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# Identification of chemical constituents of *Cynanchum sarcomedium* by HR-LC/MS and evaluation of anti-proliferative activity on human MCF-7 and HCT-15 cancer cell lines: *In vitro* mechanistic evidences

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## Abstract
The plant *Cynanchum sarcomedium* is an underutilized, xerophytic perennial shrub that belongs to Apocynaceae. The present study aims at evaluating the effects of methanolic extract of *C. sarcomedium* on breast cancer and colon cancer cell lines. Cell viability was assessed by MTT assay and apoptosis was determined by morphological observation through DAPI staining. Mitochondrial membrane potential was evaluated by JC-1 staining. Cell cycle analysis and western blot analysis on the expression of proteins associated with mitochondrial cascade was also carried out. In MTT assay of MCF-7 cell lines, maximum cytotoxicity was observed at 50 µg/ml for 48 h by the methanolic extract. So, further studies have been carried out under this experimental condition. Whereas in HCT-15 cell lines, 100 µg/ml for 48 h was the effective experimental condition. Flow cytometricanalysis of treated MCF-7 and HCT-15 cell lines demonstrates that cells were arrested at the G0/G1 phase and western blot analysis indicates the activation of proteins in the mitochondrial signaling pathway. Thus, the study suggests C. *sarcomedium* as a promising candidate having significant anti-proliferative activity. Hence, it merits further exploration as a potential therapeutic drug.

**Keywords:** *Cynanchum sarcomedium*, anti-proliferative, apoptosis, western blot, MCF-7, HCT-15

## Introduction
Despite the leading progress in the discovery and development of various novel drugs, cancer remains the second major cause of death in the world. Prostrate cancer, lung cancer, skin cancer, colorectal cancer, breast cancer etc. are common types of cancers. Among them, breast cancer is a frequent type of cancer occurring among women. As per the SEER stat fact sheet of National Cancer Institute, USA (NCI), the number of new cases of female breast cancer was 125.0 per 100,000 women per year. The number of deaths was 21.5 per 100,000 women per year while prostate, lung, bronchus, and colorectal cancers account for 44 % of all cases in men. Three types of standard treatment are used: Surgery, Radiation therapy, and Chemotherapy. Even though various types of drugs are available for the cancer treatment, due to their deleterious side effects; search for a new molecule is still in the pipeline. The induction of apoptosis or exhaustion of cancer cells without causing excessive damage to normal cells by any natural compound is a key target for identifying methods of cancer prevention and therapy (Taraphdar *et al*., 2001) [23].
Natural products have been considered as a vast source of various bioactive compounds that targets various diseases. Of all the drugs screened by NCI, about 40 percent come from industry, and the rest primarily come from academic collaborators. Another source of novel compounds is the Natural Products Branch of NCI that collaborates with agencies throughout the world to collect thousands of plant and marine organisms to screen for potential anticancer compounds. Plants have long been used for millennia in traditional medicine against cancer. Instead of a conventional single compound-single target approach, a consortium of bioactive molecules against multiple targets is gaining more attention nowadays. The synergistic action of various phytochemical compounds acts on various targets for cancer development, thus increasing therapeutic efficacy and eliminating side effects (Cilla *et al*., 2015) [21]. Plants are considered as an inexhaustible pool of a broad array of phytochemicals like phenols, terpenes, alkaloids etc. that can be exploited for the development of new drugs (Gali-Muhtasib *et al*., 2015) [22].
*Cynanchum sarcomedium* (Apocynaceae) is a xerophytic, laticiferous, perennial shrub; least explored with regard to their bioactivities. Earlier, the genus *Cynanchum* had been deeply nested in the genus *Sarcostemma* and recent molecular studies resulted in the taxonomic dissolution of *Sarcostemma* into *Cynanchum* (Meve & Liede, 2012) [15]. The genus is commonly known as Caustic wine, found in the drier parts of the Old World from South Africa through the Arabian Peninsula and India to Australia. The species of this genus are under 'least concern' status in Red List of South African Plants. Pre-apoptotic, genotoxic and modulatory activities of the plant on *Allium cepa* have been reported earlier (Bhagyanathan & Thoppil 2015; Bhagyanathan &Thoppil 2016) [2, 3, 4]. Also, the plant is rich in phytochemical constituents like terpenoids, fatty acid esters etc. (Bhagyanathan &Thoppil, 2015) [2, 3]. To date, there is no ever report utilizing the anti-proliferative potential of the shrub on breast cancer and colon cancer cells.
Hence, the present study has been designed to evaluate the non-volatile constituents of the plant by HR-LC/MS and the anti-proliferative potential of the plant, *C. sarcomedium* on breast cancer (MCF-7) and colon cancer (HCT-15) cell lines.

## Materials and Methods

### Plant material

*Cynanchum sarcomedium* Meve & Liede was collected from Wayanad, Kerala, India (Coordinates: 11.605 °N 76.083 °E). The specimen was authenticated and a voucher specimen (CALI No. 123741) was deposited at the Herbarium of Department of Botany, University of Calicut, Kerala, India.

### Plant extract preparation

10 g of the ground plant materials were subjected to sequential extraction in n-hexane to remove non-polar components followed by 100 ml methanol. A stock solution of plant extract was prepared in PBS at a concentration of 1 mg/ml. From the stock, respective concentrations of methanolic extracts (50, 100, 200 and 400 µg/ml) for the experiments were prepared using the appropriate volume of cell culture working medium DMEM, and RPMI respectively.

