Anti-Cancer Potential of Vateria Indica L. Stem Bark Enriched Fractions in EAC and C6 Cell lines—an In-Vitro Study

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Abstract

The western Ghats of India present Pharmacognosists and botanist a rich source of biological diversity to explore their unearthed potential. V. indica, one among the thousands of such plant growing in the southern states of India especially in Kerala, Karnataka and Tamil Nadu, at about 750 m altitude is a slow growing plant of Dipterocarpaceae family. The present study was aimed to investigate the anticancer potential of stem bark of Vateria indica by trypan blue exclusion method and Sulphorhodamine B assay against C6 and EAC cell lines respectively. From the studies it was found that the acetone fraction of V. indica demonstrated high activity whereas ethanolic and petroleum fraction were moderately active against C6 cell lines respectively. It was also observed that the ethanolic and ethyl acetate fraction of V. indica were highly active whereas petroleum ether extract was moderately active against EAC cell lines.

Keywords: Vateria indica, Anticancer potential, SRB assay.

Introduction

Multiple alterations in gene expression can be a leading factor in deregulating the balance of cell proliferation as well as cell death thus evolving into a gamate of cells which could invade tissues and metastize to distant sites causing significant mortality rates. Such abnormal growth of cells is basically termed as cancer [1]. Under normal situation; the cells grow followed by its division and eventually die in a systematic manner. In cancer condition however, the cells in a part of the system begin to grow in an uncontrollable manner[2]. According to the NCRP, that is National Cancer Registry Program of Indian Council of Medical Research, it is estimated that there could be about 5,34,353 men and about 6,14,404 women associated cancer cases by 2020. It could literally mean that the number of cancer cases could go up from 9, 79, 786 in 2010 to 11, 48, 757 by 2020 [3]. With the threat of cancer cases looming large and the number of cases increasing by the year, it is imperative to find a solution towards the prevention and treatment patterns associated with it. Nature provides us with one such opportunity in tackling this issue. The role of plants in treatment of human beings as well as animals is recognized and documented since time immemorial. Currently, the need for basic scientific investigation of medicinal plants using indigenous medical system has become more interesting and relevant [4]. Phytochemicals have been proposed to offer protection against a variety of diseases including...
obesity, cardiovascular diseases and cancer[5].The U.S national cancer Institute way back in 1950’s had recognized the potential of natural products as anticancer agents. A number of groups having anticancer activity or property are derived from plant source and include alkaloids, terpenoids [6] etc. The ongoing research for new molecules as anticancer agents from plant source is among promising strategies for its prevention [7].

One such plant which has recently gained attention in this field is Valeria indica. The plant, popularly known as white dammar belonging to the family Dipterocarpaceae grows in abundance in the evergreen west coast forests of Karnataka, Tamil Nadu as well as Kerala [8].

The bark is found to possess DL-epicatechin (Fig 1.), afzetechin, fischinidol, resveratrol (Fig 2.), stilbenoids along with different flavones (Fig 3.) and phenols (Fig 4.). The extract from Vateria indica has been used to treat ailments like chronic bronchitis, boils, diarrhoea and several related diseases. Plants and herbs used in traditional medicine have been accepted currently as among the prominent source of chemo preventive drug discovery and development [9]. The present study was thus envisaged to study the anticancer properties of Vateria indica Linn. Through in vitro cancer screening models.

Materials and Methods

Source

Stem bark of Vateria indica Linn. was collected between October to November, 2011 from End Point area, near Swarna River, Manipal, Udupi District of Karnataka. Dr. Richard Lobo, Associate Professor at the department of Pharmacognosy authenticated the plant and deposited a voucher specimen (No. PP 584) at Pharmacognosy department of Manipal college of Pharmaceutical sciences, Manipal University.

Extraction and Fractionation of Plant Material

Extraction

The stem bark of V. indica was dried in shade and then coarsely powdered (500g). Alcoholic extract was prepared using ethanol in a soxhlet apparatus.

The crude ethanolic extract was concentrated, dried and stored in a desiccator. The yield of crude ethanolic extract was 29.6%.

Plant powder (1250g) was kept for cold maceration for a week using water: chloroform in the ratio 99:1 as solvent. The crude aqueous extract was concentrated on water bath and stored in a desiccator after drying. The yield of crude aqueous extract was 6.032%.

