one (as negative control), incubated for 24 hrs. Then the extracts were removed from each well and the cells were washed with 500 µl PBS. Later, PBS was removed, and 180 µl of the trypsin-EDTA solution was added and incubated at 37°C for 3-4 minutes. Again, the culture medium was poured into each respective wells, and harvested into 12 x 75 mm polystyrene tubes. The tubes were centrifuged for five minutes at 300 x g at 25°C, and the supernatant was discarded. Later the cell pellet was washed twice with PBS and used for further analysis.

Cell cycle analysis by propidium iodide (PI) staining [8]

The cell pellet thus obtained was fixed in 1ml cold 70% ethanol by adding drop wise with constant vortexing to prevent clumping and leave the preparation for at least 30 minutes on ice. Later, the contents were centrifuged at 5000 rpm and washed twice with PBS. 50 µl of Ribonuclease A (RNase A) was added directly to the pellet to remove RNA. To this 400µl PI stain was added to stain only DNA. The PI stained cells were incubated for 5 to 10 mins at room temperature and the reaction mixture was analysed using flow cytometry.

Detection of Apoptosis by Annexin V-Propidium Iodide (PI) staining (Rieger *et al*., 2011) [9]

The programmed cell death is called apoptosis which alters the cell morphologically and molecular content. In the early apoptotic stages loss of cellular membrane asymmetry is a significant event where phosphatidylserine (PS) is externalised. Annexin V binds to the PS and can be detected by flow cytometry. PI is a membrane-impermeable red fluorescence nuclear dye. PI in combination with fluorescein isothiocyanate Annexin V is used to study apoptosis. The cell pellet was re-suspended in 1X Binding Buffer at a concentration of 1 x 106 cells/ml. 100 µl of the solution (1 x 106 cells) was transferred to a 5 ml culture tube, and 5 µl of FITC Annexin V was added. The cells were gently vortexed and incubated at room temperature in dark for 15 min. Later 5 µl of PI and 400 µl of 1X Binding Buffer was added to each tube and vortexed gently. Immediately the reaction mixture was analysed to detect the apoptosis by flow cytometry.

Caspase 3 activity by flow cytometry

A family of cysteine proteases called caspases are essential in inducing apoptosis (Crowley and Waterhouse, 2016) [10]. Many broken fragments resulted from the activity of caspases remain intact during apoptosis which can be detected by using specific antibodies. And this can be quantified by flow cytometry. Here CASPASE 3-FITC antibodies are used to analyse the caspase 3 expression in A431 cells after treating with *M. alliaceae* leaf extracts

0.5 mL BD Cytofix/Cytoperm solution was added to the cell pellet in a polystyrene tube. After 10 minutes, the tubes were washed with 0.5% bovine serum albumin (BSA) in 1X PBS and 0.1% sodium azide. 20 µL of Anti–Caspase-3 antibody was added and mixed thoroughly, incubated for 30 minutes in the dark at room temperature. Later the reaction mixture was washed with 1X PBS with 0.1% sodium azide, 0.5 mL of PBS was added, mixed thoroughly, and analysed for caspase 3 expression using flow cytometry.

Results and Discussion

***In vitro* cytotoxicity MTT assay**

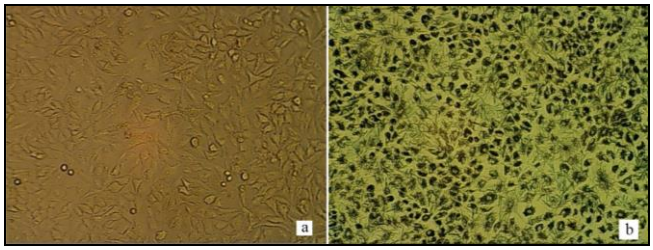

Fig 1: (a) A431 cells before adding MTT reagent (b) A431 cells after adding MTT reagent showing the formation of formazan in the live cells

In vitro cytotoxicity MTT assay was performed on A431 cells along with the standard camptothecin the results are represented in fig. 2. The morphology of A431 cells before adding MTT reagent (Fig.1a) are normal but irregular in shape and adhere to the walls of the 96 well plates, the change in colour of the cells (Fig.1b) after adding MTT reagent is due to the formation formazan which exhibit blue colour in the living cells.