### HR-LC/MS analysis

Chromatographic separations were performed on an HR-LC/MS Q-TOF (Agilent, USA) equipped with an electrospray ionization source. The column employed for separation is CAPCELL C18, MG-II type, 4.6 mm × 250 mm dimensions with a particle size of 5 µm; by applying the following gradient at a flow rate of 0.5 ml/min. Isocratic elution was performed using a mobile phase consisting of 95 % A (water) and 5 % B (acetonitrile); 1-3 min linear from 5 to 95 % A; isocratic 10 % A. The injection volume was 5 µl (full loop injection) and the total run time was 45 min. Eluted compounds were detected with MS Q-TOF equipped with an electrospray ion source in positive ion modes using the following operating parameters: nebulizer gas: nitrogen; 13 l/min; gas temperature: 250 °C; nozzle voltage: 1000 V.

### Cell culture

MCF-7 and HCT-15 cell lineswere procured from National Centre for Cell Science (NCCS), Pune, India and grown as a monolayer in Dulbecco's Modified Eagle's Medium(DMEM) containing HEPES and sodium bicarbonate supplemented with 10 % Fetal Bovine Serum (FBS) and 1% antibiotic–antimycotics. Cells were maintained in a tissue culture flask kept in a humidified incubator (5 % $CO_2$ in air at 37 °C) with a medium change every 2–3 days. When the cells reached 70–80 % confluence, they were harvested with trypsin-EDTA (ethylene diaminetetra acetate) and seeded into a new tissue culture flask.HCT-15 cell line was grown as monolayer in rosewell park memorial institute (RPMI) medium supplemented with 10 % FBS and 1 % antibiotic-antimycotic solution. Cells were maintained in tissue culture flask and harvested as mentioned before.

### Cytotoxicity assay

The cytotoxicity was assessed by MTT assay which determines the metabolically active mitochondria of intact cells. MCF-7 and HCT-15 cells were seeded in 96-well plates with $5\times10^3$ cells/100 µl and incubated for 24 h at 37 °C. The MCF-7 cells were then treated with plant extracts (50 µg/ml, 100 µg/ml, 200 µg/ml, and 400 µg/ml) and incubated for another 24 h at 37 °C in a 5 % $CO_2$ atmosphere. 5-Flurouracil (5-FU) (50 µg/ml) and DMSO (0.1 % v/ v) were served as positive and negative controls. 100 µg/ml, 200 µg/ml, and 400 µg/ml were the concentrations of plant extract used for cytotoxic evaluation on HCT-15 cell lines. The assay was performed by the addition of premixed MTT reagent, to a final concentration of 10 % of total volume, to culture wells containing various concentrations of the test concentrations of plant extract and incubated for a further 4 h. During the 4 h incubation, living cells converted the tetrazolium component of the dye solution into a formazan product. 100 µl of the solubilization solution was then added to the culture wells to solubilize the formazan product and the absorbance at 570 nm was recorded using a 96-well plate reader (Bio-Rad, Hercules, CA, USA).Percentage inhibition was calculated using the formula,

$$\text{Cytotoxicity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

### Morphological detection of apoptosis

DAPI staining was applied for determining the apoptotic cells. MCF-7 cells and HCT-15 cells were cultured in a 24-well tissue culture grade plate for 24 h. After incubation with different concentrations of the extract, and DMSO (0.1 % v/ v) for 48 h, cells were washed with PBS, fixed with 2 % paraformaldehyde for 15 min followed by washing in PBS and were treated with 0.2 % Triton X-100 in PBS for 15 min at room temperature. Cells after washing with PBS were stained with DAPIand incubated in dark for 30 min. 5-FU (50 µg/ml) was used as the standard drug control. The cells were then examined and photographed using a fluorescent microscope (IXL 40, Labovision, India).

### Determination of mitochondrial membrane integrity

The mitochondrial membrane potential ($\Delta\Psi m$) was assayed using JC-1 (contains the cationic dye tetraethylbenzimidazolylcarbocyanine iodide) mitochondrial potential sensor (Invitrogen, USA), according to the manufacturer's directions. Briefly, MCF-7 and HCT-15 cells were incubated for 24 h in 24-well plates (Greiner, Germany) and the cells were treated with different concentrations of the extract, 5-FU (50 µg/ml) and DMSO (0.1 % v/ v) for 48 h. The treated cells were washed with PBS and incubated for 30 min in 10 % DMEM medium without phenol red containing JC-1. The cells were then examined and photographed using a fluorescent microscope under green excitation filter.

### Cell cycle analysis

The cells were treated as previously described. After treatment for 48 h, the cells were trypsinized and harvested. Cells were repeatedly washed with PBS and fixed with ice-cold 70 % ethanol (1 ml) at -20 °C overnight. Prior to analysis, ethanol was removed (3000 rpm, 5 min) and washed twice with cold PBS. Subsequently, 250 µl of cell cycle reagent was added. This was incubated at dark for 30 minutes (which is light sensitive). After this, it was analyzed using MuseTM Cell Analyzer (Merck-Millipore, Germany). Gating was performed with reference to untreated control cells and samples.

### Western Blot

Western blotting was performed to detect the proteins of beta-actin, caspase-3, caspase-9 and Poly ADP ribose

polymerase (PARP). MCF-7 and HCT-15 cells ($1.5 \times 10^6$) were seeded onto culture dishes in the presence or absence of extract and 5-FU, and were treated for 48 h. Cells were washed twice with ice-cold PBS-EDTA and incubated in Radio immunoprecipitation assay buffer (RIPA). The lysates were centrifuged at 3018.6 g for 10 min at 4 °C, and supernatants were used as the cell protein extracts. Each extract were applied to 12 % SDS polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane, which was previously activated, and then blocked for 1 h using 3 % Bovine Serum Albumin (BSA) in Tris Buffered Saline with Tween 20 (TBST). After washing the cells in TBST for 3times, primary antibodies against β-actin, caspase-3, caspase-9 and PARP were added at a (v/v) ratio of 1:1000. After overnight incubation at 4 °C, the primary antibodies were washed away and secondary antibodies were added for 1 h incubation at room temperature. Finally, 5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium (BCIP-NBT) staining was employed to develop the signal of the membrane.

