In vitro anticancer potential of Anaphyllum wightii Schott. against Dalton’s lymphoma ascites cell lines and molecular docking studies of β-sitosterol

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Cancer is the second most life threatening noncommunicable disease in humans that challenges the mankind with its multifactorial etiology. Even though various treatment strategies such as radiation therapy, chemotherapy, etc. are commonly used for this disease, researches are being focussed on developing plant-derived novel anticancer compounds that lack side effects. There were no previous reports on the anticancer potential of the ethnomedicinally important plant Anaphyllum wightii Schott. (Araceae). Hence, in the present study, we evaluated the in vitro cytotoxic effects of A. wightii on Dalton’s lymphoma ascites (DLA) tumor cell line. Interestingly, the acetone extract of rhizome showed much lower LC50 value (14.27 μg/mL) for the DLA cell line compared to that of normal rat spleen cells (1189.23 μg/mL), revealing its significant anticancer potential. The molecular docking analysis showed β-sitosterol, present in the rhizome, as a promising lead molecule for the development of cytochrome P450 1 A1 inhibitors, which may provide potential anticancer agents.

Keywords: Cancer, Keerikkizhangu, Noncommunicable disease (NCD), Tumor, Wight's Twisted Arum

Cancer is a deadly disease characterized by abnormal and uncontrolled cell growth which destroy body tissues. It is one of the major non communicable diseases affecting 8.1 million patients causing 16% deaths worldwide next only to cardiovascular diseases (31%). Asia is reported to be leading both in cancer incidence (57.3%) as well as in cancer mortality (48.4%). In the Age-Standardized Rate (ASR) of cancer incidence and mortality globally (24 world areas) for all cancers combined, India ranks 18th in cancer incidence with ASR 279.8 and the least (24th) in mortality with ASR 123. The United States is projected to record 1.81 million new cancer cases and 0.61 million deaths in 2020; approximately, 4950 new cases and 1660 deaths each day. Even though early detection of various cancers helps in proper treatment and curing, the side effects associated with the treatment procedures such as radiation therapy, chemotherapy, etc. make cancer the most threatening disease. Treatment methods such as chemotherapy and radiotherapy result in severe side effects on patients, ranging from nausea, allergic reactions, decreased immune response to bleeding and toxicity. Patients also endure from the chance of metastasis, with future complications like formation of tumours in other parts of the body.

Plants provide a hope as a dependable resource of anticancer compounds without any side effects unlike synthetic drugs. For mankind, there is an urgent need to explore newer resources to develop therapeuticsto overcome such a dreaded disease. Hence, researchers are focussed on developing such novel anticancer compounds from medicinal plant resources.

Anaphyllum wightii Schott., belonging to Araceae (Arum family), commonly called Wight's Twisted Arum, and locally in Kerala as Keerikkizhangu, is an ethnomedicinal plant used by tribal communities of Kerala such as Kani, Kadars, Madhuvars, etc. It is a tall herbaceous plant belonging to the family Araceae which is an endemic and threatened species of South India. The plant is having a rhizomatous stem, pinnately compound leaves, with two major variations, either broad or narrow and spadix inflorescence with a characteristically twisted spathe. Tribal people use the rhizome of this plant as an antidote against snake bite and also as food. The rhizome is reported to have various pharmacological properties such as antibacterial, anthelmintic, antioxidant, hepatoprotective, anti-inflammatory,
and antidiabetic activities which support the ethno-
botanical evidences\textsuperscript{13}.

\(\beta\)-Sitosterol, a potential anticancer compound\textsuperscript{17},
already considered as a standard reference for herbal
drug comounds\textsuperscript{18}, has been reported from other
Araceae members\textsuperscript{19}. However, there are no previous
reports available on the anticancer potential of
\textit{Anaphyllum wightii}. Hence, in the present study, we
assessed the \textit{in vitro} cytotoxic effects of this plant on
Dalton’s lymphoma ascites (DLA) cell lines and
thereby evaluate the anticancer property of the plant.
This study also involved the molecular docking
analysis of \(\beta\)-sitosterol against target proteins
involved in cancer development.

\textbf{Materials and Methods}

\textbf{Plant material}

Both broad and narrow-leaved varieties of
\textit{Anaphyllum wightii} were collected from Kallar,
Kerala, identified by a taxonomic expert from the
University of Kerala, and were planted in the garden
of Dept. of Botany, University of Kerala. The leaves
of both varieties and the rhizome of only the broad-
leaved variety, which is commonly used in tribal
medicine, were used for the \textit{in vitro} cytotoxicity
assays.