The A431 cells were treated with five different leaf extracts (Fig. 2 c-g) along with positive (Fig. 2 b) and negative controls (Fig. 2 a), when observed under an inverted light microscope exhibit irregular morphological changes such as detachment of cells from the substratum, reduction in the size, fragmentation, and the cells gradually became shrunken and appears as small bodies. This is clearly attributed to the nature and differential features occurred in the cancer cells which is a typical characteristic feature of cell death.

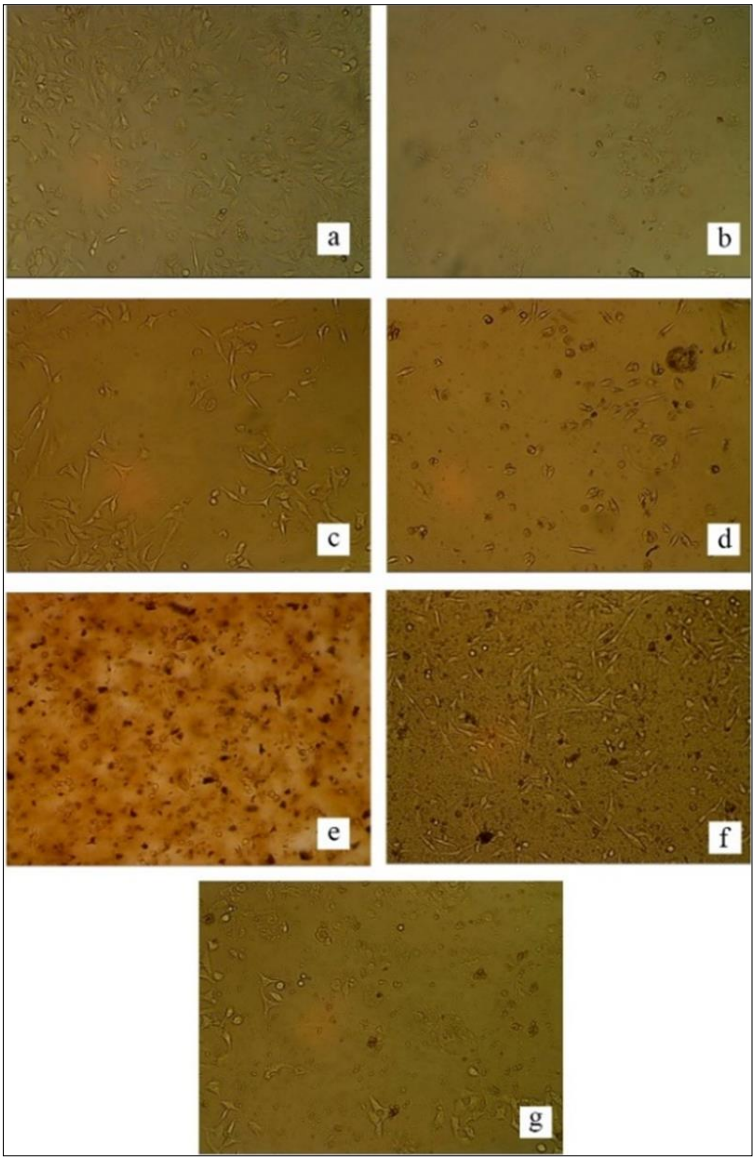

Fig 2: A431 cells treated with (a) untreated cells (negative control) (b) standard camptothecin (positive control) (c) petroleum ether leaf extract (d) chloroform leaf extract (e) ethyl acetate leaf extract (f) ethanolic leaf extract and (g) aqueous leaf extract of M. alliaceae