**Results**

The present study evaluates the non-volatile components present in the methanolic extract of *C. sarcomedium* and its *in vitro* anti-proliferative potential on breast cancer and colon cancer cell lines.

**HR-LC/MS analysis**

Non-volatile phytoconstituents in the two species of *Cynanchum* were identified by HR-LC/MS. The identities, retention time, mass-to-charge ratio and observed molecular mass for individual components are presented in Table 1 and the liquid chromatogram is also given as Fig. 1. Metabolite assignments were made by comparing retention time and MS data (accurate mass and mass-to-charge ratio) of the compounds. Identifications were confirmed with standard compounds. Ten non-volatile components were detected in the *C. sarcomedium* extract, tentatively identified and compared with the results of standard compounds. D-mannitol, clocortolonepivalate, traumatic acid, epiafzelechintrimethyl ether, phloionolic acid, α-erythroidine, eupatoriochromene, 4-oxo-9Z, 11Z, 13E, 15Eoctadecatetraenoic acid and gedunin were the major compounds identified by HR-LC/MS analysis. Interestingly, 2- amino-3-methyl-1-butanol, an alcoholic compound having a molecular mass of 103.099 and [M+H]+ at *m/z* as 104.1062, detected in both plant species as a strong signal. Clocortolonepivalate having molecular mass of 494.228 and [M+H]+ at *m/z* as 477.2245 was found as next higher peak (peak 3) with a retention time of 12.396 min. Eupatoriochromene was identified as peak 8 at a retention time of 23.417 min. and [M+H]+ at *m/z* is 201.0902. Thus, HR-LC/MS analysis provided a vivid picture of various non-volatile constituents in the methanolic extract of *C. sarcomedium.*

**Inhibition of proliferation of MCF-7 and HCT-15 cell lines by *C. sarcomedium***

MTT assay was carried out to analyse the cytotoxicity on MCF-7 cell lines and HCT-15 cell lines treated with the methanolic extract of *C. sarcomedium*. In the case of MCF-7 cell lines, 50, 100, 200 and 400 µg/ml of methanolic plant extracts and treatment period of 24 and 48 h was taken as the experimental condition. 50 µg/ml of 5-Fluro uracil was kept as the positive control. Maximum cytotoxicity was observed at 50 µg/ml concentration of methanolic extract of *C. sarcomedium* (64.25 ± 0.71 %) at 48 h of treatment period (Fig. 2a). At 100 µg/ml of the extract of *C. sarcomedium*, the cytotoxicity percentage was 44.89 ± 0.94 %. A toxicity of 56.71 ± 0.98 % was observed in cells treated with 5-FU for 48 h. Therefore, the results were found to be significant when comparing to 5-FU. Interestingly, dose-dependent decrease in cytotoxicity was observed in the MTT assay. In the highest concentration (400 µg/ml), cytotoxicity was quite lower than other concentrations; 26.78 ± 0.82 % for *C. sarcomedium* at 48 h treatment. In 24 h of incubation period also, dose-dependent decrease in cytotoxicity was observed. Briefly, 50 µg/ml was the effective concentration and 48 h is the optimum time period for maximum cytotoxic activity by *C. sarcomedium* on MCF-7 cell lines.

In the case of HCT-15 cell lines, experimental conditions chosen were 100, 200 and 400 µg/ml for 24 h. In HCT-15 cell line also, dose-dependent decrease in the inhibition of cell proliferation was noted. At 100 µg/ml of concentration, the cytotoxic percentage was 77.56 ± 0.96 % for *C. sarcomedium* methanolic extract at 24 h (Fig. 2b). And so, 100 µg/ml was the optimum concentration of *C. sarcomedium* for inhibition of cell proliferation of HCT-15 cell lines at 24 h.

**Morphological detection of apoptosis**

To identify whether these cytotoxic results are due to apoptosis, cells (treated and control) were subjected to DAPI staining. After DAPI staining, MCF-7 cells treated with the plant extract (50 µg/ml and 100 µg/ml) showed distinct nuclear fragmentation, nuclear shrinkage and chromatin condensation which are clear indications of apoptosis (Fig. 3a). In HCT-15 cells treated with methanolic extract of *C. sarcomedium* (100 µg/ml), DAPI staining unraveled various hallmarks of apoptosis. *C. sarcomedium* induced apoptosis on HCT-15 cell lines by inducing various nuclear deformities like blebs and condensation (Fig. 3d). Other morphological changes include a reduction in the size of the cells, the cells gradually become flat as well as shrunken with the appearance of small vesicle bodies (apoptotic bodies) and karyopycnosis were also observed. Polymorphic nuclei were seen in various treated cells. 5-FU treated cells also showed pronounced symptoms of apoptosis by DAPI staining (Fig. 3b; 3e). Whereas, in untreated cells, DAPI stained nuclei were rounded and homogenously stained (Fig. 3c; 3f).

**Determination of mitochondrial membrane potential**

To study the mitochondrial membrane integrity, cells were treated with plant extract and stained with JC1. Loss of ΔΨm is evidenced by the decrease in red fluorescence and the aggregation of green fluorescence. As seen in the Fig. 4, cells treated with plant extract (50 µg/ml and 100 µg/ml) indicated the marginal shift in ΔΨm by the decrease in red/green fluorescence intensity ratio as opposed to untreated cells (more red fluorescence).HCT-15 cells were treated with plant extracts and stained with JC-1 to determine the mitochondrial membrane potential. Normal cells showed aggregation of red fluorescence, while treated cells showed a decreased red fluorescence, which indicated a reflective breakdown in the mitochondrial trans membrane potential. 5-FU treated HCT-15 cells showed an extreme

reduction in red fluorescence. To sum up, the results suggested that the plant extract exhibited ant proliferative activity on breast cancer cells and colon cancer cells mediated by apoptosis through the depolarisation of mitochondrial membrane potential. To sum up, the results suggested that the plant extract exhibited anti-proliferative activity on breast cancer and colon cancer cells mediated by apoptosis through the depolarisation of mitochondrial membrane potential.

