

Research Article

In Vitro Cytotoxicity of Four Folklore Medicinal Herbs of Kodanthur Tribal Village at Chinnar, Udumalpet, Tamil Nadu, India

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Abstract

Four plant species *Solanum trilobatum*, *Spathodea campanulata*, *Syzygium jambos* and *Tylophora indica* leaf extracts extracted with aqueous, methanol, ethanol and chloroform were screened for cytotoxicity in MCF-7 cells. Among 16 extracts tested significant ($p < 0.01$) cytotoxicity was exhibited by SJCLE (74.4%), and TICLE (64.04%) with an IC50 of 95.2 $\mu\text{g/mL}$ and 119 $\mu\text{g/mL}$ respectively. The extract SJELE was found to exhibit 70.6% cytotoxic to MCF 7 cells but IC50 was found to higher 123.4 $\mu\text{g/mL}$ compared to SJCLE and TICLE. The other extracts SCCLE, TIMLE, SJMLE, TICLE and TIELE exhibited above 60% anti-proliferative activity with their IC50 concentrations ranging from 98 $\mu\text{g/mL}$ -132 $\mu\text{g/mL}$. Furthermore, other extracts such as STELE, STMLE, and SCELE unveiled 50%-58% cytotoxicity against MCF-7 cell line at the highest concentration 300 $\mu\text{g/mL}$.

Keywords: Anticancer; Apoptosis; Cytotoxicity; Membrane blebbing

Introduction

According to estimates from the World Health Organization (WHO) in 2019, 1 cancer is the first or second leading cause of death before the age of 70 years in 112 of 183 countries and ranks 3rd or 4th in a further 23 countries. Cancer's rising prominence as a leading cause of death partly reflects marked declines in mortality rates of stroke and coronary heart disease, relative to cancer, in many countries [1]. Overall, the burden of cancer incidence and mortality is rapidly growing worldwide; this reflects both aging and growth of the population as well as changes in the prevalence and distribution of the main risk factors for cancer, several of which are associated with socioeconomic development [2]. In the development of formularies and pharmacopoeias, data on medicinal plants drive towards a new lane in the discovery of anti-cancer therapeutic agents. Herbal remedy has been approved worldwide where the plants and plant metabolites are used in the treatment of cancer. Records on plant dependent anticancer drugs from published and unpublished sources were first circulated in

December 1967 by Hartwell with monumental information of 3000 species of plants with anticancer property [3]. The National Cancer Institute ratified 114,000 anticancer plant extracts from 35,000 plant species from 20 countries [4]. The isolation of the vinca alkaloids, vinblastine and vincristine from the *Catharanthus roseus* hosted a novel era in the use of plant constituents as anticancer agents with a paramount significance applied in the treatment of cancer at clinical level [5-7]. The discovery has paved a track to phytotherapy in cancer regimens with an advent of anticancer camptothecin derivatives, topotecan, irinotecan and etoposide considerably examined in cancer rehabilitation [8,9]. With a basic concept from the literature reviews cited above the present investigation was carried out to rule out the cytotoxic and anticancer efficacy of four medicinal plants *S. trilobatum*, *S. campanulata*, *S. jambos* and *T. indica* prevalent in the Western Ghats of Tamil Nadu.

Materials and Methods

Collection of plant material

The selected plants leaf samples were collected based on the information's obtained from tribal communities of Kodanthur tribal village at Chinnar forest, Udumalpet and around the Western Ghats of Coimbatore district, Tamil Nadu. The voucher specimens *Solanum trilobatum* L. (No.1269), *Spathodea campanulata* P. Beauv. (No.1371), *Syzygium jambos* L. Alston (No.1408) and *Tylophora indica* (Burm.f.) Merr (No.1194) were deposited in the Department of Microbiology, RVS College of Arts and Science, Sulur for future references.

