ABSTRACT

Objective: To find out the cytotoxicity, anti-tumor and anti-inflammatory activities of six species of plants belonging to Viscaceae family available in Western Ghats, India.

Methods: Cytotoxicity of Viscum extracts was studied by trypan blue exclusion and MTT assay using various cell lines. Anti-tumor activity was determined using Ehrlich ascites carcinoma (EAC) and Dalton’s lymphoma ascites (DLA) cells in mice. Anti-inflammatory activities of Viscum extracts were studied using carrageenan and dextran induced mouse paw edema models.

Results: Viscaceae plant extracts studied were cytotoxic towards transformed cells DLA and EAC as well as to MCF-7, MDA-MB-231 and SKBR3 cell lines. Extracts of V. orientale, V. nepalense and V. ramosissimum, V. trilobatum were cytotoxic towards normal cells while V. angulatum and V. capitellatum were found to be nontoxic. Excepting V. angulatum all the other species selected here showed toxicity to animals. Administration of nontoxic concentration of extracts of Viscaceae species significantly (P<0.001) increased the lifespan of ascites tumor bearing animals and reduced DLA cells induced solid tumor development. All these plants except V. capitellatum and V. trilobatum showed significant (P<0.001) anti-inflammatory activity against carrageenan and dextran models and reduced pro-inflammatory cytokine levels.

Conclusion: Four plants of Viscum species studied were cytotoxic to tumour cells and inhibited tumour development. Of the six species studied V. angulatum was non-toxic to animals and showed maximum efficiency as an antitumour agent. These plants showed significant anti-inflammatory activity and reduced inflammatory markers.

Keywords: Semiparasitase plants, Viscum species, Tumour reducing activity, Anti-inflammatory activity, Pro-inflammatory cytokines

INTRODUCTION

**Viscum** is a genus of about 70-100 species of mistletoes, native to temperate and tropical regions of Europe, Africa, Asia and Australasia. Mistletoes constitute the predominant group of angiosperm shoot or stem hemi-parasites, which grow on the branches of host trees or shrubs and take water, water-conducted nutrients and organic solutes from the host's vasculature. Historically speaking, mistletoe was considered as an antidote for poisons as well as a remedy for barrenness and constipation [1]. Rudolf Steiner, the founder of anthroposophical medicine, introduced mistletoe for cancer treatment [2]. Significant work has been done on one of the species of Viscaceae family, Viscum album L. which grows in European countries. Iscador, an aqueous extract of *V. album* has been widely used as an anti-cancer drug for several decades [3]. Anti-tumor [4], anti-carcinogenic [5], anti-metastatic [6], chemotherapeutic and radio protective activities [7] of *V. album* have been reported. *V. album* contains several active components such as mistletoe lectins [8] viscotoxins [9], alkaloids [10] and polysaccharides [11] which are reported to show anti-tumor properties by causing cell cycle delay or arrest and induction of apoptosis [12]. It inhibits tumor angiogenesis [13, 14] and exerts immune potentiating activities that enhance the host defense system against tumors [15, 16]. Compounds of mistletoe origin are also been reported to show in vitro inhibitory potential on multidrug resistance protein (MDR1) [17]. The analysis of clinical studies suggests that adjuvant treatment of cancer patients with mistletoe extracts is associated with a better survival, a reduction of side effects of conventional therapy and with an increase of quality of life [18-21].

