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Original Article

POTENTIAL ANTI-TUMOR AND ANTI-INFLAMMATORY ACTIVITY OF SIX MISTLETOE PLANTS IN THE FAMILY *VISCACEAE* PRESENT IN WESTERN GHATS, INDIA

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ABSTRACT

Objective: To find out the cytotoxicity, anti-tumor and anti-inflammatory activities of six species of plants belongs to *Viscaeceae* 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: *Viscaeceae* 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 *Viscaeceae* plants 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. capitallatum* 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

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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 mistletoes 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], chemo 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 exert 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 capitallatum* 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 berry. 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 flowers. 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 epiphyllous stamens with sessile anthers. The ovary is obovoid 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 trillobatum* are hyper coparasities. Former growing on *Dendropnthoe falcota* (parasitic plant on *Terminalia tomento*) and *Viscum trillobatum* growing on *Macrosoten capitellata* (parasitic plant on *Mangifera indica*). *Viscum trillobatum* 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 Scientfic 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 Adayar 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 Belgam, India. Identification of the plants was done by Dr. Shivamurthy, G. R, Controller of Examinations, JSS College for Women, Saraswathypuram, Mysore. The species of plants and their host trees are given in table 1.

Table 1: Name of plants, their host trees

Plants	Host tree
Viscum orientale Wild	*Pongamia pinnata*
Viscum nepalense Spiens	*Zizyphus oenoplea*
Viscum ramosissimum Well	*Ficus bengalensis*
Viscum angulatum Heyneex DC	*Schrebera swietenioides*
Viscum capitallatum Sm	*Terminalia tomentosa*
Viscum trilobatum Talbot	*Mangifera indica*

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

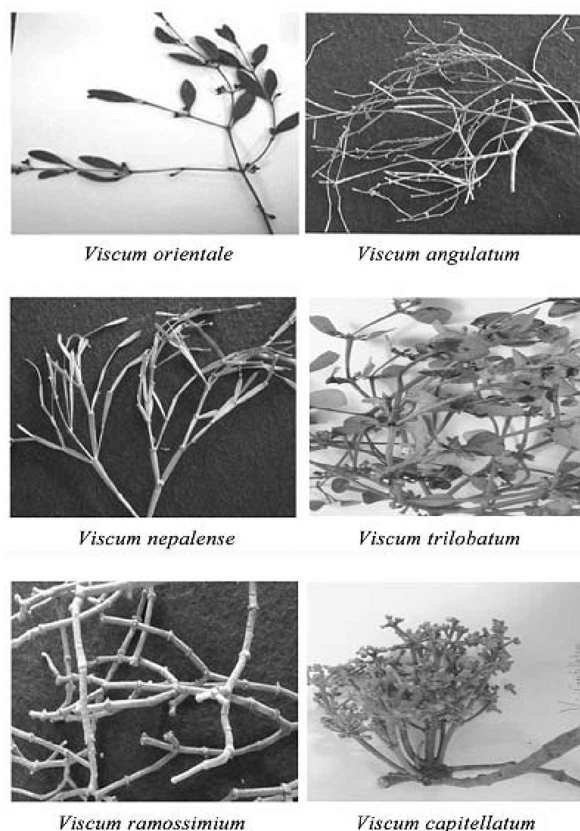

Fig. 1: Photos of six hemi-parasitic sub-herbs in the family *Viscaceae* collected from Western Ghats

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 (1x10^6cells/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

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

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 cell lines L929, MCF-7 and MDA-MB-231 (5×10^3cells/well) were seeded in 96 well flat bottom plates and incubated for 24 h at 37 °C under 5% CO_2 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–

$$\%\ Cell\ viability = 100 - \left(\frac{A570nm\ of\ untreated\ cells - A570nm\ of\ treated\ wells}{A570nm\ of\ untreated\ cells} \times 100\right)$$

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* extracts, *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 Vtrillobatum* extracts at doses of 16 mg/kg b. wt, 8 mg/kg b. wt and 3.3 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 to eight groups (n=10/group). All of the animals were injected intraperitoneally with 1 x 10^6 EAC cells to induce ascites tumor. Animals in group I were inoculated with EAC cells alone and kept as untreated control. Animals in group 2-6 were treated with different *Viscum* extracts 3.3 mg/kg. in the case of *V. orientale, V. nepalense, V. ramosissimum, V. Capitollatum and V. trillobatum* and 8 and 16 mg for *V. angulatum* for 10 consecutive days. The death pattern of animals due to the tumor burden was noted and the percentage increase in life span was calculated using the formula

$$\%\ Increase\ in\ life\ span = \frac{T - C}{C} \times 100$$

Where 'T' represent the number of days extract treated animals survived and 'C' represent the number of days tumor control animals survived **[**28**]**.