Test leaf extract preparation

Powdered and defatted fresh leaves were shade dried and

further extracted with aqueous and organic solvents viz. methanol, ethanol and chloroform by cold maceration method in room temperature. Crude herb extracts were obtained after a week of soaking, filtration *via*. whatmann filter paper no.1, and concentration through rotary vacuum evaporator. The concentrated crude extracts were stored at 4°C for further analysis.

Cytotoxic assay

Cell line maintenance: Human breast adenocarcinoma cells (MCF-7) obtained from National Centre for Cell Sciences (NCCS), Pune was maintained in Eagles Minimum Essential Medium (EMEM) in a CO₂ incubator under 37°C, 5% CO₂, 95% air and 100% relative humidity. The cells were maintained by weekly passage by changing the culture medium twice a week.

Cell suspension: Single cell suspension was obtained by detaching monolayer cells with trypsin-Ethylenediaminetetraacetic Acid (EDTA). With an aid of hemocytometer, viability of cells was counted and diluted with a medium containing 5% FBS to obtain 1 × 10⁵ cells/mL. In a 96 well microplates about 100 µL of cell suspension with plating density of 1 × 10⁴ cells/well was plated and incubated at 37°C providing 5% CO₂, 95% air and 100% relative humidity enabling for the attachment of cells. Following 24 h of incubation, the cells in the microplates were treated with different concentration of plant extracts.

Testing for cytotoxicity: About 100 µL of plant extract prepared with varying concentration ranging from minimum 300 µg/mL to 18.75 µg/mL was dispensed in to appropriate wells containing 100 µL of cell suspension followed by an additional incubation of the plates for 48 hrs. The cell suspension with DMSO and Cytosin (CTX) (10 µg/mL) served as negative and positive controls respectively, and triplicates were maintained for all concentrations.

MTT assay: Anti-proliferative effect of test extracts on MCF-7 cells was measured using the standard colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay. After 48 hours of incubation, 15 µL of MTT (5 mg/mL) in Phosphate Buffered Saline (PBS) was added to each well and incubated at 37°C for 4 h until purple precipitates were obviously visible under microscope. The medium together with MTT was then flicked, formed formazan crystals were solubilized in 100 µL of DMSO and absorbance recorded at 570 nm using a micro plate reader. The MTT assay measures the cellular mitochondrial enzymes (succinate dehydrogenases) that reduces (cleaves tetrazolium ring) the yellow water-soluble tetrazolium salt dye MTT into insoluble purple formazan. The quantity of formazan formed is directly proportional to the number of viable cells (existence of mitochondrial enzymes in the live cells) present in the plates. The percentage anti-proliferation (cytotoxicity) was determined by the following formula:

Statistical analysis

Nonlinear regression graph was plotted using drug concentration against percentage proliferation; a linear

regression with trend line was used to predict the IC₅₀ concentration of extracts that inhibits 50% proliferation of MCF-7 cells. Statistical significance was determined by one-way Analysis of Variance (ANOVA) by Dunnett's multiple comparison tests with control (DMSO) using GraphPad Prism.

Morphological observation of MCF-7 cells

The morphological observation of cells was performed with an aid of Phase-Contrast Inverted Microscope as described [11,12]. In brief, the MCF-7 cells were plated at a density of 1 × 10⁶ cells/mL into 6-well plates and incubated for 24 h to adhere followed by the treatment with plant extracts of determined IC₅₀ concentrations and incubated at 37°C for 24 h. The cells were then centrifuged (300 × g for 10 min) and washed twice with Phosphate Buffered Saline (PBS) by discarding the supernatant and remaining media. About 10 µL of fluorescent dye acridine orange (AO) (10 µg/mL) was added into the cellular pellet at equal volume, kept undisturbed for 2 h and a drop of stained cell suspension was observed under fluorescent (UV) microscope within an hour. The live viable, early and late apoptotic and secondary necrotic cells were determined based on the morphology under staining. The AO is an intercalating nucleic acid-specific fluorochrome crosses the cell membrane of live and early apoptotic cells with an emission of green fluorescence, when bound to DNA. The criteria for cellular morphological identification are: Viable cell nucleus will appear green with unbroken structure, early apoptosis with bright-green condensed chromatin in nucleus, late apoptosis have dense orange areas of chromatin condensation and orange intact nucleus depicts secondary necrosis.