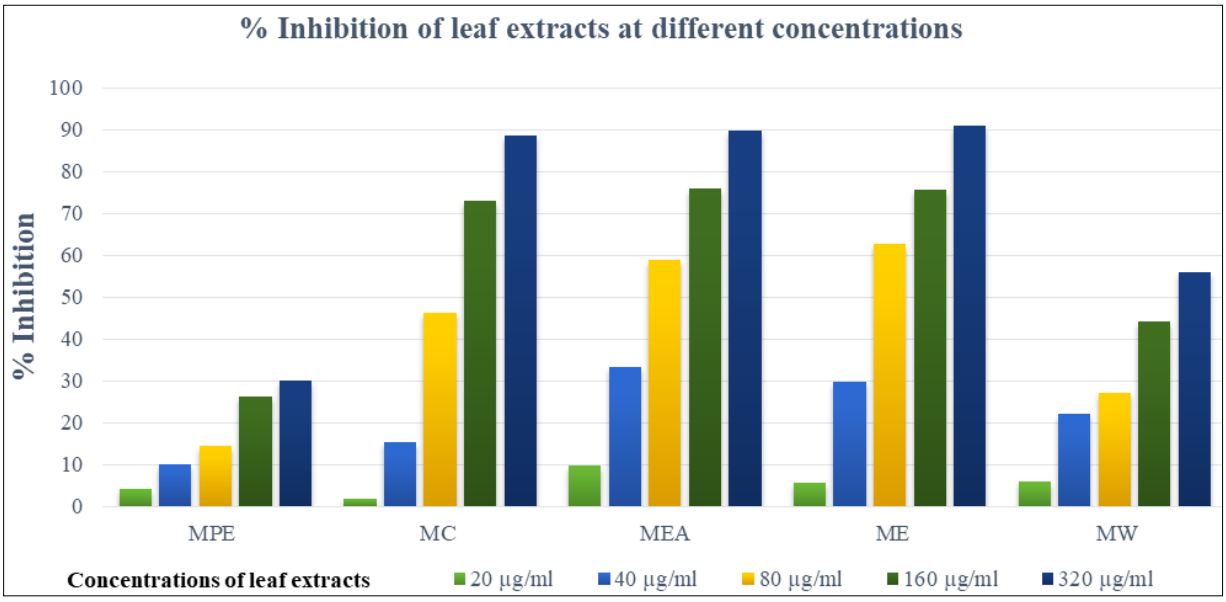

Fig 3*:* Percentage inhibition of Petroleum ether leaf extract (MPE), Chloroform leaf extract (MC), Ethyl acetate leaf extract (MEA), Ethanolic leaf extract (ME) and Aqueous leaf extract (MW) of *M. alliaceae* against A431 cell line at different concentrations

The percentage inhibition of five different leaf extracts of *M. alliaceae* at different concentrations exhibited against the A431 cell line are depicted in the fig.3. Ethyl acetate and ethanolic leaf extracts are effectively inhibited the cell's growth than the other three-leaf extracts. Based on the results obtained the IC 50 value was calculated, and the same is represented in fig. 4. The IC 50 values of MC is 93.46 µg/ml, MEA is 71.31 µg/ml, ME is 73.32 µg/ml and MW is 236.49 µg/ml. MEA and ME with lesser IC 50 values are considered as the best inhibitors of A431 cells. Hence, MEA and ME are used for further analysis.

