In vitro cytotoxicity and anticancer activity of four folklore medicinal plants used among tribal communities of Western Ghats, Coimbatore, Tamil Nadu

Rangasamy Dhanabalan1,*, Muthusamy Palaniswamy2, Joseph Devakumar1
1*Department of Microbiology, Rathnavel Subramaniam College of Arts and Science, Coimbatore-641402, Tamil Nadu, India
2Department of Microbiology, School of Life Sciences, Karpagam University, Coimbatore-641021, 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 their anticancer activity in MCF-7 cells. Importantly, among the 16 extracts tested significantly reliable (p<0.01) antiproliferation was exhibited by SJCLE (92.79%) and TICLE (79.97%) with IC50 of 84.3 µg/mL and 90 µg/mL respectively. The SJELE and STCLE displayed antiproliferative effect of 74.8% and 71% with IC50 concentrations 132.53 µg/mL and 203.23 µg/mL respectively. A total of eight extracts such as SCMLE, SJALE, SCELE, STMLE, STELE, TIELE, SCCLE and SJMLE unveiled 41.56-69.28% cytotoxicity against MCF-7 cell line at the highest concentration 300 µg/mL. Four extracts namely TIMLE, STALE, SCALE and TIALE showed least cytotoxicity 26.14%, 15.6%, 14.3% and 7.81% respectively at 300 µg/mL.

Key words: Anticancer, apoptosis, cytotoxicity, membrane blebbing

1. INTRODUCTION

Worldwide among leading causes of death in 2012, cancer accounts for 8.2 million demises and figures as second foremost reason of death in the United States.1 Aggressive existences of lung, colorectal, stomach, liver and breast cancers are the chief cancerous deaths occurring annually. Every year more than 60% of the world’s new cancer cases occur in Africa, Asia and South and Central America accounting for 70% of the world’s cancer expiries. It is anticipated that in the next two decades the world’s annual cancer cases will rise from 14 million to 22 million cases.2

In the development of formularies and pharmacopoeias, data’s on medicinal plants drive towards a new lane in the discovery of anticancer 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 Records on plant dependent anticancer drugs from published and unpublished sources were first circulated in December 1967 by Hartwell with monumental information of 3000 species of plants with anticancer property.3 The National Cancer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, vinblastine5 and vincristine6 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.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 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.

2. MATERIALS AND METHODS

2.1. Collection of plant material

The plant samples were collected from different areas around Western Ghats, Coimbatore, Tamil Nadu and authenticated by Dr. G.V.S. Murthy, Scientist ‘F’ and Head, Botanical Survey of India, Southern Regional Centre, Coimbatore. 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.
plant leaves were collected based on the information’s obtained from tribal communities such as Malasars, Irulas and Konars living around the Western Ghats.

2.2. Preparation of leaf extracts

The fresh disease free shade dried powdered leaves were defatted with petroleum ether and extracted with aqueous and organic solvents viz. methanol, ethanol and chloroform by cold maceration method. After a week of soaking, filtration was conducted with whatmann filter paper no.1, and concentrated via. rotary vacuum evaporator. The concentrated crude extracts were stored at 4°C for further analysis.

2.3. In vitro cytotoxicity assay

2.3.1. Maintenance of cell lines

Human breast adenocarcinoma cells (MFC-7) were obtained from National Centre for Cell Sciences (NCCS), Pune and preserved in Eagles Minimum Essential Medium (EMEM) incubating under 37°C, 5% CO₂, 95% air and 100% relative humidity. The cell suspension with DMSO by an additional incubation of the plates for 48 hrs at 37°C, 5% CO₂, 95% air and 100% relative humidity allowing for the attachment of cells. After 24 hrs of incubation the cells in the plates were treated with different concentration of plant extracts.

2.3.2. Preparation of cell suspension

The monolayer cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) to make a single cell suspension. The viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x10⁴ cells/mL. About 100 µL of cell suspension was seeded into 96-well plates at a plating density 1x10⁴ cells/well and incubated at 37°C provided with 5% CO₂, 95% air and 100% relative humidity for the attachment of cells. After 24 hrs of incubation the cells in the plates were treated with different concentration of plant extracts.