Results and Discussion

In vitro cytotoxicity assay

Using human mammary cancer cells (MCF-7), a total of 16 extracts from 4 plant species were evaluated for their *in vitro* cytotoxic activity (anti-proliferation). The assay results with significant IC₅₀ values are depicted in Figure 1. A complete growth arrest was noticed in positive control treated with Cytosin (Cyclophosphamide) under a minimum concentration of 18.75 µg/mL, where the drug was able to debar 92% (p<0.001) proliferation of MCF-7 cells in the culture media.

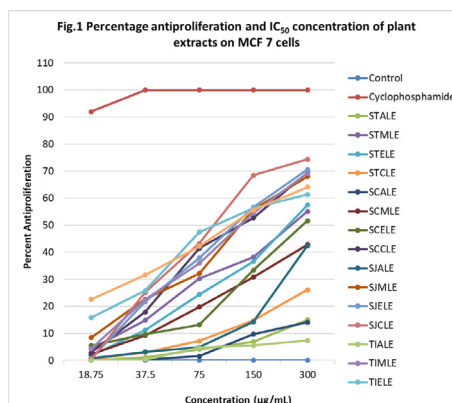


Figure 1: Graphical representation of dose dependent anti-proliferation

of plant extracts

Worth noting anti-proliferation has been identified in SJCLE (74.4%), SJELE (70.6%), SCCLE (69.52%), TIMLE (69.3%), SJMLE (67.99%), TICLE (64.04%), and TIELE (61.32%) at the concentration of 300 µg/mL, where their IC₅₀ concentrations ranged between 95 µg/mL to 132 µg/mL, though fairly high still point subtly towards selective activity. A dose dependent anti-proliferation of plant extracts was represented and the percentage of anti-proliferation were further examined by Analysis of Variation (ANOVA) with Dunnett's multiple comparison test for the statistical significance. The extracts SJCLE and TICLE represented a highest significance ($p < 0.00$) of anti-proliferation in the MCF 7 cell line. Furthermore, the other group of extracts SCCLE, SJMLE, SJELE, TIMLE, and TIELE showed a significant ($p < 0.05$) anti-proliferation but not comparable to Cytoxan, which could be due to the less active metabolites arresting cell proliferation. In the entire study the cell suspension with DMSO maintained as a negative control showed cell line proliferation in an uncontrollable manner. Among 4 plants the *Syzygium jambos* and *Tylophora indica* extracts excluding aqueous form were found to be more effective on cell lines inhibiting proliferation.

Cytotoxic potential of STELE and STMLE produced 57.48% and 55.11% cell death at 300 µg/mL with an IC₅₀ concentration of 192 µg/mL and 203 µg/mL respectively. The cell proliferation restriction of STALE and STCLE indicated that much more concentration of >300 µg/mL of extract is required for significant anti-proliferative effect of MCF 7 cells.

In the entire study the highest anti-proliferative property has been observed from the extracts of *S. jambos* > *T. indica* > *S. campanulata* > *S. trilobatum* and our findings could be a comparable report with other reports previously published. Our result concurs with the antiproliferative property of *S. trilobatum* ethanolic leaf extracts on Ehrlich Ascites Carcinoma (EAC) cells and inhibition of mice melanoma and metastasis, which could be due to the presence of flavonoid quercetin in the plant [13-15]. The antiproliferative property of *S. trilobatum* was substantiated by the American cancer society which booms flavonol 'Quercetin' and its significance in anticancer therapy [16]. Epidemiological studies recommends dietary flavonoids and its role in reducing the risk of tumors in colon, breast, prostate, lung and pancreas [17,18]. A study submits the Saponin Fractions from *S. trilobatum* (SFST) has a dose-dependent suppressive effect in cell proliferation [19]. A partially purified Sobatum from *S. trilobatum* proved cytotoxic in Dalton's Lymphoma ascites, Ehrlich ascites and tissue culture cells (L929 and Vero) [20].