**Cell cycle analysis**

The cell cycle distributions in MCF-7 cells treated with methanolic extract of *C. sarcomedium* (50 µg/ml) for 48 h were analyzed by flow cytometry. The percentage of cells in each phase of cell cycle in treated cells and negative control (0.1 % v/v DMSO) were determined and the results are shown in Fig. 5a& 5b. At 48 h of treatment with 50µg/ml of extract, the enhancement in cell population in $G_0/G_1$ stage was accompanied by a decrease in number of cells in S phase. An increase in number of cells a G-Phase, indicating cell cycle arrest is associated with $G_0/G_1$phase and subsequent apoptosis. Percentage of cells in $G_0/G_1$phase was found to be 59.4 % when treated with plant extract and 54 % in DMSO (negative control). S-phase values of 27.1 % and 23.3 %were observed respectively in treated cells and negative control. The cell count at $G_2/M$ phase was found to be 9 % and 15.4 % respectively in extract treated breast cancer cells and in DMSO control. Apoptotic cells were distinguished by their decreased DNA content, as shown by their weaker staining intensity in the area of the G0/G1 phase.

The effect of methanolic extract of *C. sarcomedium* (100 µg/ml) on the cell-cycle progression of HCT-15 cell lines was studied after 24 h by cell cycle analysis. Untreated cells showed a normal cell cycle progression. The cell count of HCT-15 cells treated with plant extracts is depicted in Fig. 5c & 5d. Upon treatment with methanolic extract of *C. sarcomedium* caused a significant inhibition of cell cycle progression by blocking the transition of the G0/G1 phase of cell cycle. The treatment of HCT-15 cell lines with *C. sarcomedium* extract showed significant drift in cell distribution. There was approximately 50.09 ± 1.12 % increases in G0/G1 phase cells depicting retention of cells in G0/G1 phase. The results suggested potent inhibition of cells at the G0/G1 phase and hence *C. sarcomedium* showed mitotic inhibition, which could be considered significant. In untreated cells, progression is quite normal with cell population of 53.47 ± 1.12 % in G0/G1, 2.85 ± 0.98 % in S phase and 18.65 ± 0.45 % G2/M phase respectively.

**Western blot**

As disruption in mitochondrial membrane potential is clearly observed by JC-1 staining, western blot had been attempted using cascade of proteins in mitochondrial pathway of apoptosis, in order to verify whether the apoptosis is mitochondria-mediated. After exposure to plant extract (50 µg/ml) for 48 h, procaspase-9 (43 kDa) was fully cleaved into caspase-9 (37, 35 kDa).Also, the plant extract induced to activate the cleavage of procaspase-3 (32 kDa) into caspase-3 (15 kDa).Along with these, PARP (116 kDa) also has been degraded to cleaved PARP of 89 kDa (Fig. 6).Similar pattern was observed in MCF-7 cells treated with 5-FU (positive control). The equivalent amount of protein was verified by reprobing the blots with anti-β-actin antibody. Treatment with plant extract induced the proteins in a cascade. DMSO- based experiment did not showed the activation of apoptotic proteins.

Furthermore, in HCT-15 cell lines also both plant extracts was found to activate the cascade of proteins (caspase-3, caspase-9, and PARP) in the mitochondrial signaling pathway (Fig. 7). β-actin was used as a negative control to ensure equal loading. Same cleavage pattern of the proteins was observed as in the MCF-7 cell lines but the expression level of apoptotic proteins in HCT-15 cell lines treated with plant extract is higher than those noted in the MCF-7 cell lines. These results strongly suggest that apoptosis induced by *C.sarcomedium* in MCF-7 and HCT-15 cells occurred via mitochondria-dependent signal pathway.

**Discussion**

Various plants have recently gained widespread attention since numerous works have delineated their potential role against various diseases including cancer and other dreadful diseases. Breast cancer is the second important cause of death in women worldwide. Accumulative evidences have been shown that the bio-active compounds in plants have the key role in targeting the cancer (Cattaneo *et al*., 2015; Najmuddin *et al*., 2016) [5, 24].

Methanolic extract of *C. sarcomedium* consists of 10 non-volatile compounds as detected by HR-LC/MS (Fig. 1). Clocortolonepivalate is a steroid type compound detected at a retention time of 12.396 min. with a molecular mass of 494.228. It was identified in the hydrodistilled oil and supercritical fluid $CO_2$ extract of *Valerianawallichi* and its antioxidant potential was evaluated (Pandian & Nagarajan, 2015) [6]. Likewise, D-mannitol was detected at a retention time of 10.363 min. It was reported to be present in *Nyctanthesarbortristis* and evaluated for its anticancer potential on HeLa, HepG2 cell lines, without much adverse effects on the survival of normal human lymphocytes by MTT assay, EB/AO Staining and caspase-9 activity assay and obtained promising results for the activity (Timsina & Nadumane, 2016) [19].