2.3.3. Cell treatment with test plant extracts

About 6 mg of plant extract was dissolved in 2 mL of serum free sterile DMSO to obtain a stock concentration 6000 µg/2 mL. One mL of the stock plant extract was kept undispensed and another 1 mL was serial two fold diluted in sterile tubes containing 1mL of DMSO, so as to obtain a drug concentration ranging from 3000, 1500, 750, 375 and 187.5 µg/mL. About 100 µL of diluted plant extract was dispensed to the appropriate wells containing 100 µL of cell suspension followed by an additional incubation of the plates for 48 hrs at 37°C, 5% CO₂, 95% air and 100% relative humidity. The cell suspension with DMSO and Cytoxan (CTX) (10 µg/mL) served as negative and positive controls respectively, and triplicates were maintained for all concentrations.

2.3.4. MTT assay

The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay for measuring the activity of cellular mitochondrial enzymes (succinate dehydrogenases) reduces (cleaves the tetrazolium ring) the yellow water soluble tetrazolium salt dye MTT into insoluble purple colored formazan. Therefore, the quantity of formazan produced remains directly proportional to the number of viable cells or the intensity of purple colored insoluble formazan formed remains directly proportionate to the live cells/existence of mitochondrial enzymes in the live cells. After 48 hours of incubation, 15µL of MTT (5mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hrs until purple precipitates were obviously visible under microscope. The medium together with MTT was then flicked, formed formazan crystals were solubilized in 100µL of DMSO and absorbance recorded at 570 nm using a micro plate reader. The percentage antiproliferation (cytotoxicity) was determined by the following formula:

\[
\text{Percentage antiproliferation} = \frac{\text{Average Abs of control wells} - \text{Average Abs of plant extract wells}}{\text{Average Abs of control wells}} \times 100
\]

2.4. 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 Graph Pad Prism software.

2.5. Morphological observation of MCF-7 cells

The cellular morphological observation was performed using Phase-Contrast Inverted Microscope as described. In brief, the MCF-7 cells were plated at a density of 1×10⁶ cells/mL into 6-well plates and incubated for 24 hrs to adhere followed by the treatment with plant extracts of determined IC₅₀ concentrations and incubated at 37°C for 24 hrs. 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 10µL of fluorescent dye acridine orange (AO) (10µg/mL) was added into the cellular pellet at equal volume, kept undisturbed for 2 hrs 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

2.3. In vitro cytotoxicity assay

A total of 16 extracts from 4 plant species were screened for their cytotoxic activity (antiproliferation) against human mammary cancer cells (MCF-7) and the cytotoxic assay results with significant IC_{50} values are depicted in Table 1. A complete growth arrest was noticed in positive control treated with Cytoxan (Cyclophosphamide) and it was worth noting that IC_{50} values ranged between 84-275µg/mL, though fairly high, still point subtly towards selective activity. A dose dependent antiproliferation of plant extracts were represented in Figure 1-4 and the percentage of antiproliferation were further examined by analysis of variation (ANOVA) with Dunnett’s multiple comparison test for the statistical significance.

Table 1. Percentage antiproliferation and IC_{50} concentration of plant extracts on MCF 7 cells