The other herb *S. campanulata* proved to be moderately anti-proliferative on MCF and cells. Six common human dietary sources of anticancer flavonols from methanolic leaf extracts of *S. campanulata* such as 1-O-caffeoyl-β-D-glucopyranoside kaempferol 3-O-(2-O-β-D-xylopyranosyl)-β-D-galactopyranoside,

kaempferol 3-O-(6-O-β-L-rhamnopyranosyl)-β-D-galactopyranoside, acteoside, kaempferol 3-O-(6-O-α-L-rhamnopyranosyl)-α-D-glucopyranoside and quercetin 3-O-(2-O-β-D-xylopyranosyl)-β-D-galactopyranoside has been reported [21]. The dietary sources of flavonols in *S. campanulata* might attribute to the antiproliferation in synergistic or distinctive action.

In vitro cytotoxic activity of compounds was evaluated by using SRB assay against human leukemia cancer cell lines (HL-60) [22]. The researcher isolated Stigmasta-5,22-dien-3-ol, octadecenamide, and umbelliferone from chloroform extract that showed cytotoxicity with decreasing cell viability in a dose-dependent manner, but it was found low as compared to positive control, i.e., Adriamycin against HL-60 cell lines.

Among all extracts subjected in the study the chloroform leaf extract of *S. jambos* (SJCLE) revealed highest cytotoxicity against MCF-7 cells. Besides the SJELE and SJMLE unveiled cytotoxicity with IC₅₀ of 123.4 µg/mL and 131.4 µg/mL respectively. Previous reports on methanolic extracts of *S. jambos* Linn leaves were studied *in vitro* on Hep G2 cells, and the results demonstrated their cytotoxic effect with well-established indication of programmed cell death with the fragmentation of chromatin into units of one or more nucleosomes that form the nucleosomal DNA ladder on an agarose gel. The study's gels unequivocally demonstrated that, at the optimal concentration, the extract-treated cells genomic DNA was significantly fragmented. The control cells were not treated and did not exhibit any fragmentation [23]. The research confirmed the extract acted on a cellular level positively affecting the apoptotic cell cycle pathway *via* Bcl-2 and Bax gene expression. According to, the N-heptane fraction of *S. jambos* reduced all cell lines viability in comparison to the negative control, which consisted of untreated cells [24]. In a previous study examined the cytotoxicity of many methanol extracts from several *Syzygium* species, noting that the MCF-7 cell line did not exhibit any cytotoxicity from the methanolic extract of *S. jambos* at 100 µg/mL [25]. Since the extract's chemical makeup is mostly composed of phenolic chemicals, in the present *in vitro* study, the cytotoxicity of the chloroform, methanol, and ethanolic extracts was greater. Two acylated flavonol glycosides myricitin and myricetin in addition to quercetin were reported from the leaves of *S. jambos* [26,27]. Reports recommend the inhibitory property of flavonol quercetin against mice melanoma and invasive [14,16].

At 300 µg/mL the leaf extract of *T. indica* (TICLE) disclosed pronounceable antiproliferation on MCF-7 cells. Effective anticancer activity of *T. indica* acetone and ether extracts on BHK-21 cell line was reported [28]. Unique class of antitumor tylophorine analogs differing in mode of action from known antitumor drugs has been reported [29]. Polar Phenanthrene-Based Tylophorine derivatives (PBTs) N-(2,3-methylenedioxy-6-methoxy-phenanthr-9-ylmethyl)-5-aminopentanol and N-(2,3-methylenedioxy-6-methoxy-phenanthr-9-ylmethyl)-l-2-piperidinemethanol

has been proved to be potentially cytotoxic against A-549 human cancer cells [30].