No systematic work has been done on semi-parasitic plants other than *Viscum album*. In the present study six plants belonging to the family Viscaceae viz Viscum orientale Wild, Viscum nepalense Spring, Viscum ramosissimum Wall and Viscum angulatum Heyne ex DC Viscum capitellatum Sm and Viscum trilobatum Talbot collected from Western Ghats, India were checked for their anti-tumour and anti-inflammatory activity. *V. orientale* is a large hemi-parasitic, much branched shrub with opposite and oblanceolate leaves. Flowers are produced in dichasial cymose triad clusters developing into ovoid to sub-globose berries. The plant is reported to have medicinal applications mainly in neuralgia, diabetes and in the treatment of itching [22]. *V. nepalense* is a leafless hemi-parasitic mistletoe found growing on the branches of various trees. The branches are long, flat, with pendulous tufts and internodes being variable in length, usually a trifle wider at the distal end and striate. The leaves are visible only in the very young internodes as small bracts below the ovary. It has been reported that this species possesses a number of therapeutic properties and is used for the treatment of many diseases in traditional medicine [23]. *V. ramosissimum* is a slender, pendulous, leafless, yellowish-green plant with rounded internodes. Inflorescence may be dichasia or modified dichasia with pistillate flowers or sometimes solitary representing a reduced dichasium. Sometimes the dichasia are complete, bearing a terminal pistillate flower and two lateral staminate flowers. The berry is ovoid and pale green in colour. *V. angulatum* is a leafless hemiparasitic shrub with four-angled branches which are slightly broadened near the apex of the internode and smooth. Flowers are seen as solitary or in groups either with all female flowers or with a single female flower surrounded by male flowers. Male flowers have four triangular perianth lobes with four ephippial stamens with sessile anthers. The ovary is ovoid with a short style in female flowers. The berry is globose and yellowish. This plant is traditionally used in Asian countries for the treatment of hypertension [24]. *Viscum capitellatum* and *Viscum trilobatum* are hyper coparasitics. Former growing on *Dendropthoe falcata* (parasitic plant on *Terminalia tomento*) and *Viscum trilobatum* growing on *Macrozeten capitellata* (parasitic plant on *Mangifera indica*). *Viscum trilobatum* is an...
evergreen plant grows up to 25 cm long. The present work was aimed to determine cytotoxic, anti-tumor and anti-inflammatory activities of the plants of the *Viscaceae* present in Western Ghats in India along with their toxicity profiles.

**MATERIALS AND METHODS**

**Animals**

Swiss albino mice (male, 4–6 w old, 20-25 g b. wt) were obtained from small Animal breeding station, Kerala Veterinary University, Thrissur. The animals were kept in ventilated cages in air-controlled room and fed with mouse chow-(Krish Scientific Shopee, Bangalore, India) and water *ad libitum*. All animal experiments were performed as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (No.149/PO/ Rc/S/1999/CPCSEA), Ministry of Environment and Forest, Government of India, and implemented through the Institutional Animal Ethical Committee of Amala Cancer Research Centre.

**Chemicals**

Minimum Eagle's Medium (MEM) was purchased from Hi-Media, Mumbai, India. Fetal calf serum was purchased from Biological Industries, Israel. Carrageenan, dextran and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals, St. Louis, USA. All other chemicals used were of analytical reagent grade.

**ELISA kits**

Highly specific ELISA kits for Interleukin-1β (IL-1β), Interleukin-6 (IL-6), C-reactive protein (CRP), and tumor necrosis factor-α (TNF-α) were purchased from Pierce Biotechnology (Rockford-Illinois, USA).

**Cell lines**

Transformed cells, L929 (murine lung fibroblast cell line), MCF-7 (human breast cancer cell line), and MDA-MB-231 (human breast cancer cell line) were obtained from National Cell Science Centre, Pune, India and maintained in MEM supplemented with 10% fetal calf serum (FCS) and antibiotics. Ehrlich ascites carcinoma (EAC) and DLA cells were originally procured from Adyar Cancer Institute, Chennai, India and were maintained in the peritoneal cavity of Swiss albino mice. Sheep red blood cells (SRBC) were collected from a local slaughter house and preserved in Alsever’s solution.

**Collection of plants and their identification**

Hemi-parasitic sub-herbs in the family *Viscaceae* were collected as per Good Collection practice from the Chammundi Hills, Bandipur forest area Mysore as well as from Belagavi, India. Identification of the plants was done by Dr. Shivamurthy, G. R, Controller of Examinations, JSS College for Women, Saraswathy Puram, Mysore. The species of plants and their host trees are given in table 1.