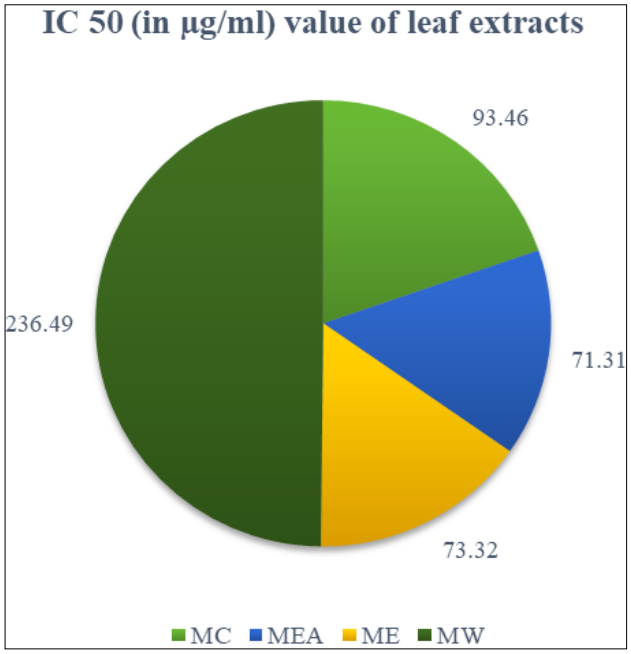

Fig 4: IC 50 (in µg/ml) value of Chloroform leaf extract (MC), Ethyl acetate leaf extract (MEA), Ethanol leaf extract (ME) and Aqueous leaf extract (MW) of *M. alliaceae*

Mohansrinivasan *et al.*, (2015) [11] analysed the cytotoxicity against skin cancer cell line A431 using petroleum ether seed extract of grapes by MTT assay and reported the maximum cell lysis at the concentration of 500 µg/ml, having the IC 50 value of 480 µg/ml indicated that such concentration could lyse 50 % of skin cancer cell lines. A detailed investigation on *in vitro* and *in vivo* anticancer activity of *Aristolochia rigens* root extracts was performed against the A431 cell line along with other human cancer cell lines by following SRB assay to determine *in vitro* cytotoxicity. The IC 50 value for the A431 cell line was 28 µg/ml (Akindele *et al.*, 2015) [12]. The anticancerous activity of a natural flavonoid, caffeic acid n-butyl ester (CAE), on various cell lines was reported by Zeng *et al.*, (2018) [13]. According to them, CAE exhibited significant anticancer activity against A431 cells with an IC 50 value of 20 µM. IC 50 value of *Vanilla planifolia* ethanolic leaf extract was calculated by MTT assay against A431 cells was found to be 31.2 µg/ml (Vijaybabu & Punnagal, 2019) [14]. Yan *et al.*, (2021) [15] synthesized and evaluated the anticancer activity of dendrocandin analogue against six human cancer cells by MTT assay. The synthesized compound showed potent cytotoxicity with the IC 50 value of 16.27 ± 0.26 µM. During the present investigation effective cytotoxicity of *M. allieace* ethyl acetate and ethanolic leaf extracts against A431 cells with IC 50 values of 71.31 and 73.32 µg/ml are in conformity with the earlier workers.

Cell cycle analysis by propidium iodide (PI) staining

To check the anticancerous potential of *M. allieace* leaf extracts, A431 cells were exposed to ethyl acetate (MEA) and ethanolic (ME) leaf extracts (extracts with better cytotoxicity against A431 cells), and then the percentage of cells arrested at different stages of the cell cycle was analysed using flow cytometry (fig. 5). The results were compared with that of the standard camptothecin.