α-erythroidine was identified at a retention time of 21.034 min and a molecular mass of 273.1354. It belongs to an alkaloid group of a novel class of phytoestrogens. In an earlier report, α-erythroidine was structurally characterized from the methanolic extract of the stem bark of *Erythrinapoeppigiana*. The estrogenicity of the isolated erythroidine was assayed in various estrogen receptor-(ER)-dependent test systems, including receptor binding affinity, cell culture based ER-dependent reporter gene assays, and gene expression studies in cultured cells using reverse transcription polymerase chain reaction techniques. α-erythroidine induced the enhanced expression of the specific α- ER dependent genes trefoil factor-1 and serum/glucocorticoid regulated kinase 3 in MCF-7 cells, confirming its estrogenicity (Djiogue *et al*., 2014). Thus, it may be correlated with the ant proliferative activity of *C. sarcomedium* on MCF-7 cell lines, observed in the present study.

Gedunin is a limonoid compound detected in the HR-LC/MS analysis with a prominent peak retention time 28.708 min. It is a highly potent compound earlier isolated from *Xylocarpusgranatum*, which inhibits the growth of CaCo-2 colon cancer cell line *in vitro* with an IC50 value of 16.83 µM (Uddin *et al*., 2007) [20]. Also in another study, gedunin inhibits the proliferation of ovarian cancer cells. *In*

*vitro* treatment of ovarian cancer cell lines with gedunin produced up to 80% decrease in cell proliferation (Kamath *et al*., 2009) [10]. The mechanism of action of this compound is well known; it induces cancer cell death by apoptosis through inactivation of p23 and activation of caspase 7, which cleaves p23 at the C-terminus (Patwardhan *et al*., 2013) [17]. Moreover, modulation of T-lymphocyte responses and amelioration of allergic responses were also shown by the natural product, gedunin (Ferraris *et al*., 2012) [27]. These evidences corroborate its presence in *C. sarcomedium* with the anticancer activity observed in the present study.

Several anticancer drugs exert their cytotoxic effects by inducing apoptosis mediated by intrinsic pathway (Jo *et al*., 2005; Xu *et al*., 2009). Consistent with these studies, the present study reports the ant proliferative effect of *Cynanchum* species on MCF-7 and HCT-15 cell lines *via* apoptosis and intrinsic pathway of cell death. Initially, MTT assay was carried out in order to screen the cytotoxic potential of *Cynanchum* species. As a first step, inhibition of cell proliferation was evaluated using methanolic extract of *C. sarcomedium* by MTT assay. In the present study, cytotoxic potential of *C. sarcomedium* on MCF-7 and HCT-15 cells were determined based on the reduction of the yellow coloured MTT to a purple formazan by mitochondrial succinate dehydrogenase which reflects the normal function of mitochondria and cell viability (Lau *et al*., 2004) [26]. The cells were then solubilised with an organic solvent. The released as well as the solubilized formazan reagent is measured spectro-photometrically. Reduction of MTT can only occur in metabolically active cells whereas dead cells do not undergo the reduction.

In order to evaluate the cause of growth inhibition by the *C. sarcomedium* methanolic extract, morphological assessment of apoptosis by DAPI staining was performed. Apoptosis or Programmed cell death is a dismantled process triggered by the cell by its own mechanism to kill individual components, when subjected to any stress; thus exerting no toxicity to surrounding normal cells (Machana *et al*., 2011) [14]. Nuclear fragmentation, shrinkage, membrane blebbing etc. are the hall mark characteristics of cells undergoing apoptosis (Pattanayak *et al*., 2015) [16] which are clearly observed by DAPI staining in treated cells (Fig. 3). Additionally, apoptotic bodies and condensed chromatin are the typical features of early and late stage of apoptosis (Machana *et al*., 2011) [14]. Initiation of nuclear buds were also noticed, by polyploidization processes whose exceeding genetic material may tend to be eliminated from the nucleus in the form of micronuclei in *Allium cepa* test system (Fernandes *et al*., 2007) [8].

Mitochondria play an important role in apoptosis triggered by an intrinsic mechanism. The dysregulation of mitochondrial membrane integrity is considered as an early event in apoptosis mediated by the intrinsic apoptotic pathway (Esmaeili *et al*., 2016) [25]. In the present study, mitochondrial depolarization was assessed using JC-1 staining; detects the loss of mitochondrial potential by the reduction of red/green fluroscence intensity proportion. Methanolic plant extract treated cells exhibited the extreme disruption of mitochondrial function as determined by the reactivity of fluorescent dye, JC-1 with mitochondria (Fig. 4). In control cells, JC-1 enters into the mitochondrial matrix and stains red to form J-aggregates. Any process that alters the mitochondrial polarization, disrupts the accumulation of dye in the matrix, it spreads in the entire cell and results in the shift of red fluorescence to green (JC-1 monomers) (Ahamad *et al*., 2014) [1]. The stress stimuli can elicit ROS and induce the opening of mitochondria membrane transition pores, which facilitate the release of apoptotic molecules such as cytochrome c in to the cytosol which may stimulate apoptotome assembly (Chen *et al*., 2014). The released cytochrome c then interacts with specific adapter, such as Apaf-1, which in turn activates a cascade of apoptotic proteins. Also, increase in intracellular Ca level is also a prominent factor for the dysfunction of mitochondrial membrane integrity, expansion of the matrix and rupture of outer mitochondrial membrane ultimately leading to cell death (Koppikar *et al*., 2010) [12].