Fractionation

For obtaining fractionation of V. indica, the dried crude extract (50g) was redispersed in distilled water. This was followed by successive extraction with solvents of increasing polarity index like petroleum ether, ethyl acetate and acetone .concentrated using Rotary evaporator, under reduced pressure, dried and stored in desiccator.

Fractions of crude ethanolic extract were named as VIP (Vateria indica Petroleum ether), VIE (Vateria indica ethyl acetate) and VIA (Vateria indica acetone). For in vitro studies, all the extracts were completely dissolved in DMSO.

Maintenance of Cell Cultures

Thawing and Recovery of Cells [10]

Vial from liquid nitrogen freezer was removed and immediately warmed by rubbing between palms. The vial was agitated continuously until medium was thawed. Cells should be thawed as soon as possible to prevent formation of ice crystals that can cause cell lysis. Top of the vial was wiped with 70% ethanol before opening. The thawed suspension was transferred into a sterile centrifuge tube containing 4 mL warm complete medium (with 10% FBS). The content was centrifuged at 1200 rpm for a period of 5 min. at room temperature and supernatant was discarded. The cell pellets were gently resuspended in 1 mL complete medium and transferred to properly labeled culture plates containing appropriate amount of medium. The culture was finally checked after 12 hrs to ensure viability and attachment of cells to the plate.
Incubation Conditions

The cell cultures were incubated in an incubator with temperature of 37°C and 5% CO₂.

Passaging

Many adherent cell cultures would cease proliferating when they become confluent and the remaining could die if they are left in a confluent state for too long. These cell cultures therefore need continuous passaging, meaning, once the cells are confluent, a segment of the cells are required to be transferred to a fresh cell culture vessel. These cells were then maintained in a T-25 tissue culture flask.

The medium from tissue culture flask was aspirated and discarded. The cells were washed with 5 mL HBSS, aspirated and discarded. To this, 0.5 mL of pre warmed 1x solution of trypsin-EDTA was added covering the monolayer. Incubation in a CO₂ incubator at a temperature of 37°C for 3-5 min was carried out.

The cells were observed in a phase contrast microscope to ensure detached rounded cells. After dislodging, the cells were resuspended in growth medium comprising 10% FBS. The cells were gently pipetted up and down, thus disrupting cell clumps following which suitable volume of resuspended cells were transferred to a new cell culture vessel which contained pre warmed growth medium. The vessel was moderately swirled to help the cells mix with medium.

Cell Freezing

Cells were checked for health, contamination and morphological characteristics before freezing at log phase. The cells were harvested by trypsinization, resuspended in medium containing 10% FBS, centrifuged at 1200 rpm for 4 min. and then cells were resuspended in freezing mixture at a density of 3-5 x 10⁶ cells/mL.

Cell suspension of 0.5 mL was transferred to each freezing vial. The vials were then labeled with name of cell line, date, passage number as well as growth medium. The cryovials were stored in liquid nitrogen at -196°C.

Type of cell lines used

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type of cancer</th>
<th>Tissue type</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAC cells</td>
<td>Mice liquid tumor</td>
<td>Peritoneal fluid cancer</td>
</tr>
<tr>
<td>C6</td>
<td>Rat glioma cells</td>
<td>Gliona (Glial cells)</td>
</tr>
</tbody>
</table>

Table 1: Category of cell lines used in the study

In-Vitro Cytotoxicity Screening SRB Assay [11]

The assay measures whole culture protein content which is directly proportional to the cell number. Sulphorhodamine B (SRB), an amino xanthene dye that is bright pink, under mild acidic conditions fixes to basic amino acid residues in trichloro acetic acid bound cells. The amount of SRB bound is proportional to the number of live cells left in a culture after the drug exposure. The colour developed with this assay is rapid, stable and visible, the intensity of which is directly proportional to the number of viable cells that can be measured at 540/630 nm.

Cell lines in exponential phase were harvested from T-25 tissue culture flasks. A stock suspension was then prepared in suitable media supplemented with 10% serum. Following procedure was followed for the assay.

A tissue culture plate with 96 well with flat bottom was seeded with 100 µL of stock cell suspension of suitable concentration. Attachment was allowed for 24 hrs.

Test compounds were prepared prior to investigation in 0.5% DMSO. Serial dilution with suitable medium was performed to get different concentrations of 25, 50, 100 and 200 µg. (C6 cell lines).