Herbal metabolites are always considered as a natural medication for various ailments. Natural agents from plant sources inducing apoptosis by impeding the proliferation of malignant cells can be used in cancer chemotherapy and chemoprevention. Therapeutic plants have posed a great attention in the treatment of various types of cancer considering as safer and effective anticancer agents [31]. The consumption of flavonoid rich fruits and vegetables remained to reduce the risk of cancer [32-34]. Due to additive or synergistic and rival or confounding activity of plant extracts on tumor cell lines provide an vital model for the analysis of biological mechanisms produced in clinical effects [35-37]. Similar kind of anticancer studies has aided in the identification of plant metabolites with effective anticancer activity on cancerous cells deprived of provoking effects on normal cells [38].

Morphological observation of MCF-7 cells

With the aid of microscopic technique, the original morphological criteria of apoptosis can be perceived and appraised determining the structural alterations in cells [39,40]. The microscopic examination remained the gold standard for the precise detection of apoptosis with comprehensive information about cell lines [41]. Normal inverted microscope was applied to observe the morphological changes of MCF-7 cells in the control and test plates after 72 h post treatment under 400X magnification. The present study demonstrates that the highest antiproliferative property was exhibited by *S. jambos* straggled by *T. indica*>*S. campanulata* and *S. trilobatum* via induction of membrane blebbing on MCF-7 cells inducing apoptosis. The micrographs of cells treated with higher concentration of extracts exposed distinctive morphological membrane blebbing, a process associated with apoptosis such as cell shrinkage, chromatin condensation, DNA fragmentation and apoptotic body formation (Figures 2 and 3). The apoptotic micrographs endorse the apoptosis is a consequence of chromatin margination and cytoplasm condensation with typical compaction and segregation of the nuclear chromatin.

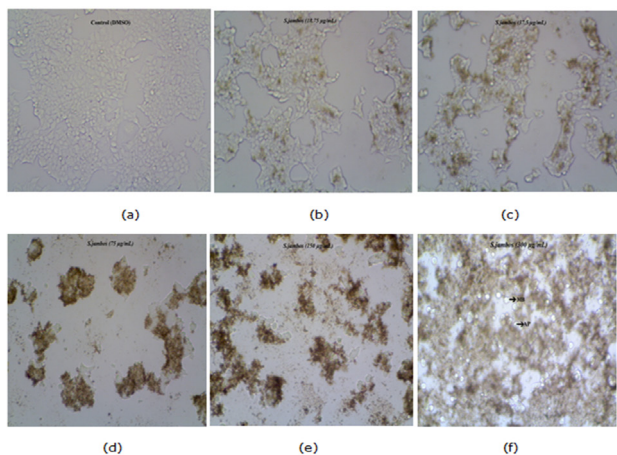


Figure 2: Micrographs of MCF 7 cells treated with chloroform leaf extracts of *S.jambos* examined after 48 hrs, (a) Plate 1a Control (DMSO)

with confluent growth of cells; (b) Plate 1b Progression of proliferation inhibition with increase in concentration of plant extract; (c) Plate 1c Progression of proliferation inhibition with increase in concentration of plant extract; (d) Plate 1d Progression of proliferation inhibition with increase in concentration of plant extract; (e) Plate 1e Progression of proliferation inhibition with increase in concentration of plant extract; (f) Plate 1f Progression of proliferation inhibition with increase in concentration of plant extract