Determination of the effect of *Viscum* species on DLA cells induced solid tumor development

Swiss albino mice were divided into eight groups (n=10/group). Solid tumor was induced by injecting DLA cells (1×10^6cells/animal) subcutaneously into the right hind limbs of animals in all groups. Animals in group I were kept as tumor control. Animals in groups 2-8 were treated with different *Viscum* extracts for 10 consecutive days (concentration as given above). The radii of developing tumors were measured from two directions using vernier calipers at three day intervals for one month and tumor volume was calculated using the formula

$$Tumor\ volume\ (V) = \frac{4}{3}\pi r_1^2 \times r_2$$

Where 'r_1' and 'r_2' represent the major and minor diameter, respectively [29].

Determination of anti-inflammatory activity of Viscum species against carrageenan induced inflammatory model

Swiss albino mice were divided in to 6 groups. Animals in group 1 were kept as untreated control. Animals in group 2-6 were treated with different concentrations of *Viscum* extracts for five consecutive days. Acute inflammation was induced by injecting 50 µl of freshly prepared 1% suspension of carrageenan in normal saline on sub plantar region of 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 and 48 h.

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

$$\%\ Inhibition\ of\ paw\ thickness = \frac{(tCn - tCo) - (tTn - tTo) \times 100}{tCn - tCo}$$

Where tCn=paw thickness at particular time point of control animal; tC0=paw thickness before induction; tTn=paw thickness at particular time point of treated animal; and tT0=paw thickness before induction.

Determination of anti-inflammatory activity of aqueous extracts of *Viscum* species against dextran 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 2-6 were treated with *Viscum* extracts (i. p) for five consecutive days. Acute inflammation was induced by injecting 50 µl of freshly prepared 1% suspension of dextran in normal saline on the sub plantar region of 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 h 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 3 h after carragenine 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 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. trillobatum* (2.6 gm) from 10 gm of the each crude powder.

Cytotoxic acivity 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 20µg/ml, for *V. nepalense* was 15µg/ml, for *V. ramosissimum* was 17µg/ml and for *V. angulatum* was 22µg/ml. Aqueous extracts of *Viscaceae* family also showed cytotoxicity towards Ehrlich ascites (EAC) cells. The IC50 value for *V. orientale* was 35µg/ml, for *V. nepalense* was 19µg/ml, for *V. ramosissimum* was 90µg/ml and for *V. angulatum* was 18µg/ml (table 2).

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

Viscum plant	IC_{50} (µg/ml)	
	DLA cells	EAC cells
Viscum orientale	20	35
Viscum nepalense	15	19
Viscum ramosisimium	17	90
Viscum angulatum	22	18
Viscum capitellatum	>500	>250
Viscum trilobatum	>500	>500

Compared to other Viscum species, *V. capitallatum* and *V. trilobatum* were less ctotoxic. IC_{50} of *V. capitallatum* extract to DLA cells was>500 µg and EAC cells was>250 µg/ml. In the case of *V. tribolatum* IC_{50} to DLA and EAC cells were more than 500 µg/ml

Cytotoxic acivity 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= 17 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= 42µg/ml), MDA-MB-231(IC 50 = 28 µg/ml) and SKBR3 (IC 50 = 20 µg/ml). Compared to other extracts *V. capitallatum* and *V. trilobatum* extracts were less cytotoxic to cancer cells. IC_{50} to all the cells studied were more than 25 ug/ml.

V. orientale, V. nepalense, V. ramosissimum and V. capitallatum 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

Viscum plants	IC50 (µg/ml)				
	Transformed cells			Normal cells	
	L929 cells	MCF-7cells	MDA-MB-231cells	SKBR3	Vero cells
Viscum orientale	9	29.5	20	11.5	>50
Viscum nepalense	12.5	17	15	10	>50
Viscum ramosissimium	22.5	25.5	22	14	>50
Viscum angulatum	20	42	27.5	20	Non-toxic
Viscum capitellatum	>25	>25	>25	>25	>25
Viscum trilobatum	>25	>25	>25	>25	Non-toxic

Toxicity of *Viscum* extracts

Administration of aqueous extracts (single dose) of *V. orientale, V. nepalense, V. ramosissimum and V. trilobattum* at different concentrations such as 330, 115, 58, 33 mg/kg b. wt showed observable toxicity including weight loss and mortality. All 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. trilobattum* died within 5 d. *V. capitallatum* 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 capitallatum* 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