### Table 1: Name of plants, their host trees

<table>
<thead>
<tr>
<th>Plants</th>
<th>Host tree</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Viscum orientale</em> Wild</td>
<td><em>Pongamia pinnata</em></td>
</tr>
<tr>
<td><em>Viscum nepalense</em> Spiens</td>
<td><em>Zizyphus oenoplea</em></td>
</tr>
<tr>
<td><em>Viscum ramosissimum</em> Well</td>
<td><em>Ficus bengalensis</em></td>
</tr>
<tr>
<td><em>Viscum angulatum</em> Heyne  DC</td>
<td><em>Schrebera swietenioides</em></td>
</tr>
<tr>
<td><em>Viscum capitallatum</em> Sm</td>
<td><em>Terminalia tomentosa</em></td>
</tr>
<tr>
<td><em>Viscum trilobatum</em> Talbot</td>
<td><em>Mangifera indica</em></td>
</tr>
</tbody>
</table>

Photograph of the plants collected are given in fig. 1.

![Photograph of the plants collected](image)
Preparation of aqueous extract

Plants were washed in running water, rinsed with autoclaved double distilled water, air dried and powdered. Aqueous extracts of each plant were prepared by mixing 10 g of plant powder with 100 ml of autoclaved double distilled water and stirred overnight. The supernatant obtained by centrifugation was dried by lyophilisation.

**Determination of in vitro cytotoxicity of aqueous extracts of Viscaceae species**

In vitro cytotoxic activity of the extracts of Viscaceae species was determined by the trypan blue dye exclusion method [25]. Tumor cells DLA and EAC (1x10⁶/cells/0.1 ml) were mixed with different concentrations (10-500 µg/ml) of the plant extracts and incubated for 3 h at 37° C. After incubation, 0.1 ml of 1% trypan blue solution was added to each tube mixed well and kept for 2-3 min and loaded on a haemocytometer. Viable cells exclude trypan blue dye, while non-viable cells take up the dye and thus appear blue in colour. The number of stained and unstained cells was counted separately and percentage cell death was determined using the formula

\[
\text{% of Cell death} = \frac{\text{Number of dead cells}}{\text{Total number of cells}} \times 100
\]

**Determination of the effect of aqueous extracts of Viscaceae species on viability of different transformed cells by MTT assay**

The effect of the aqueous extracts on the viability of different transformed cells was determined using MTT assay [26-27]. Tumor cells lines L929, MCF-7 and MDA-MB-231 (5x10⁵/cells/well) were seeded in 96 well flat bottom plates and incubated for 24 h at 37° C under 5% CO₂ atmosphere. After incubation, different concentrations of plant extracts (5-50 µg/ml) were added to the wells and the incubation was continued for 48 h with or without plant extracts.

The medium was aspirated 4h before the completion of incubation and 20 µl of MTT (5 mg/ml) solution was added to each well and incubated at 37° C for 2 h. After incubation, plates were centrifuged, supernatant was removed and 100 µl of DMSO was added to each well. The plates were then incubated at room temperature for 15 min and the optical density was measured at 570 nm. Percentage viability was expressed as (Abs (570 nm) of untreated wells – Abs (570 nm) of treated wells) / Abs (570 nm) of untreated wells * 100%

**Toxicity studies of Viscum extracts in animals**

Swiss albino mice were used for this study. Different groups of mice (6 mice/group) were treated intraperitoneally with a single dose of an extract of V. orientale, V. nepalense, V. ramosissimum and V. angulatum, V. capitallatum and V. trillobatum at concentrations 330 mg/kg b. wt, 115 mg/kg b. wt, 58 mg/kg b. wt, 33 mg/kg b. wt, 16 mg/kg b. wt respectively and all the animals were monitored for one month for change in body weight, mortality and any adverse reactions.

In another experiment V. orientale, V. nepalense, V. ramosissimum, V. angulatum, V. capitallatum and V. trillobatum at concentrations 330 mg/kg b. wt, 115 mg/kg b. wt, 58 mg/kg b. wt, 33 mg/kg b. wt, 16 mg/kg b. wt were administered intraperitoneally for 5 consecutive days and the body weight, mortality and any adverse reaction were monitored for one month.