AP: Apoptosis, MB: Membrane blebbing

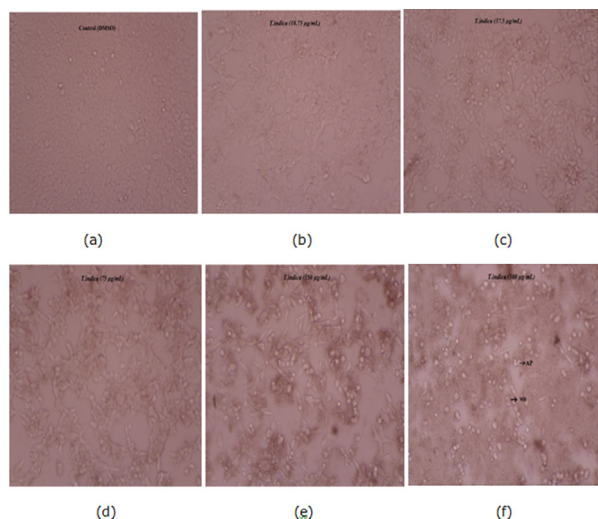


Figure 3: Micrographs of MCF 7 cells treated with chloroform leaf extracts of *S.jambos* examined after 48 hrs, (a) Plate 2a Control (DMSO) with confluent growth of cells; (b) Plate 2b Progression of proliferation inhibition with increase in concentration of plant extract; (c) Plate 2c Progression of proliferation inhibition with increase in concentration of plant extract; (d) Plate 2d Progression of proliferation inhibition with increase in concentration of plant extract; (e) Plate 2e Progression of proliferation inhibition with increase in concentration of plant extract; (f) Plate 2f Progression of proliferation inhibition with increase in concentration of plant extract

AP: Apoptosis, MB: Membrane blebbing

The morphological observation of treated cells exposed extremely impaired extra and intracellular structures by reduction in number of viable cells, in accordance with the cytotoxic property exhibited by the plant fractions; however the controls remained with confluent growth throughout the incubation period. Clear signs of apoptosis in our investigation concur with the study determining the progression of cells internal environment condensation under treatment with plant extracts [42,43]. The condensation remains accompanied by nuclear and cell outline convolutions resulting in a breach of nucleus into detached ruins to produce membrane bounded apoptotic bodies by budding of the cell. The anti-proliferative property of extracts can be evaluated by counting viable cancer cells, while the apoptogenic property shall be determined by observing typical morphological changes on apoptosis specifically membrane blebbing. Apoptosis was well evident in the micrographs of cell lines treated in a dose dependent manner of all the four plant extracts.

In the process of development and homeostasis in multicellular organisms, apoptosis remains exceedingly controlled and organized cell death process regulating variety of physiological and pathological conditions. The cytotoxicity micrographs in the present study exposed

the morphological characteristics of apoptotic cells containing chromatin condensation, fragmented nuclei and DNA concurring with the previous report determining the apoptotic cellular morphological changes are due to condensation of chromatin and oligonucleosomal DNA cleavage [44]. The present study outcome indicates clearly that the selected plants *S. trilobatum*, *S. campanulata*, *S. jambos* and *T. indica* used as a folklore healthcare in traditional medicine acts *via* programmed cell death. Out of 4 plants selected the *Syzygium jambos* and *Tylophora indica* was found to be potent in anti-proliferative activity on MCF-7 cell lines. Additional studies are crucial to define the molecular mechanisms and its pathways to evaluate potential *in vivo* anticancer activity of the selected plant fractions along with active components.

Conclusion

The impact of drug efficacy in therapy always remains instigated from *in vitro* level before its execution to *in vivo* models. Plant flavonoids play a major role in disease prevention and therapy, henceforth the flavonoid rich plants are explored with great implication in the field of rehabilitation. Since the phytochemical analysis of *S. trilobatum*, *S. campanulata*, *S. jambos* and *T. indica* leaf extracts revealed the existence of flavonoids; the present cytotoxic study was executed in accordance with a concept "flavonoids as anticancer agents." The MTT assay was instigated in the present study to accept or rule out the cytotoxic efficacy of selected plant extracts, as the method provides an accurate and reliable quantification both in cancer and noncancerous cell lines in terms of cell viability. The chloroform leaf extracts of *S. jambos* was found to be potent antiproliferative but not up to the level of Cytoxan. In the present study the IC₅₀ concentration of all the active extracts with more than 60% anti-proliferation ranged from 95.2 µg/mL-132 µg/mL and the "high" IC₅₀ values are likely may be due to very low concentration of compounds of interest, which could considerably enrich the bioactivity in the cytotoxic assay. Our study could further aim for the anticancer compound identification with an execution on *in vivo* models.

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Conflict of Interest

We declare that, we all authors have no conflict of interest.

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