\textbf{Soxhlet extraction}

About 12 g of the powdered rhizome (of broad-
leaved variety) and two leaf varieties were subjected
to serial soxhlet extraction for about 6-8 h using
120 mL each of the solvents such as petroleum
ether, chloroform, acetone, methanol and distilled
water in the increasing order of polarity. Then
methylanic extracts of the two leaf varieties and the
methanolic, as well as acetone extracts of the
rhizome, were selected for the \textit{in vitro} cytotoxicity
assay since, in case of the rhizome, acetone extract
was more active than the methanolic extract. Only
the acetone and methanolic extracts were selected
for the study because these two were found to
have higher antioxidant potential compared to
other solvent extracts in our previous study
(Unpublished data).

\textbf{\textit{In vitro} cytotoxicity assay}

The short term \textit{in vitro} cytotoxic effects of the
rhizome and leaf extracts were studied using Dalton’s
lymphoma ascites (DLA) cell line by trypan blue
exclusion method\textsuperscript{20} using a hemocytometer. The
tumor cells aspirated from the peritoneal cavity of
tumor-bearing mice were washed thrice with
phosphate-buffered saline (PBS) or normal saline.
Viable cell suspension (1x10\textsuperscript{6} cells in 0.1 mL) was
added to the tubes with different concentrations of the
extracts and later made up to 1 mL using PBS. The
tube containing only cell suspension (without the
extract) was used as the control. These assay mixtures
were incubated for three hours at 37°C. Further, the
cell suspension was mixed with 0.1 mL of 1% trypan
blue, loaded on a hemocytometer after 2-3 min. Live
cells do not absorb the blue colour of trypan blue but
dead cells take up the colour of the dye. The number
of stained and unstained cells were counted
separately, and the percentage of cell death was
calculated. The assay was done using normal rat
spleen cells also.

\textbf{HPTLC Fingerprinting}

About 25 µL each of the sample extracts (100 µg/µL)
and standards (1.0 mg/mL) were applied as bands of
width 8 mm on silica gel 60 F\textsubscript{254}
pre-coated aluminium sheets through CAMAG microliter syringe
using Automatic TLC Sampler 4 (ATS4). After
sample application, the plate was introduced vertically
in a CAMAG developing chamber (10x10 cm)
pre-saturated with the mobile phase. A number of solvent
systems were tried, and a system that gave the
maximum resolution was selected as the solvent
system for the extract. The optimum separations of
constituents were achieved using toluene: ethyl
acetate: formic acid (5: 3: 0.1) as the mobile phase.
The developed chromatogram was air-dried to
evaporate solvents from the plate, and the plate was
kept in CAMAG Visualizer, and the images were
captured under UV light at 254 and 366 nm\textsuperscript{18}.

\textbf{Post chromatographic derivatization}

The plate was derivatized using vanillin-sulphuric
acid reagent, heated at 105°C by placing on CAMAG
TLC plate heater till the colour of the bands appeared.
Then the plate was visualized under white light, and
the chromatograms were documented.

\textbf{Molecular docking analysis}

Molecular docking analysis of \(\beta\)-sitosterol was
done using Autodock software against the target
Cytochrome P450 1 A1. The docked conformation of
the ligand-protein complex was visualized using the
Discovery studio visualizer.

\textbf{Statistical analysis}

The values are expressed as mean ± SE. A value of
\(P <0.05\) was considered significant.
Results and Discussion

The in vitro cytotoxicity assays can be considered as the preliminary screening methods to evaluate the anticancer potential of plant extracts. In the present study, the short term in vitro cytotoxic effects of acetone, as well as methanolic extracts of the rhizome and the methanolic extracts of the two leaf varieties, were done using Dalton’s lymphoma ascites (DLA) cell line. Lymphoma cancer, a heterogeneous group of malignant disease with a wide spectrum of illnesses, comprises 70 different subtypes, and is observed most commonly in children, next to Leukemia21. Dalton’s lymphoma is a tumor that originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, US, in 1947. Subsequently, an ascites form was developed by repeated intraperitoneal transplantation of tumors. It is highly invasive in nature, and kills the host in a concise period of life span22.

The percentage of cytotoxicity was calculated by the trypan blue exclusion method. Trypan blue is a blue-coloured dye that can penetrate into dead cells and stain them, whereas living cells will remain unstained. Thus after treating the cell lines with the extracts, the exact number of dead and viable cells can be counted by using this dye23. The percentage of cell death effected by the four extracts at different concentrations are represented in Table 1.