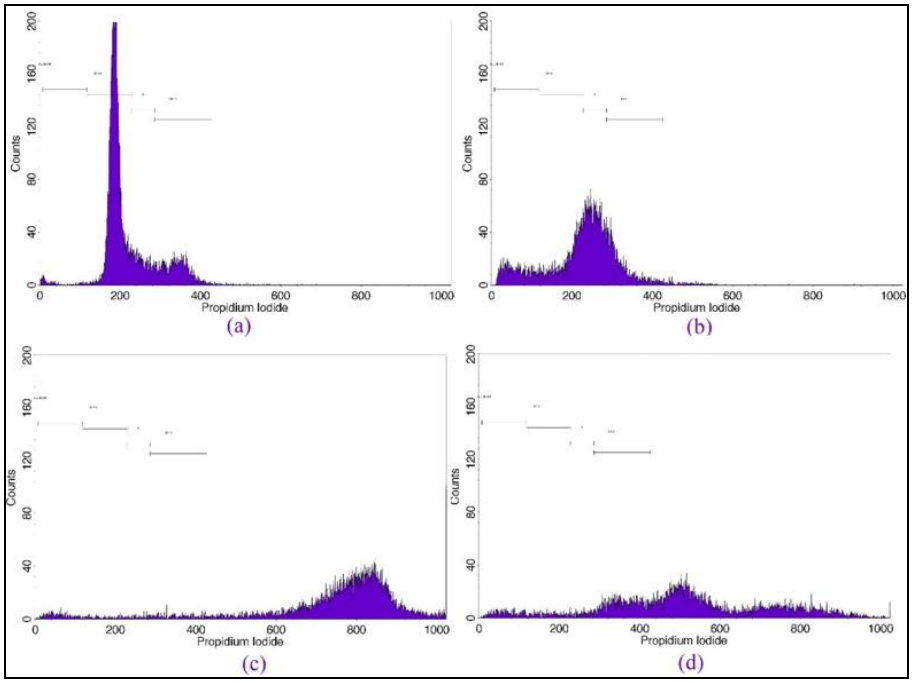

Fig 5: Cell cycle analysis of *M. alliaceae* leaf extracts against A431 cells. Propidium Iodide (PI) histograms of the gated cell singles distinguished cells at the Sub G0/G1, G0/G1, S and G2/M phases. (a) untreated cells (b) cells treated with standard Campothecin (c) cells treated with ethyl acetate leaf extract, and (d) cells treated with ethanolic leaf extract.

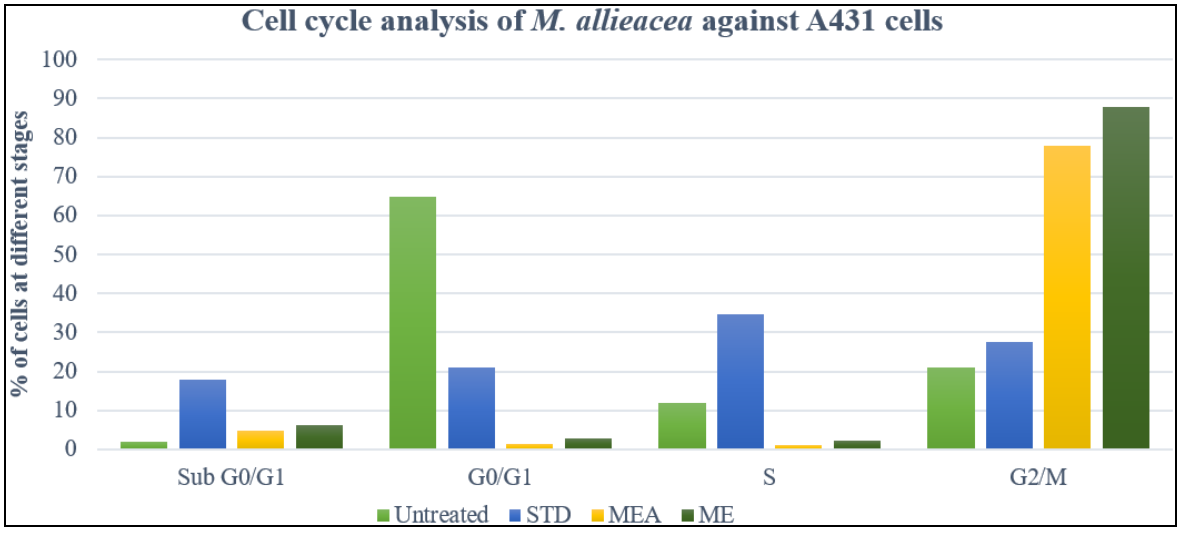

Fig 6: Percentage of A431 cells arrested after treating with M. allieace leaf extracts. Percentage of cells arrested at different cell cycle stages after treating with (a) untreated (b) standard Campothecin (STD) (c) ethyl acetate leaf extract (MEA) and (d) ethanolic leaf extract