<table>
<thead>
<tr>
<th>Plant extract / Drug</th>
<th>Plant extract concentration (µg/mL)/</th>
<th>Percentage antiproliferation</th>
<th>IC_{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STALE</td>
<td>0</td>
<td>1.93 3.02 6.88 15.66</td>
<td>ND</td>
</tr>
<tr>
<td>STMLE</td>
<td>4.245</td>
<td>14.82 27.1 44.32 53.12</td>
<td>242.3</td>
</tr>
<tr>
<td>STELE</td>
<td>1.809</td>
<td>11.2 24.35 35.36 57.48</td>
<td>247.48</td>
</tr>
<tr>
<td>STCLE</td>
<td>4.245</td>
<td>13.44 25.88 41.89 71.05</td>
<td>203.23</td>
</tr>
<tr>
<td>SCALE</td>
<td>0</td>
<td>0.48 1.32 9.74 14.3</td>
<td>ND</td>
</tr>
<tr>
<td>SCMLE</td>
<td>2.227</td>
<td>9.25 17.93 30.82 41.56</td>
<td>&gt;300</td>
</tr>
<tr>
<td>SCELE</td>
<td>5.428</td>
<td>9.67 13.22 29.5 51.63</td>
<td>275</td>
</tr>
<tr>
<td>SCCLE</td>
<td>8.42</td>
<td>15.57 32.15 55.81 67.98</td>
<td>130.49</td>
</tr>
<tr>
<td>SJALE</td>
<td>0.765</td>
<td>2.38 1.79 14.26 41.93</td>
<td>&gt;300</td>
</tr>
<tr>
<td>SJMLE</td>
<td>0.765</td>
<td>7.86 21.38 38.48 69.28</td>
<td>210.96</td>
</tr>
<tr>
<td>SJELE</td>
<td>0.696</td>
<td>6.68 28.18 56.34 74.8</td>
<td>132.53</td>
</tr>
<tr>
<td>SJCLE</td>
<td>0.139</td>
<td>25.12 43.21 90.88 92.79&quot;</td>
<td>84.3&quot;</td>
</tr>
<tr>
<td>TIALE</td>
<td>0.128</td>
<td>0.19 4.88 5.53 7.81</td>
<td>ND</td>
</tr>
<tr>
<td>TIMLE</td>
<td>0.45</td>
<td>2.96 7.21 14.74 26.14</td>
<td>&gt;300</td>
</tr>
<tr>
<td>TIELE</td>
<td>6.76</td>
<td>15.84 25.75 57.69 65.03</td>
<td>131.94</td>
</tr>
<tr>
<td>TICLE</td>
<td>28.07</td>
<td>36.31 46.03 65.87 79.977&quot;</td>
<td>90&quot;</td>
</tr>
<tr>
<td>Cytoxan</td>
<td>97&quot;**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in the parenthesis represents the percentage antiproliferation (cytotoxicity), ND-Not detected, IC_{50}-50% inhibitory concentration, *** and ** represents the statistical significance of treatment groups at the level of p<0.001 and p<0.01 respectively compared with the negative control by Dunnet's multiple comparison test.

STALE, STMLE, STELE & STCLE-S. trilobatum aqueous, methanol, ethanol and chloroform leaf extracts respectively
SCALE, SCMLE, SCELE & SCCLE-S. campanulata aqueous, methanol, ethanol and chloroform leaf extracts respectively
SJALE, SJMLE, SJELE & SJCLE-S. jambos aqueous, methanol, ethanol and chloroform leaf extracts respectively
TIALE, TIMLE, TIELE & TICLE-T. indica aqueous, methanol, ethanol and chloroform leaf extracts respectively

Fig. 1 Dose dependent antiproliferation of Solanum trilobatum leaf extracts on MCF 7 cells

Fig. 2 Dose dependent antiproliferation of Spathodea campanulata leaf extracts on MCF 7 cells

Fig. 3 Dose dependent antiproliferation of Syzygium jambos leaf extracts on MCF 7 cells
The chloroform leaf extracts of S. tilobatum exhibited 71% antiproliferation, STELE and STMLE displayed 57.48% and 53.12% antiproliferation on MCF-7 cells at 300 µg/mL. Our result concurs with the antiproliferative property of S. tilobatum ethanolic leaf extracts on Ehrlich Ascites Carcinoma (EAC) cells\(^8\) and inhibition of mice melanoma and metastasis,\(^4\) which could be due to the presence of flavonoid quercetin in the plant.\(^5\) The antiproliferative property of S. tilobatum was substantiated by the American cancer society which booms flavonol ‘Quercetin’ and its significance in anticancer therapy.\(^6\) Epidemiological studies\(^7\)–\(^8\) recommends dietary flavonoids and its role in reducing the risk of tumors in colon, breast, prostate, lung and pancreas. A study submits the saponin fractions from S. tilobatum (SFST) has a dose-dependent suppressive effect in cell proliferation.\(^9\) A partially purified Sobatum from S. tilobatum proved cytotoxic in Dalton’s Lymphoma ascites, Ehrlich ascites and tissue culture cells (L929 and Vero).\(^10\)