The cell cycle control is considered as a vital event in the co-ordination and regulation of cell division. In order to understand the mechanism of arrest of cell proliferation, cell cycle analysis was carried out using flow cytometry analysis following the treatment of MCF-7 cells with 50 µg/ml of extract for 48 h. The number of cells in the G0/G1 is increased with the concomitant decrease in the cell count in S and G2/M phase (Fig. 5). This indicates that cells were blocked from exiting G1and would have led to the accumulation of cells in G0/G1 and thus cells were not progressing to subsequent phases of cell cycle. Taken together, these findings suggest that cell cycle arrest was occurred in G0/G1 phase of the cell cycle. The results reported herein are consistent with the previous reports in various cell lines (Dogra *et al*., 2016; Yan *et al*., 2016) [7, 29]. The chemical compounds present in the plant at subtoxic levels may have inhibited the proliferation of MCF-7 cells by blocking the progression of cell cycle at G0/G1 phase. Research has proved that arrest of cells at major checkpoints of cell cycle, as an event proceeding to the detection of apoptosis (Foo *et al*., 2015; Shoja *et al*., 2015) [9, 18]. The mechanism of mitotic arrest and the role of cell cycle proteins in the cell cycle regulation by the treatment of methanolic extract of *C. sarcomedium* deserve further investigations.

With regard to mechanism of anti-cancer activity, apoptosis is a controlled process mediated by two important signaling pathways viz., extrinsic and intrinsic pathways. Extrinsic pathways involves the death receptors which initiate procaspase-8 and ultimately results in its cleavage and apoptosis (Liu *et al*., 2016) [13]. Intrinsicpathway involves the pivotal role of mitochondria by the release of cytochrome c. In the present study, mitochondrial membrane potential was found to be disrupted as evidenced by JC-1 staining. Thus, it is assumed that proteins in the mitochondrial pathway would have been dysregulated their functioning and it may be the cause of anti-proliferation of breast cancer cells. Due to which, three major proteins in the mitochondrial cascade (Caspase-3, Caspase-9 and PARP) have been selected for the western blot analysis. Cytochrome c after being released into cytosol forms a complex with caspase-9, Apaf-1 and dATP and in turn activates caspases-9, caspase-3 and caspase-7 (Kong *et al*., 2009). Here, in the present work; it was observed that the collapse of mitochondrial membrane potential was induced by methanolic extract of *C. sarcomedium* (50 µg/ml) for 48 h. Moreover, it is found that the precursor forms of caspases (-3, -9) decline, suggesting their activation. Caspases are a member of aspartic acid-specific cysteine proteases involved in the initiation and execution of apoptosis.Caspase-9 is expressed initially in the apoptotic

cascade by the release of apopotogenic molecules into cytoplasm, which initiates the sequential proteolytic cleavage of other downstream proteins like caspase-3 (Liu *et al*., 2016) [13]. The observed cleavage of PARP after *C. sarcomedium* extract treatment of the breast cancer cells indicated that caspase-3 activation (and subsequent PARP cleavage) may substantiate the process of apoptosis in these cells (Fig. 6 & 7). Caspase-3 is an important executioner of apoptosis in caspase family, as it is either partially or totally responsible for the proteolytic cleavage of many specific substrates like PARP, lamin, focal adhesion kinase (FAK) resulting in apoptosis (Kim *et al*., 2011) [30].

**Table 1:** Non-volatile chemical constituents of *C. sarcomedium* as analysed by HR-LC/MS

| S. No. | RT (min) | Compound | Class | Molecular mass | $[M+H]^+$ (*m/z*) |
|---|---|---|---|---|---|
| 1. 1 | 4.593 | 2- amino-3-methyl-1-butanol | Alcohol | 103.099 | 104.1062 |
| 2. 4 | 10.363 | D-mannitol | Sugar alcohol | 182.0793 | 165.0761 |
| 3. 6 | 12.396 | Clocortolonepivalate | Steroid | 494.228 | 477.2245 |
| 4. 8 | 14.165 | Traumatic acid | Dicarboxylic acid | 228.1352 | 211.1322 |
| 5. 12 | 17.931 | Epiafzelechintrimethyl ether | Flavonoid derivative | 316.131 | 299.1277 |
| 6. 13 | 20.538 | Phloionolic acid | Hydroxy fatty acid | 332.2543 | 355.2436 |
| 7. 14 | 21.034 | α-erythroidine | Alkaloid | 273.1354 | 256.1321 |
| 8. 15 | 23.417 | Eupatoriochromene | Chromene | 218.0936 | 201.0902 |
| 9. 16 | 23.671 | 4-oxo-9Z,11Z,13E,15E octadecatetraenoic acid | Fatty acid | 290.1868 | 291.1941 |
| 10. 18 | 28.708 | Gedunin | Terpene | 482.2281 | 465.2246 |