Fig. 6 shows the percentage of cells arrested at various stages of the cell cycle. Around 10,000 cells were subjected for analysis. In sub G0/G1 stage, 2.11%, 17.97%, 4.73% and 6.39% of cells get arrested in cells untreated, cells treated with standard camptothecin (CPT), MEA and ME leaf extracts, respectively. In G0/G1 phase, 64.84%, 20.95%, 1.33% and 2.81% of cells get arrested in cells untreated, cells treated with CPT, MEA and ME, respectively. In the S phase, 11.87%, 34.77%, 1.21%, and 2.21% of cells get arrested in cells untreated, treated with CPT, MEA and ME, respectively. Whereas, in the G2/M phase, 21.16%, 27.58%, 78.03% and 87.84% of cells get arrested in cells untreated, cells treated with CPT, MEA and ME, respectively**.** In comparison with a standard, MEA and ME were able to arrest more cells at the G2/M phase.

Naranmandura *et al.*, (2007) [16] assessed the toxicity of dimethylmonothioarsinic acid (DMMTA) towards A431 cells by MTT assay and cell cycle arrest analysis by flow cytometry. They reported that DMMTA significantly arrested the A431 cells at S and G2/M phases. They also noted the significant decrease in cell population in G1 phase by 38.1%, but there was increase in S and G2/M phase from 7.3 and 12.2% to 41.3 and 20.6%, respectively. Zeng *et al.*, (2018) [13] repoted the gradual increase of cell population in G2 phase in a dose-dependent manner leading to cell arrest. In the present investigation also the percentage of cell arrest was increased at the G2/M phase.

Detection of Apoptosis by Annexin V-PI staining

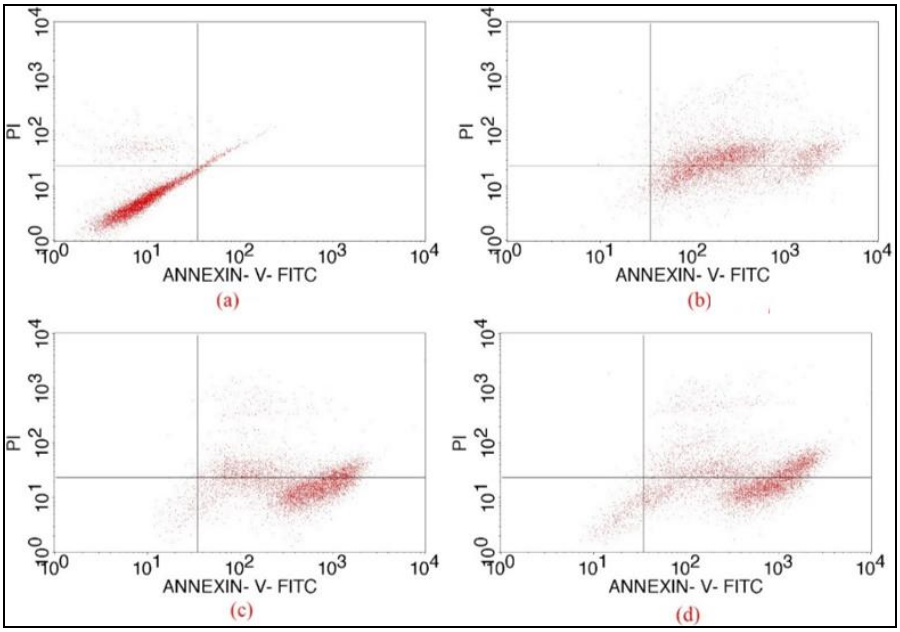

Fig 7: Annexin V - PI expression study of *M. alliacea* on A431 cells. Quadrants showing expression of (a) untreated cells (negative control) (b) standard Campothecin – 15 µM (positive control) (c) ethyl acetate leaf extract (MEA) – 71 µg/ml and (d) ethanolic leaf extract (ME) – 75 µg/ml treated cells against the FTIC-Annexin V and Propidium Iodide (PI) stain

The quadrants in Fig. 7 shows the effect of *M. alliaceae* leaf extracts on A431 cells against Annexin V and PI staining along with negative and positive controls. The upper left quadrant represents the percentage of necrotic cells, the lower left quadrant represents the percentage of viable cells, the upper right quadrant represents the percentage of late

apoptotic cells, and the lower right quadrant represents the percentage of early apoptotic cells.