**Determination of the effect of Viscum species on EAC cells induced ascites tumor development**

Swiss albino mice were divided into 6 groups (6 mice/group). Animals in group 1 were kept as untreated control. Animals in group 2-6 were treated with different concentrated extracts for 5 consecutive days. Acute inflammation was induced by injecting 50 µl of freshly prepared 1% suspension of carrageenan in normal saline on the right paw of mice one hour after the last dose of extract administration [30]. The paw thicknesses of all the animals were measured using a vernier caliper before and after carrageenan injection and continued for 6 h with 1 hour intervals followed by 24 h and 48 h.

The percentage inhibition of paw thickness was calculated using the formula:

\[
\text{% Inhibition of paw thickness} = \left(\frac{tCn - tE0}{tCn - tC0}\right) \times 100
\]

Where tC0=paw thickness before induction; tCn=paw thickness at particular time point of control animal; and tE0=paw thickness before induction.

**Determination of anti-inflammatory activity of aqueous extracts of Viscum species against carrageenan induced acute inflammatory model**

Swiss albino mice were divided into 8 groups (6 animals/group). Animals in group 1 were kept as untreated control. Animals in group 2-8 were treated with different concentrations of Viscum species for five consecutive days. Acute inflammation was induced by injecting 50 µl of freshly prepared 1% suspension of dextran in normal saline on the right paw of mice 1 h after the last dose of extract administration [31]. The paw thickness of all the animals was measured using a vernier caliper before and after dextran injection and continued for 6 h. At 1 hour intervals followed by 24 h and 48 h. The percentage inhibition of paw thickness was calculated using the formula as mentioned above.

**Effect of aqueous extracts of Viscum species on pro-inflammatory cytokine levels during carrageenan induced paw edema formation**

Blood was collected at 5 min after carrageen injection with and without treatment and Serum was separated from animals in the above experiment and used for the estimation of various pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) by ELISA method with NO estimation was done by Griess reagent method [32].

**Statistical analysis**

The values are expressed as mean±standard deviation (SD). The mean values were statistically analyzed by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnett’s
multiple comparison test) using Graph Pad Instat 3 Software (Graph Pad Software, Inc. La Jolla, California, USA). Significant levels of control groups were determined by comparing with those of normal group, whereas significant levels of Viscum-treated groups were determined by comparing with those of control groups. P value<0.05 was considered to be statistically significant. The P-value considered as significant are indicated by “*” “**” and “***” for p<0.05, p<0.01 and p<0.001 respectively.

RESULTS

Yield of the extract

The extracted solid yield from V. orientale was 3 gm, V. nepalense 1.5 gm, V. ramosissimum 5.5 gm and V. angulatum 3.2 gm V. capitallatum (3 gm). V. trilobatum (2.6 gm) from 10 gm of the each crude powder.

Cytotoxic activity of Viscum species on DLA and EAC Cells

Short term cytotoxicity studies using different Viscum species by trypan blue-dye exclusion assay showed that V. orientale, V. nepalense, V. ramosissimum and V. angulatum were cytotoxic to Dalton’s lymphoma ascites (DLA) cells. The IC50 value for V. orientale was 20 µg/ml, for V. nepalense was 15 µg/ml, for V. ramosissimum was 17 µg/ml and for V. angulatum was 22 µg/ml. Aqueous extracts of Viscaceae family also showed cytotoxicity towards Ehrlich ascites (EAC) cells. The IC50 value for V. orientale was 35 µg/ml, for V. nepalense was 19 µg/ml, for V. ramosissimum was 90 µg/ml and for V. angulatum was 18 µg/ml (table 2).