Groups	Mean survival days	% increase in life span
Control	16.8±1.4	-
Viscum orientale (3.3 mg/kg b. wt)	18.3±1.2**	9.12
Viscum nepalense (3.3 mg/kg b. wt)	22.17±1**	42.8
Viscum ramossimium (3.3 mg/kg b. wt)	22.67±1.4**	34.9
Viscum angulatum (8 mg/kg b. wt)	22.17±1**	31.9
Viscum angulatum (16 mg/kg b. wt)	26.16±1.6**	55.72
Viscum capitallatum	20±2.19	23.76
Viscum trilobatum	17.83±1.83	10.33

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. capitalatum* (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. ramosissimum and V. angulatum* were found to be significantly lower than that of untreated controls. The tumor volume of the untreated control on the 30thday was 3.11 cm^3 whereas the tumor volume of animals treated with *V. orientale* was 1.07 cm^3, *V. nepalense* 0.43 cm^3, *V. ramosissimum* 1.42 cm^3 and *V. angulatum* was 0.337 cm^3on 30th day. In the case of *V. capillatum* and *V. trillobatum* tumour volume are day 30 was 2.4 cm^3 and 2.5 cm^3which was not significant. *V. angulatum* showed highest anti-tumor activity *i.e.* 89% reduction in the tumor volume on 30th day (fig. 2).

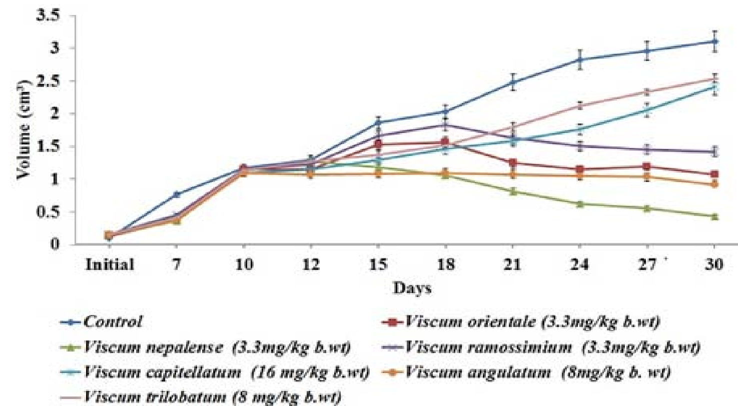

Fig. 2: Anti-tumor effect of *Viscum* extracts on solid tumor development, (Values are mean±SD of six animals)

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 3rdh. 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. ramosissimum* 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.

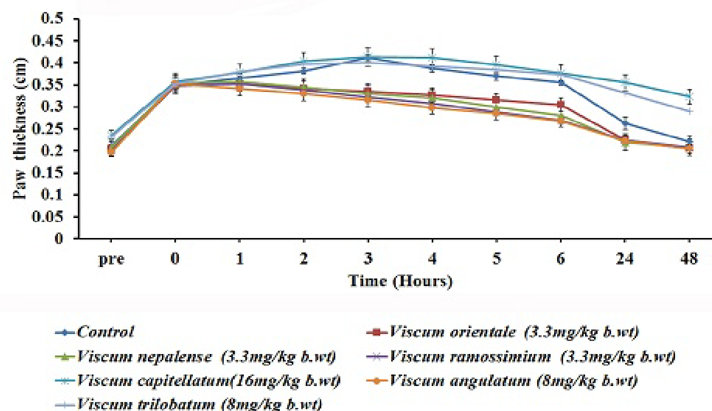

Fig. 3: Anti-inflammatory effect of *Viscum* extracts on carrageenan induced inflammatory model, (Values are mean±SD of six animals)

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 0.415 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.

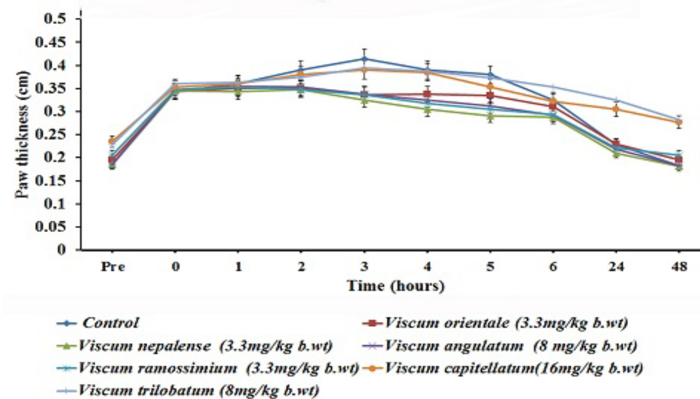

Fig. 4: Anti-inflammatory effect of *Viscum* extracts on dextran induced inflammatory model, (Values are mean±SD of six animals)

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).