The chloroform leaf extracts from S. campanulata (SCCLE) exposed 67.98% antiproliferation, whereas the SCELE and SCMLE were effective at 51.63% and 41.56% on MCF-7 cells at 300 µg/mL. Six common human dietary sources of anticancer flavonoids from methanolic leaf extracts of S. campanulata such as 1-O-caffeoyl-\(\beta\)-D-glucopyranoside kaempferol 3-O-(2-O-\(\beta\)-D-xylopyranosyl) - \(\beta\)-D-galactopyranoside, kaempferol 3-O-(6-O-\(\beta\)-L-rhamnopyranosyl)-\(\beta\)-D-galactopyranoside, acetyoside, kaempferol 3-O-(6-O-\(\alpha\)-L-rhamnopyranosyl)-\(\alpha\)-D-glucopyranoside and quercetin 3-O-(2-O-\(\beta\)-D-xylopyranosyl)-\(\beta\)-D-galactopyranoside has been reported.\(^11\) The dietary sources of flavonoids in S. campanulata might attribute to the antiproliferation in synergistic or distinctive action.

The chloroform leaf extract of S. jambos (SJCLE) revealed highest cytotoxicity 92.79% (p<0.01) against MCF-7 cells with an IC\(_{50}\) 84.30 µg/mL. Besides the SJELE and SJMLE unveiled 74.8% and 69.28% cytotoxicity with IC\(_{50}\) of 132.53 and 210.96 µg/mL respectively. Two acylated flavonol glycosides myricitin and myricetin\(^22\) in addition to quercetin\(^22\) were reported from the leaves of S. jambos. Reports recommend the inhibitory property of flavonol quercetin against mice melanoma and invasive metastasis.\(^14\)^\(^16\)

At 300 µg/mL chloroform leaf extract of T. indica (TICLE) disclosed 79.97% (p<0.01) and TIELE revealed 65% antiproliferation on MCF-7 cells with IC\(_{50}\) values 90 µg/mL and 131.94 µg/mL respectively. Effective anticancer activity of T. indica acetone and ether extracts on BHK-21 cell line was reported.\(^24\) Unique class of antitumor tylophorine analogs differing in mode of action from known antitumor drugs has been reported.\(^25\) 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)-1,2-piperidinemethanol has been proved to be potentially cytotoxic against A-549 human cancer cells.\(^26\) 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.\(^27\) The consumption of flavonoid rich fruits and vegetables remained to reduce the risk of cancer.\(^28\)^\(^29\) 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.\(^31\)^\(^33\) 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.\(^34\)

2.4. 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. The microscopic examination remained the gold standard for the precise detection of apoptosis with comprehensive information about cell lines. Normal inverted microscope was applied to observe the morphological changes of MCF-7 cells in the control and test plates after 72 hrs post treatment under 400X magnification. The present study demonstrates that the highest antiproliferative property was exhibited by SJCLE (92.79%) straggled by TICLE (79.97%)>SJJELE (74.8%)>STCLE (71%)>SJMLE (69.28%)>SCCLE (67.98%)>TIELE (65%)> STELE (57.48%)>STEMLE (53.12%), SCELE (51.63%)>SJALE (41.93%) and SCMLE (41.56%) 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 (Fig. 5a-f & 6a-
f), a process associated with apoptosis such as cell shrinkage, chromatin condensation, DNA fragmentation and apoptotic body formation. The apoptotic micrographs endorse the apoptosis is a consequence of chromatin margination and cytoplasm condensation with typical compaction and segregation of the nuclear chromatin.

**Fig. 5a-f.** Micrographs of MCF 7 cells treated with chloroform leaf extracts of *S.jambos* examined after 48 hrs

**Fig. 5a.** Control (DMSO) with confluent growth of cells

**Fig. 5b-f.** Progression of proliferation inhibition with increase in concentration of plant extract **AP**: Apoptosis, **MB**: Membrane blebbing

**Fig. 5f**

**Fig. 6a-f.** Micrographs of MCF 7 cells treated with chloroform leaf extracts of *T.indica* examined after 48 hrs

**Fig. 6a.** Control (DMSO) with confluent growth of cells
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\textsuperscript{35-36} determining the progression of cells internal environment condensation under treatment with plant extracts. 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 antiproliferative 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. 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 four plants selected the Syzygium jambos and Tylophora indica was found to be potent in antiproliferative 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.

3. 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 implemented in the present study to 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 IC_{50} concentration of all the active extracts ranged from 84-275 µg/mL with exception of STALE, SCALE, SCMLE, SIAL, TIALE and TIMLE. The “high” IC_{50} 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.

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

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