Table 2: Cytotoxicity of Viscum extracts on Dalton’s Lymphoma ascites (DLA) and Ehrlich carcinoma cells (EAC)

<table>
<thead>
<tr>
<th>Viscum plant</th>
<th>IC50 (µg/ml) DLA cells</th>
<th>IC50 (µg/ml) EAC cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. orientale</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>V. nepalense</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>V. ramosissimum</td>
<td>17</td>
<td>90</td>
</tr>
<tr>
<td>V. angulatum</td>
<td>&gt;500</td>
<td>&gt;250</td>
</tr>
<tr>
<td>V. capitellatum</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>V. trilobatum</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

Compared to other Viscum species, V. capitellatum and V. trilobatum were less cytotoxic. IC50 of V. capitellatum extract to DLA cells was >500 µg and EAC cells was >250 µg/ml. In the case of V. trilobatum IC50 to DLA and EAC cells were more than 500 µg/ml

Cytotoxic activity of Viscum species to cancer cells in culture

V. orientale showed cytotoxicity towards transformed cells L929 (IC 50= 9 µg/ml), MCF-7 (IC 50= 29.5 µg/ml), MDA-MB-231 (IC 50= 20 µg/ml) and SKBR3 (IC 50= 12 µg/ml). V. nepalense showed cytotoxicity towards transformed cells L929 (IC 50= 13 µg/ml), MCF-7 (IC 50= 22 µg/ml), MDA-MB-231 (IC 50= 15 µg/ml) and SKBR3 (IC 50= 10 µg/ml). V. ramosissimum showed cytotoxicity towards transformed cells L929 (IC 50= 23 µg/ml), MCF-7 (IC 50= 26 µg/ml), MDA-MB-231 (IC 50= 22 µg/ml) and SKBR3 (IC 50= 14 µg/ml). V. angulatum showed cytotoxicity towards transformed cells L929 (IC 50= 20 µg/ml), MCF-7 (IC 50= 42 µg/ml) and SKBR3 (IC 50= 20 µg/ml). Compared to other extracts V. capitellatum and V. trilobatum extracts were less cytotoxic to cancer cells. IC50 to all the cells studied were more than 25 µg/ml.

V. orientale, V. nepalense, V. ramosissimum and V. capitellatum also showed cytotoxicity towards normal cell like Vero cells. However, V. angulatum showed selective cytotoxicity towards transformed cells and no toxicity was observed to normal cells (table 3).

Table 3: Comparative cytotoxicity of different Viscum extracts to transformed cells and normal cells

<table>
<thead>
<tr>
<th>Viscum plants</th>
<th>IC50 (µg/ml) Transformed cells</th>
<th>IC50 (µg/ml) Normal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L929 cells</td>
<td>MCF-7 cells</td>
</tr>
<tr>
<td>V. orientale</td>
<td>9</td>
<td>29.5</td>
</tr>
<tr>
<td>V. nepalense</td>
<td>12.5</td>
<td>17</td>
</tr>
<tr>
<td>V. ramosissimum</td>
<td>22.5</td>
<td>25.5</td>
</tr>
<tr>
<td>V. angulatum</td>
<td>20</td>
<td>42</td>
</tr>
<tr>
<td>V. capitellatum</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>V. trilobatum</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
</tbody>
</table>

Toxicity of Viscum extracts

Administration of aqueous extracts (single dose) of V. orientale, V. nepalense, V. ramosissimum and V. trilobatum at different concentrations such as 3.3, 8 and 16 mg/kg b. wt were given to mice for 5 consecutive days and all the animals were monitored for 1 mo. All the animals treated with V. orientale and V. ramosissimum died within 9 d. V. nepalense treated animals died within 12 d. Similarly all the animals treated with V. trilobatum died within 5 d. V. capitellatum was less toxic. V. angulatum treated groups did not show mortality or any adverse effects even at concentrations of 330 mg/kg b. wt.

In another study, lower concentrations of Viscum extracts like 3.3, 8 and 16 mg/kg b. wt were given to mice for 5 consecutive days and all the animals were monitored for 1 mo. All the animals treated with V. orientale and V. ramosissimum at a concentration of 16 mg/kg b. wt died within 4 d and V. nepalense treated animals died within 15 d. Viscum capitellatum treated animals did not show any toxicity (up to 58 mg/kg) while V. trilobatum showed toxicity at 33 mg/kg body weight. Although there was a reduction in body weight, animals treated continuously with all the Viscum extracts at a concentration of 8 mg/kg b. wt did not show mortality. Continuous administration of V. orientale, V. nepalense and V. ramosissimum at a concentration of 3.3 mg/kg b. wt showed significant increase in body weight and there was no mortality of animals. So further in vivo studies using V. orientale, V. nepalense and V. ramosissimum were done using 3.3 mg/kg b. wt. of the extracts. Continuous administration of V. angulatum to mice at concentrations 3.3, 8 and 16 mg/kg b. wt for 5 consecutive days did not show any mortality, body weight reduction or any other adverse effects. Thus concentrations of 8 and 16 mg/kg, b. wt. were used for in vivo studies with V. angulatum.