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

	TNF-α(pg/ml)			IL-1β(pg/ml)		
	Initial	3rd h	24th h	Initial	3rd h	24th h
Control	22.3±0.8	272.5±5.2	33.3±3.9	18.6±0.1	49.3±1.6	22.3±0.78
Viscum orientale(3.3 mg/kg b. wt)	23.4±0.9	268.2±4.6^{ns}	33.4±3.2^{ns}	17.2±2.3	46.9±3.2^{ns}	20.9±1.7^{ns}
Viscum nepalense(3.3 mg/kg b. wt)	22.2±0.6	125.6±3.9***	24.2±4.1***	17.2±1.6	26.6±2.6***	16.3±1.4***
Viscum ramossimium(3.3 mg/kg b. wt)	21.7±0.6	166.8±5.6***	25.2±3.5***	16.7±0.1	34.7±1.6***	19.2±2.1^{ns}
Viscum angulatum(16 mg/kg b. wt)	22.47±0.86	140.9±4.9***	22.8±4.3***	17.5±0.3	31.7±2.6***	18.1±3.2*
Viscum capitellatum	21.72±1.5	466.9±23.93	35.42±2.75	17.8±1.08	79.24±8.77ns	21.33±2.23
Viscum trilobatum	22.04±1.12	498.05±26.53	38.17±3.36	17.62±0.92	81.62±10.14ns	22.03±1.64

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

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

	IL-6 (pg/ml)			CRP (pg/ml)		
	Initial	3rd h	24th h	Initial	3rd h	24th h
Control	32.25±1	338.96±6.3	38.6±3.2	535.4±4	1171.6±26.4	731.9±25.4
Viscum oriental(3.3 mg/kg b. wt)	30.9±0.3	356.9±6*	42.5±4.8^{ns}	540.2±2	983.2±32.5*	659.5±21.4***
Viscum nepalense(3.3 mg/kg b. wt)	31.66±4	146.6±4***	38.2±4.2^{ns}	529.3±1	730.6±29.5***	542.1±17.8***
Viscum ramossimim(3.3 mg/kg b. wt)	30.2±2	213.9±5***	37.5±5.6^{ns}	530±2.9	838.8±24.7***	583.9±19.5***
Viscum angulatum(16 mg/kg b. wt)	31.32±.5	195.1±6***	36.1±3.6^{ns}	535.4±2	758.4±21.8***	555.6±16.8***
*Viscum Capitellatum(*16 mg/kg)	43.88±5.86	267.84±16.29^{ns}	44.05±3.89	526.19±31.22	1101.51±135.8^{ns}	707.18±33.75
Viscum Trilobatum(8 mg/kg)	42.29±4.02	273.61±15.01^{ns}	45.08±5.39	520.51±34.51	1179±80.27^{ns}	732.53±49.86

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. ^{ns}-Not significant (p>0.05), ***p<0.001 significantly.

Table 7: Effect of *Viscum species* on nitric oxide levels during carrageenan induced paw edema formation

	Initial	3rd h	24th h
Control	23.4±0.26	66.78±4.8	29.42±2.3
Viscum orientale(3.3 mg/kg b. wt)	22.4±0.53	67.7±3.2^{ns}	28.84±2.14^{ns}
Viscum nepalense(3.3 mg/kg b. wt)	21.2±0.63	39.69±4.8***	21.97±2.3**
Viscum ramossimium(3.3 mg/kg b. wt)	22.63±.92	58.47±3.1***	22.77±3.4**
Viscum angulatum(16 mg/kg b. wt)	22±0.53	46.15±2.7***	21.96±3.8**
Viscum capitellatum(16 mg/kg)	22.27±1.35	57.59±6.27^{ns}	22.34±1.01
Viscum trillobatum	21.98±1.57	63.98±6.42^{ns}	23.88±1.86

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. ^{ns}-Not significant (p>0.05),**p<0.01; ***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. ramosissimum and V. angulatum, V. capitallatum and V. trillobatum* 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. capitellatum* and *V. trillobatum* were less toxic. Cytotoxicity studies on normal cell like Vero cells showed that *V. orientale, V. nepalense* and *V. ramosissimum,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* tumoricidal activity of *V. orientale, V. nepalense, V. ramosissimum, 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 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. trillobatum* 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. trillobatum* 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 carragenine 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.

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