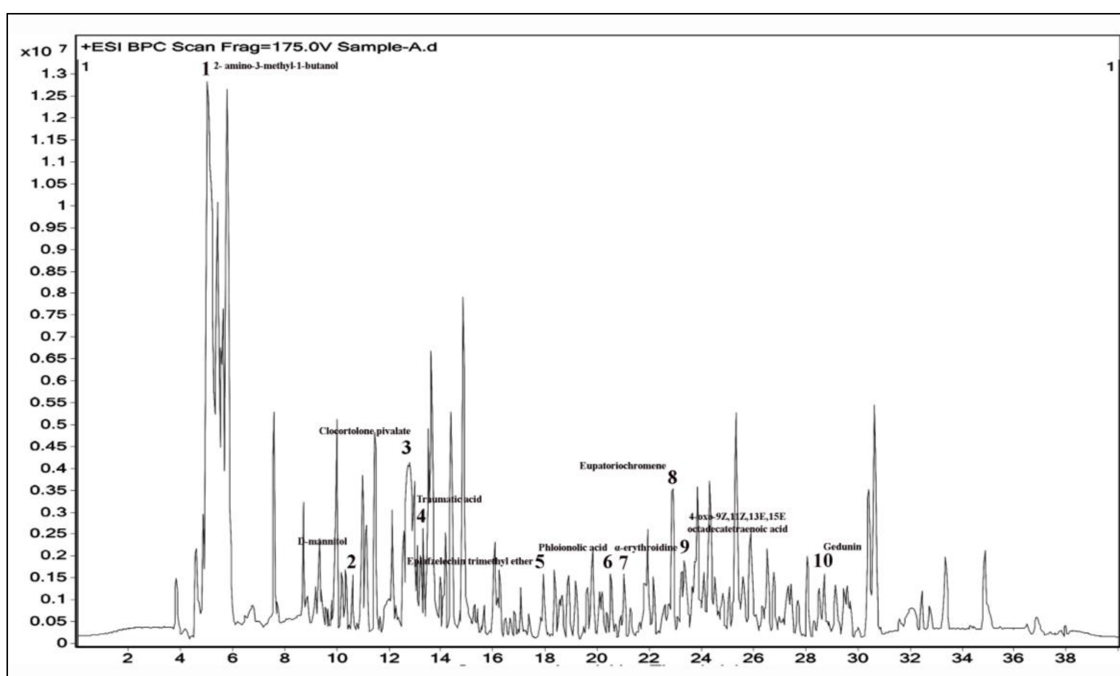


**Fig 1:** Liquid chromatogram of methanolic extract of *C. sarcomedium.* Digits on the chromatogram represent peak numbers of identified compounds.

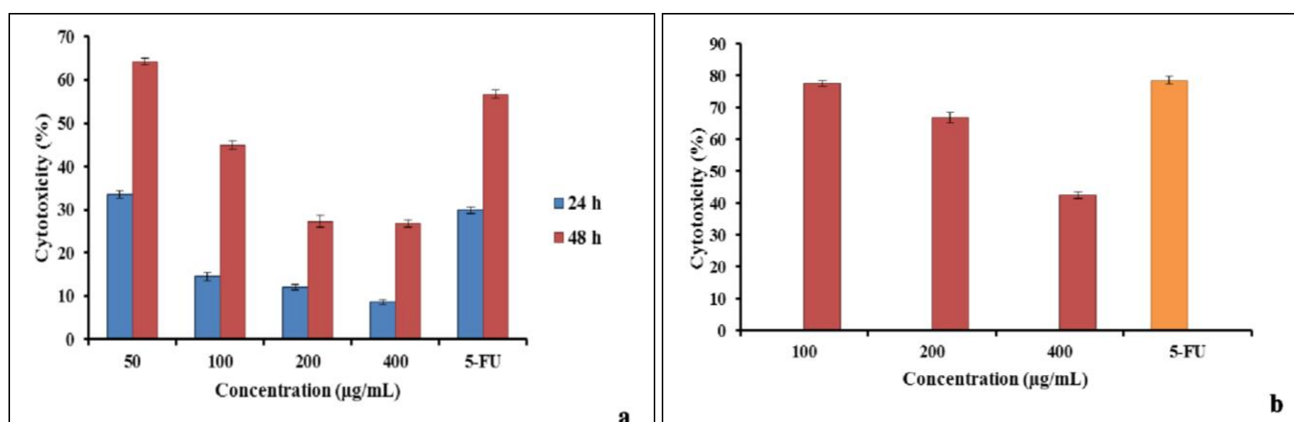


**Fig 2:** Evaluation of cytotoxicity by methanolic extracts of *C. sarcomedium*. a: MCF-7 cells; b: HCT-15 cell lines.

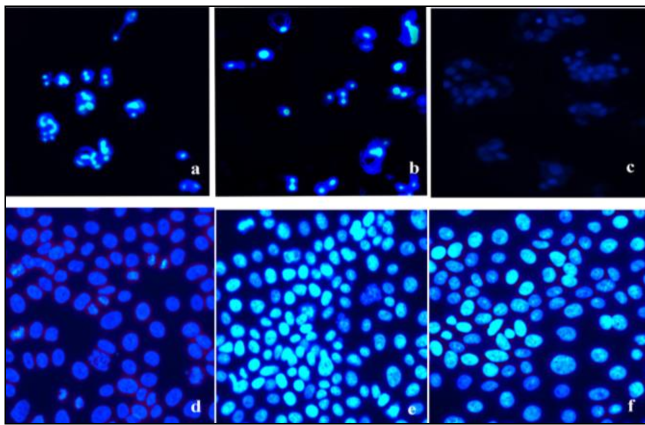


**Fig 3:** Detection of apoptosis by DAPI staining on MCF-7 and HCT-15 cell lines treated with *C. sarcomedium.* a-c: a: MCF-7 cell lines treated with plant extract, b: 5-FU, c: negative control; d-f: d: HCT-15cell lines treated with plant extract, e: 5-FU, f: negative control.

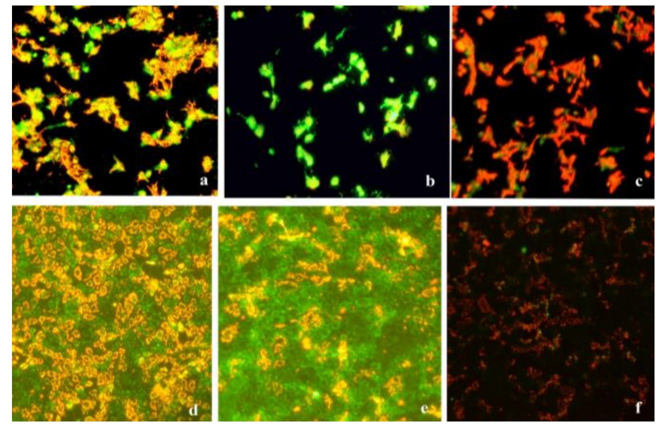


**Fig 4:** Evaluation of mitochondrial membrane potential by JC-1 staining on MCF-7 and HCT-15 cell lines treated with *C. sarcomedium.* a-c: a: MCF-7 cell lines treated with plant extract, b: 5-FU, c: negative control; d-f: d: HCT-15cell lines treated with plant extract, e: 5-FU, f: negative control.