Majority of the cells (94.8%) concentarted in lower left qudrant are viable cells (fig. 7 a), whereas the cells treated with standard (fig. 7 b), MEA (fig. 7 c) and ME (fig. 7 d) represented in lower right qudrant indicating early apoptotic cells having 74%, 79.82% and 90.42%, respectively. These results are represented in a graphical manner indicating the percentage of cells arrested in different Apoptotic stages (fig. 8).

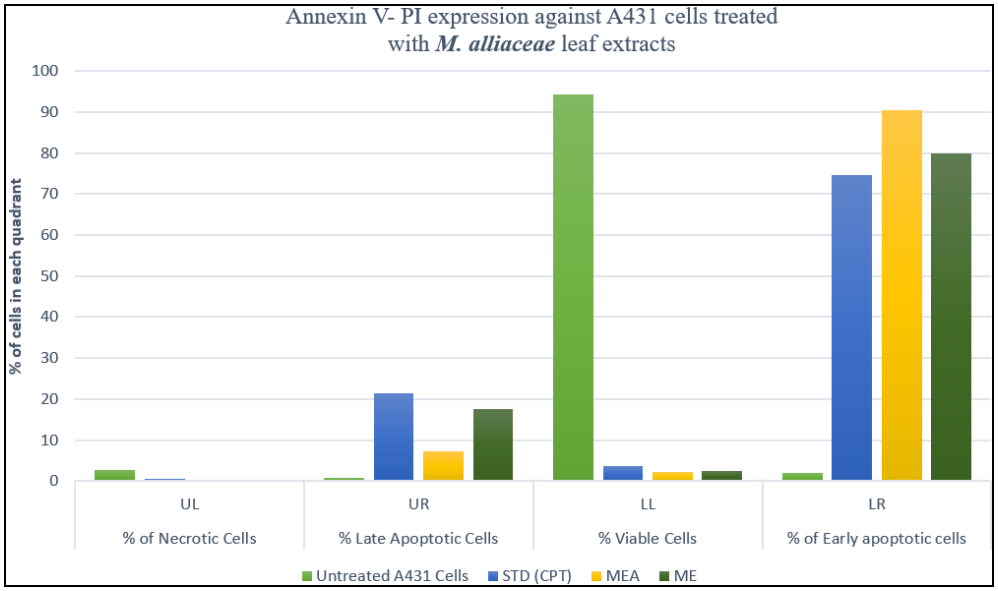

Fig 8: Percentage of cells arrested in different stages of apoptosis in the Untreated, Standrad Campothecin, Ethyl acetate leaf extracts and Ethanol leaf extracts

Li *et al.,* (2017) [17] evaluated the anticancer effects of Cantharidin (CTD) on A431 cells *in vitro* and *in vivo*. According to them CTD induced DNA fragmentation in A431 cells leading to apoptosis. Whereas, Nirmala *et al.,* (2018) [18] reported that, when A431 cells were treated with *Vitis vinifera* peel, and seed extracts could induce apoptosis, which was detected by staining with Annexin V-PI. They reported 16.32 % and 3.12 % of apoptotic cells after 24 hours of incubation with the *V. vinifera* peel and seed extracts. The results of the present investigation recorded 79.82 % and 90.42 % of cells in early apoptotic stage in ethyl acetate and ethanolic leaf extracts are better compared to other workers.

Caspase 3 Expression studies by flow cytometry

Based on the results obtained in the detection of apoptosis by Annexin V – PI staining, it is further subjected for caspase 3 expression activity to confirm the anticancerous efficacy of *M. alliaceae* leaf extracts. The Fig. 9 representing the histogram of the gated A431 singlets distinguishes cells at M1 and M2 phases, where M2 refers to negative caspase 3 expression regions, and M1 refers to positive caspase 3 expression regions.