Following 24 hrs of incubation, cells were treated with test compounds (100 µL) for 48 hrs.

Following 48 hrs of incubation, 100 µL of 10% w/v Trichloro acetic acid was added to each well without removing cell culture supernatant for fixation of cells. The plate was incubated for 60 min. at a temperature of 4°C.

Plates were then thoroughly washed 4 times with milli Q water and dried completely at room temperature. 100 µL of 0.4% SRB dye was taken and pipetted to each well. Incubation at 37°C for
30 min. was carried out in dark. The plate was immediately washed 4 times with acetic acid (1%) removing unbound dye and completely dried at room temperature.

10 mM tris base solution (200 µL) with pH of 10.5 was added to each well to solubilize the protein bound dye completely.

Using ELISA plate reader with reference wavelength 630 nm, the optical density was measured at 540 nm.

Percentage cell viability is calculated as below

\[
\text{OD of test} = \frac{\text{OD of test}}{\text{OD of control}} \times 100
\]

**Trypan Blue Exclusion Assay Method [12]**

A living cell membrane can avoid the entry of few dyes into the cell. Thus, the viable cells remain unstained by which they can easily be differentiated from dead cells which appear blue after taking up the dye when observed under light microscope.

**Cell Lines**

Cancer cell lines namely Ehrlich’s Ascetic Carcinoma (EAC) required to induce cancer in animal model was acquired from ACRC, Kerala, India. These were maintained and preserved as ascites tumor through intraperitoneal inoculation in swiss albino mice.

**Preparation of Trypan Blue Solution**

400 mg of trypan blue was dissolved in 100 ml of normal saline (0.9% NaCl) and stored at 4°C.

**Results and Discussions**

**In-Vitro cytotoxic activity of various extracts of stem bark of V. indica against C6 cell by SRB assay**

Cytotoxicity of various extracts of stem bark of V. indica bark against C6 by SRB assay is shown in table 2. The IC\textsubscript{50} value of ethyl acetate extract was found to be 332.04µg/mL whereas the acetone extract had an IC\textsubscript{50} value of 80.44µg/mL (Fig 5.) being on the highest and lowest range respectively.

**Table 2: In vitro cytotoxic activity of various extracts of stem bark of V. indica in C6 cells (Mouse ganglia carcinoma) by SRB assay at 48h of exposure**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>% Cytotoxicity</th>
<th>50 µg/mL</th>
<th>100 µg/mL</th>
<th>200 µg/mL</th>
<th>IC\textsubscript{50} value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>18.51±0.11</td>
<td>32.65±0.01</td>
<td>47.71±0.11</td>
<td>227.82</td>
<td></td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td>2.1±0.03</td>
<td>17.3±0.24</td>
<td>59.13±0.08</td>
<td>178.70</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>11.36±0.10</td>
<td>37.48±0.05</td>
<td>55.75±0.03</td>
<td>80.44</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>10.14±0.17</td>
<td>35.35±0.19</td>
<td>36.54±0.09</td>
<td>332.04</td>
<td></td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>6.43±0.08</td>
<td>50.92±0.24</td>
<td>64.85±0.06</td>
<td>110.64</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5: Effect of various fractions of *V. indica* extracts on SRB Assay in C6 cell lines. Results were expressed as mean values ± standard deviation of independent experiments performed in triplicate.

**In-Vitro cytotoxic activity of various extracts of stem bark of *V. indica* against EAC cells by trypan blue exclusion assay**

Cytotoxic activity of various extracts of stem bark of *V. indica* against EAC cells by trypan blue exclusion assay is shown in table 3. From the results it is seen that the acetone extract had an IC$_{50}$ value of 166.9µg/mL while ethanolic extract had an IC$_{50}$ value of 56.15µg/mL (Fig 6.).