Effect of Viscum species on ascites tumor development

All the Viscum species studied except V. orientale showed profound anti-tumor effect against EAC cells induced ascites tumor model. Percentage increase in life span of ascites tumor bearing mice by V. nepalense (3.3 mg/kg b. wt) was 43%, by V. ramosissimum was 35% (3.3 mg/kg b. wt) and by V. angulatum were 32% (8 mg/kg b. wt) and 56% (16 mg/kg b. wt) (table 4).
Table 4: Effect of Viscum extracts on ascites tumour development in mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean survival days</th>
<th>% increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.8±1.4</td>
<td>-</td>
</tr>
<tr>
<td>Viscum orientale (3.3 mg/kg b. wt)</td>
<td>18.3±1.2**</td>
<td>9.12</td>
</tr>
<tr>
<td>Viscum nepalense (3.3 mg/kg b. wt)</td>
<td>22.17±1**</td>
<td>42.8</td>
</tr>
<tr>
<td>Viscum ramossimum (3.3 mg/kg b. wt)</td>
<td>22.67±1.4**</td>
<td>34.9</td>
</tr>
<tr>
<td>Viscum angulatum (8 mg/kg b. wt)</td>
<td>26.16±1.6**</td>
<td>55.72</td>
</tr>
<tr>
<td>Viscum capitallatum</td>
<td>20±2.19</td>
<td>23.76</td>
</tr>
<tr>
<td>Viscum trilobatum</td>
<td>17.83±1.83</td>
<td>10.33</td>
</tr>
</tbody>
</table>

Values are mean±SD. Values were statistically analysed using one-way ANOVA followed by Dunnett multiple comparison test. ns-Not significant (p>0.05), *p<0.05; **p<0.01 significantly. Mean survival in the use of V. capitallatum (16 mg/kg) was 20 d while that of V. trilobatum (8 mg/kg) with 18 d which were not significant.

Effect of Viscum species on solid tumor development

The solid tumor volume of mice treated with V. orientale, V. nepalense, V. ramossimum and V. angulatum were found to be significantly lower than that of untreated controls. The tumor volume of the untreated control on the 30th day was 3.11 cm³ whereas the tumor volume of animals treated with V. orientale was 1.07 cm³, V. nepalense 0.43 cm³, V. ramossimum 1.42 cm³ and V. angulatum was 0.337 cm³ on 30th day. In the case of V. capitallatum and V. trilobatum tumour volume are day 30 was 2.4 cm³ and 2.5 cm³ which was not significant. V. angulatum showed highest anti-tumor activity i.e. 89% reduction in the tumor volume on 30th day (fig. 2).

![Fig. 2: Anti-tumor effect of Viscum extracts on solid tumor development, (Values are mean±SD of six animals)](image)

Effect of Viscum species on carrageenan induced inflammation model

Subplantar injection of carrageenan produced a progressive swelling of paw reaching a maximal paw thickness of 0.411 cm in the control group at 3rd h. Treatment with Viscum extracts showed a significant reduction in paw edema induced by carrageenan. Administration of V. orientale produced 30 % reduction in paw edema at 3rd h. V. nepalense produced 40% reduction in paw edema at 3rd h. V. ramossimum produced 33.5% reduction in paw edema at 3rd h and V. angulatum (16 mg/kg b. wt) produced 56.5% reduction in paw edema at 3rd h (fig. 3) indicating maximum inhibition of paw edema formation was observed in the animals treated with 16 mg/kg b. wt of V. angulatum. V. capitallatum and V. trilobatum extracts did not produce any significant reduction in the inflammation.