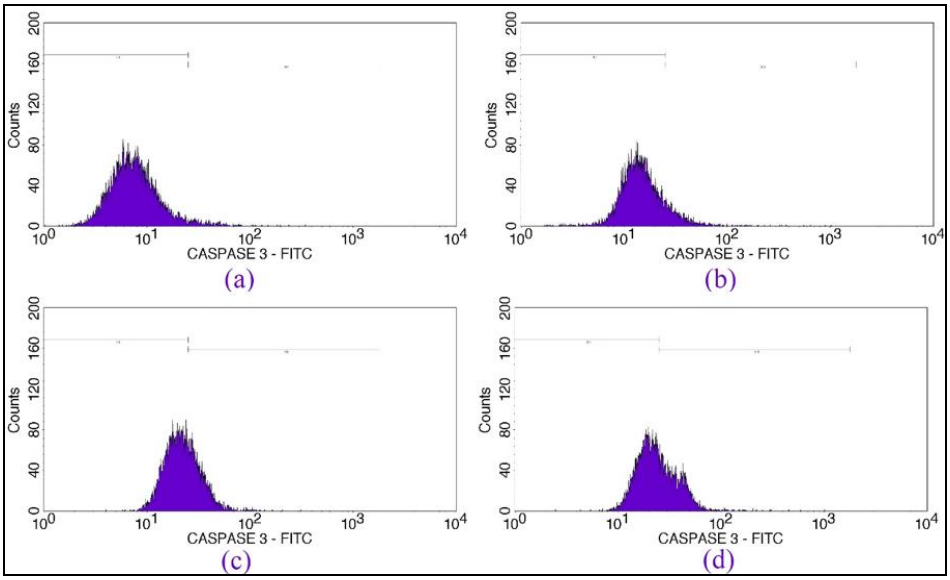

Fig 9: CASPASE 3 expression study of *M. alliaceae* leaf extracts on A431 cells. CASPASE 3 - FITC histogram of the gated A431 singlets distinguishes cells at M1 and M2 phases. (a) Untreated cells, cells treated with (b) standard camptothecin, (c) ethyl acetate and (d) ethanolic leaf extract.

A comparative account of the relative mean fluorescence intensity of untreated A431 cells and cells treated with standard camptothecin, MEA and ME leaf extracts revealed caspase 3 expression activity was found to be very low in untreated cells (9.19 MFU) compared to cells treated with camptothecin (24.25 MFU). But in ethyl acetate and ethanolic leaf extracts showing good caspase 3 expression with MFU values of 23.8 and 25.82, respectively compared to the standard (fig. 10).

Li *et al.,* (2017) [17], evaluated the anticancer effects of Cantharidin (CTD) on A431 cells *in vitro* and *in vivo*. They reported CTD stimulated specific activity of caspase 3, 9 and 8 in a time dependent manner which indicated that cell apoptosis was caspase dependent. The present work also reveals the caspase 3 expression by ethyl acetate and ethanolic leaf extracts of *M. alliacea.*

Itokawa *et al.,* (1991) [6] worked on the cytotoxic naphthoquimnones from *M. alliacea*. They isolated 9-methoxy-α-lapachone and 4-hydroxy-9-methoxy- α-lapachone which are yellow-coloured powders from the methanolic leaf extracts. These isolated compounds were cytotoxic to V-79 cells with IC 50 values of 5.6 and 6.0 µg/ml, respectively. Growth inhibition and induction of apoptosis in estrogen receptor-positive (MCF-7) and negative (MDA-MB231) human breast carcinoma cells by *Adenocalymma alliaceum* was evaluated by Dugasani *et al.,* (2009) [19]. They reported dose dependent growth inhibition with growth inhibitory concentration of 53.1 in MCF-7 cells and 29.9 µg/ml in MDA-MB231 cells. And their analysis suggests that *A. alliaceum* flower extract exerts growth inhibition on both breast cancer cells through apoptosis induction. Towne *et al.,* (2015) [20] revealed the effect of *M. alliacea* leaf extracts on embryonic and tumorigenic mouse cell lines. According to them, there was a significant decline in the number of cancer cells after treating with the leaf extracts at higher concentrations, but there was only inhibition in the growth of the non-cancerous cell growth.

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