Among the rhizome and leaf extracts selected for the assay, the acetone extract of rhizome showed the lowest LC$_{50}$ value (14.27 µg/mL) for DLA cell line followed by the methanolic extract of rhizome (39.27 µg/mL), whereas these two extracts showed higher LC$_{50}$ values for the normal rat spleen cells (1189.23 and 2468.55 µg/mL, respectively) as shown in Table 1. The LC$_{50}$ value of well-known reported anticancer compound curcumin24, was comparable to that shown by the acetone extract of rhizome of *A. wightii*. The methanolic extracts of both the narrow and broad leaves showed higher LC$_{50}$ values for the DLA cell line (910.23 and 2468.55 µg/mL, respectively) compared to that of rhizome extracts.

Lower the LC$_{50}$ value, higher will be the cytotoxicity. A potential anticancer compound should exhibit a lower LC$_{50}$ value for cancer cell lines and a comparatively higher LC$_{50}$ value for the normal cells. Thus, the results of the trypan blue dye exclusion technique indicated that both the acetone as well as methanolic extracts of rhizome could inhibit the growth of DLA cells significantly in a concentration-dependent manner and hence may contain potential anticancer compounds.

β-Sitosterol is a well-known phytosterol commonly found in the family Araceae, and it is reported as one of the major bioactive compounds in the tubers of *Colocasia esculenta*25, *Amorphophallus paeonifolius*26 and *Amorphophallus companulatus*27. Earlier studies reported that β-sitosterol has significant cytotoxic potential against the cancer cell lines HT-29 (colon cancer)28, LNCaP (prostate cancer), MDA-MB-231 (breast cancer)29, HL60 (Caucasian promyelocytic leukemia)30, U937 (human leukemic cells), COLO320 (human colorectal cancer cells)31, and MCA-102 (fibrosarcoma cells)32. However, this compound was not previously reported in this particular plant, and hence we did HPTLC analysis of the rhizome and leaf extracts using the standard β-sitosterol which confirmed the presence (Fig. 1).

Since β-sitosterol has been reported as an anticancer agent, molecular docking of the same was done against the target protein cytochrome P450 1 A1 (CYP 1 A1). CYP 1 A1 is one of the cytochrome P450 enzymes involved in the activation of carcinogenic compounds33. It can convert polycyclic aromatic hydrocarbons to carcinogenic compounds. Hence, the inhibitors of this enzyme may be useful in prevention or inhibition of various cancers. β-sitosterol showed significant interaction with the

Table 1 — In vitro cytotoxicity of rhizome, broad and narrow leaves

<table>
<thead>
<tr>
<th>Sample concentration (µg/mL)</th>
<th>Normal cell line (Rat spleen cells)</th>
<th>Dalton’s lymphoma ascites cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhizome</td>
<td>Broad leaves</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>Methanol</td>
</tr>
<tr>
<td>10</td>
<td>0±0.00</td>
<td>0±0.00</td>
</tr>
<tr>
<td>20</td>
<td>0±0.00</td>
<td>0±0.00</td>
</tr>
<tr>
<td>50</td>
<td>0±0.00</td>
<td>0±0.00</td>
</tr>
<tr>
<td>100</td>
<td>2±0.00</td>
<td>0±0.00</td>
</tr>
<tr>
<td>200</td>
<td>8±0.00</td>
<td>4±0.00</td>
</tr>
<tr>
<td>LC$_{50}$ values (µg/mL)</td>
<td>1189.23</td>
<td>2468.55</td>
</tr>
</tbody>
</table>

[Values are Mean ± Standard error; n = 3]
target protein cytochrome P450 1 A1 with the binding energy of \(-10.69\) KCal/mol. This lower binding energy indicates that the ligand (\(\beta\)-sitosterol) fits into the active site of the protein CYP 1 A1 more strongly. The docked conformation of the ligand-protein complex is represented in Fig. 2.

The docking analysis revealed that \(\beta\)-sitosterol might be a promising lead structure for the development of cytochrome P450 1 A1 inhibitors since it can interact significantly with the protein and thus may act as potential anticancer agents.

**Conclusion**

In the present study, we have determined the *in vitro* cytotoxic effects of *Anaphyllum wightii* on Dalton’s lymphoma ascites (DLA) tumor cell line and also assessed the potential of its constituent \(\beta\)-sitosterol to serve as a lead molecule for drug development. The rhizome extract of *A. wightii* showed significant *in vitro* cytotoxicity against the DLA cell line which suggests that it may contain compounds with anticancer potential which was supported by docking studies. The compound \(\beta\)-sitosterol present in the plant can act as a lead compound for the development of CYP 1 A1 inhibitors, and hence it may be useful in cancer therapy.

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**Conflict of Interest**

Authors declare no conflict of interests.

**References**