![Fig. 3: Anti-inflammatory effect of Viscum extracts on carrageenan induced inflammatory model, (Values are mean±SD of six animals)](image)
Effect of Viscum species on dextran induced inflammation model

When dextran was used as an inflammatory agent, the control animal showed a maximum paw thickness of 0.41 cm at the 3rd h. Treatment with Viscum extracts showed a significant reduction in paw edema induced by dextran. Administration of V. orientale produced 39% reduction in paw edema at 3rd h. V. nepalense produced 39.0% reduction in paw edema at 3rd h. V. ramosissimum produced 33% reduction in paw edema at 3rd h and V. angulatum (16 mg/kg, b.wt) produced maximum reduction (43%) in paw edema at 3rd h (fig.4). Both V. capitallatum and V. trilobatum extracts did not produce any observable anti-inflammatory activity.

Effect of Viscum species on the levels of pro-inflammatory cytokines and nitric oxide during carrageenan induced inflammatory model

The levels of various pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 and C-reactive protein (CRP) were markedly increased by the treatment with carrageenan. These increased levels were significantly lowered by the administration of V. orientale, V. nepalense, V. ramosissimum and V. angulatum (tables 5 and 6). Similarly Levels of NO were markedly increased by the treatment with carrageenan and the increased levels were lowered to almost normal levels by the administration of V. orientale, V. nepalense, V. ramosissimum and V. angulatum (table 7).

Fig. 4: Anti-inflammatory effect of Viscum extracts on dextran induced inflammatory model, (Values are mean±SD of six animals)

<table>
<thead>
<tr>
<th>Viscum species</th>
<th>TNF-α (pg/ml) 24th h</th>
<th>IL-1β (pg/ml) 24th h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.25±1</td>
<td>17.52±1</td>
</tr>
<tr>
<td>V. orientale</td>
<td>32.37±2</td>
<td>17.59±1</td>
</tr>
<tr>
<td>V. nepalense</td>
<td>32.54±2</td>
<td>17.62±1</td>
</tr>
<tr>
<td>V. ramosissimum</td>
<td>32.65±2</td>
<td>17.65±1</td>
</tr>
<tr>
<td>V. angulatum</td>
<td>32.76±2</td>
<td>17.66±1</td>
</tr>
<tr>
<td>V. capitallatum</td>
<td>32.87±2</td>
<td>17.67±1</td>
</tr>
<tr>
<td>V. trilobatum</td>
<td>32.98±2</td>
<td>17.68±1</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD; n=6. Values were statistically analysed using one-way ANOVA followed by Dunnett multiple comparison test. ** Not significant (p>0.05), ***p<0.001 significantly.

Table 5: Effect of Viscum species on TNF-α and IL-1β levels during carrageenan induced paw edema formation

Table 6: Effect of Viscum species on IL-6 and CRP levels during carrageenan induced paw edema formation

IL-6 and CRP levels were expressed in pg/ml. Values are expressed as mean±SD; n=6. Values were statistically analysed using one-way ANOVA followed by Dunnett multiple comparison test. **Not significant (p>0.05), ***p<0.001 significantly.

Effect of Viscum species on nitric oxide levels during carrageenan induced paw edema formation

Nitric oxide levels were expressed in pg/ml. Values are expressed as mean±SD; n=6. Values were statistically analysed using one-way ANOVA followed by Dunnett multiple comparison test. **Not significant (p>0.05), ***p<0.001 significantly.
However as in the case of inflammation both V. capitallatum and V. trilobatum extracts did not produce any significant reduction in the pro-inflammatory cytokine levels.