Table 3: In-Vitro cytotoxic activity of various extracts of stem bark of *V. indica* Linn. on EAC cells by trypan blue exclusion assay.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>% Cytotoxicity</th>
<th>25 µg/mL</th>
<th>50 µg/mL</th>
<th>100 µg/mL</th>
<th>200 µg/mL</th>
<th>IC$_{50}$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td></td>
<td>13.6±3</td>
<td>26.0±6</td>
<td>40.5±0.2</td>
<td>59.3±10</td>
<td>143.3</td>
</tr>
<tr>
<td>Ethanolic extract</td>
<td></td>
<td>28.3±4</td>
<td>51±3.3</td>
<td>48.9±13</td>
<td>39.7±2.1</td>
<td>56.15</td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
<td>20.1±0.11</td>
<td>22.3±3</td>
<td>23.1±4.9</td>
<td>65.1±10</td>
<td>166.9</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td></td>
<td>29.5±0.1</td>
<td>40.5±5.3</td>
<td>40.5±0.5</td>
<td>63.2±3.1</td>
<td>100</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td></td>
<td>20.8±2</td>
<td>36.9±2</td>
<td>44.9±0.1</td>
<td>65.5±2.7</td>
<td>103.6</td>
</tr>
</tbody>
</table>

Fig. 6: Effect of various fractions of *V. indica* extracts on Tryphan blue assay in EAC cell lines results were expressed as mean values ± standard deviation of independent experiments performed in triplicate.
Uncontrolled growth and spread of abnormal cells are the main characteristics of a group of diseases called cancer. Death can occur if the spread is not limited and controlled. Various external factors such as nicotine products, infection due to organisms and an unhealthy diet and internal factors, such as mutation that are genetically inherited, hormones and state of immune system could lead to cancer. A group of these factors in combination or in a sequential order could trigger cancer. It could take a decade or more from exposure time towards external factors and its detectable stage. Basic treatment pattern include, hormone therapy, surgery, radiation, chemotherapy, immune therapy and targeted therapy.

Dipterocarpaceous plants comprise of several oligomers of resveratrol that demonstrate a variety of biological as well as pharmacological activities, like antimicrobial and antiproliferative effects. In earlier investigations, it has been reported that vaticanol C which is a tetramer of resveratrol, exhibited strong cytotoxic effects against various cancer cell lines[13].

In the current study, the antitumor potentials of the enriched fractions of aqueous fraction, ethanolic fraction, acetone fraction, ethyl acetate fraction and petroleum ether fraction from the stem bark of Vateria indica were studied. In Ayurveda, this plant has traditionally been used for various diseases.

Phytochemicals from natural sources have become an excellent source of leads since several decades. They are unique in that they present an extensive and wide range of structural motifs and are biologically friendly [14].

In the present study the anti-cancer potentials of various enriched fractions of the bark of vateria indica in C6 and EAC cell lines by tryphan blue and SRB assay were studied.

From the results obtained it was found that all the fractions exhibited cytotoxic activity, but ethanol fraction showed significant activity with IC₅₀ value 56.15 μg/ml in EAC cells by tryphan blue method. Similarly, all the fractions were subjected to SRB assay against C6 cell lines where it was observed that except ethyl acetate fraction, all other fractions exhibited the cytotoxic activity but the acetone fraction of the bark was found to highly potent with IC₅₀ value 80.44 μg/ml.

The results obtained above suggest that the main compounds such as DL-epicatechin, afzetechin, fischinidol, resveratrol, stilbenoids along with different phenols and flavanols present in the bark could be responsible for its cytotoxic activity against EAC and C6 carcinoma cell lines.

**Conclusions**

Vateria indica belongs to the family Dipterocarpaceae and there are sufficient reports indicating the anticancer potential of plants belonging to this family. In the present study the anticancer potential of stem bark of Vateria indica against two cancer cell lines namely, Rat glioma (C6) and EAC were tested. Alcoholic and aqueous extract of stem bark of Vateria indica exhibited considerable cytotoxic activity against the cell lines in the preliminary screening. Thus a bioactivity guided fractionation scheme was adopted where in the alcoholic extract was fractionated with solvent of increasing order of polarity like petroleum ether, ethyl acetate and acetone.

These fractions were screened for their cytotoxic potential against same cell lines using two different assay methods viz a viz., SRB assay and trypan blue dye exclusion assay. The results obtained from these two basic assays demonstrate that V. indica acetone fraction was highly active whereas ethanolic and petroleum ether fraction were moderately active against C6 while ethanolic and ethyl acetate fractions were highly active against EAC cells as compared to petroleum ether fraction. The results suggest that not all fractions of the extract were active against the cell lines. Acetone, ethanolic and petroleum ether showed active cytotoxicity in cancer cells, the rest of fractions were not active. Based on the present studies it can be concluded that V. indica possess anticancer activity.

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