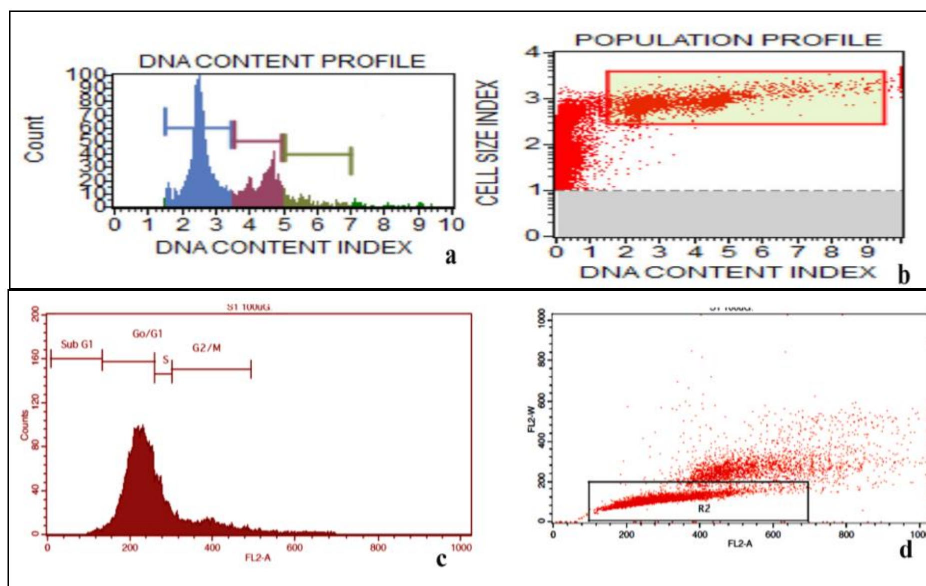


**Fig 5:** Determination of cell cycle arrest as analysed by flow cytometry. a & b: DNA content and population profile of MCF-7 cells treated with *C. sarcomedium* extract; b & c: DNA content and population profile of HCT-15 cells treated with *C. sarcomedium* extract.

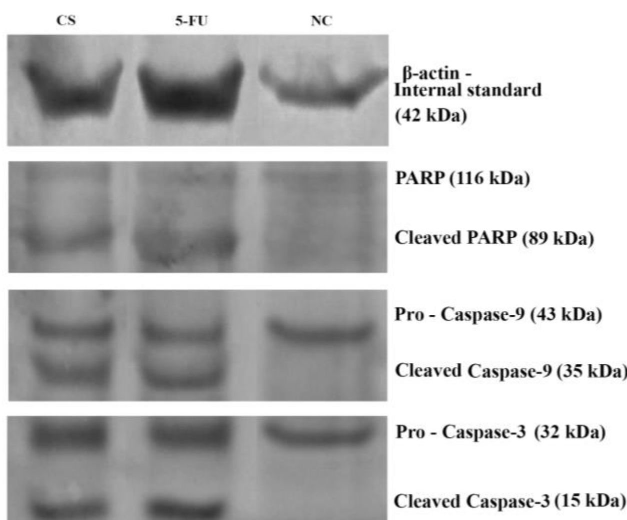


**Fig 6:** Western blot membrane showing signals: Protein extracts from MCF-7 cells treated with extract of *C. sarcomedium*, which were immunoblotted with the specified antibodies for PARP, Caspase-9 and Caspase-3. β-actin was used as an internal standard. CS: *C. sarcomedium*; 5-FU: 5-Fluoro uracil; NC: negative control.

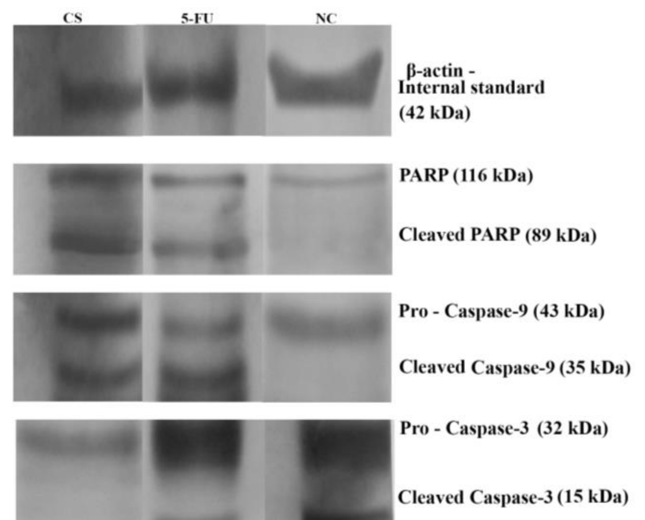


**Fig 7:** Western blot membrane showing signals: Protein extracts from HCT-15 cells treated with extract of *C. sarcomedium*, which were immunoblotted with the specified antibodies for PARP, Caspase-9 and Caspase-3. β-actin was used as an internal standard. CS: *C. sarcomedium*; 5-FU: 5-Fluoro uracil; NC: negative control.

## Conclusions

To the best of our knowledge, this is the first report on the anti-cancer mechanism of C. *sarcomedium* on breast cancer and colon cancer cell lines. The observations presented herein suggest that *C. sarcomedium* has promising anti-cancer activity which can be correlated with the presence of non-volatile constituents in the extract. While our study with MCF-7 cells and HCT-15 cells as an *in vitro* model proposes strong evidence for *C. sarcomedium* mediated apoptosis through a mitochondrial-dependent pathway, further studies with *in vivo* and clinical trials needs to be conducted to establish *C. sarcomedium* as a safe target for cancer therapy.

**Conflict of interest:** No conflicts declared

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