**DISCUSSION**

Short term cytotoxic activity of all plants of Viscaceae were studied using DLA and Ehrlich ascites carcinoma cells by trypan blue dye exclusion method. Results showed profound cytotoxicity towards both DLA and EAC cells. We also checked the cytotoxic effect of V. orientale, V. nepalense, V. ramossissimum and V. angulatum, V. capitallatum and V. trilobatum against breast cancer cell lines such as MCF-7, MDA-MB-231 and SkBR3 cell lines. MCF-7 is a human breast adenocarcinoma cell line and is useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These included the ability of MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line. Triple-negative breast cancers, to date is the highest risk breast neoplasia, so MDA-MB-231, a triple-negative (ER-ve, PR-ve, no HER2 over expression) human breast adenocarcinoma cell line was used. SkBR3 is a human breast adenocarcinoma cell line that over-expresses the HER2 gene product and has been widely used in studies seeking to overcome Herceptin resistance to HER2-overexpressing breast cancer. Four Viscum species plant extracts showed cytotoxicity towards all the four types of human breast cancer cell lines. However V. capitallatum and V. trilobatum were less toxic. Cytotoxicity studies on normal cell like Vero cells showed that V. orientale, V. nepalense and V. ramossissimum, V. capitallatum were slightly cytotoxic to normal cells. V. angulatum and V. trilobatum showed selective cytotoxicity towards transformed cells and was non toxic to normal cells.

The in vivo tumouricidal activity of V. orientale, V. nepalense, V. ramossissimum, V. angulatum, V. capitallatum and V. trilobatum were evaluated using the Ehrlich Ascites Carcinoma (EAC) induced ascites tumor model and the Dalton’s Lymphoma Ascites (DLA) cell line induced solid tumor model. EAC is referred to as an undifferentiated carcinoma and has a rapid growth rate. The present study revealed that all six plants possessed considerable anti-tumor activity against EAC cell induced ascites tumor. However, Viscum angulatum showed the highest anti-tumor activity. DLA is a transplantable and poorly differentiated malignant tumor cell. Four Viscaceae plant extracts were also found to reduce the solid tumor induced by DLA cells with Viscum angulatum showing the highest activity. V. capitallatum and V. trilobatum were non-active.

Inflammation acts at all stages of tumorigenesis. It contributes to tumor initiation through mutations, genomic instability, and epigenetic modifications. Inflammation activates tissue repair responses, induces proliferation of premalignant cells, and enhances their survival. Inflammation also stimulates angiogenesis, causes localized immunosuppression, and promotes the formation of a hospitable microenvironment in which premalignant cells can survive, expand, and accumulate additional mutations and epigenetic changes [33]. Carrageenan consists of linear sulfated polysaccharides that are extracted from red edible seaweeds. Carrageenan-induced paw edema in mice is a widely used test to determine anti-inflammatory activity. Carrageenan stimulates the release of TNF-α, which, in turn, induces IL-1β and IL-6, thus stimulating the production of COX-2 products. The present study revealed that treatment with different Viscum extracts showed a significant reduction in paw edema formation and elevated pro-inflammatory cytokine levels induced by carrageenan. Dextran is another inflammatory agent. All Viscum extracts except V. capitallatum and V. trilobatum showed a significant anti-inflammatory effect against the dextran induced inflammatory model.

Out of the six plants studied, Viscum angulatum was found to be most promising as it showed significant anti-tumor and anti-inflammatory activity combined with its non-toxicity. Presently we do not know the active ingredients responsible for the activity of V. angulatum. Lin et al. reported the presence of several flavanoid and phenolic glycosides in V. angulatum which may be contributing to the anti-tumour activity of this species [34]. Further studies on the molecular mechanism behind the anticancer effects of these plants needs to be explored.

**CONCLUSION**

Data indicated that out of six species studied four of the plants were highly toxic to animals. However at non-toxic doses they could reduce animal tumours and inhibit the inflammation induced the carrageen and dextran. V. angulatum was nontoxic and it showed significant antitumour and anti-inflammatory activity.

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**AUTHORS CONTRIBUTION**

Dr. Shivamurthy G. R. helped in collection of the plants used in the study. Dr. Girija Kuttan and Dr. Ramadasan Kuttan are the main investigators of this project.

**CONFLICT OF INTERESTS**

The authors report no declarations of interest.

**REFERENCES**

17. Engdal S, Nilsen OG. Inhibition of P-glycoprotein in Caco-2 cells: effects of herbal remedies frequently used by cancer patients. Xenobiotica 2008;38:559-73.


