Clinical Phytoscience

ORIGINAL CONTRIBUTION

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Analysis of anticancer potential of *Kingiodendron pinnatum* (DC.) Harms

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Abstract

Background The plant *Kingiodendron pinnatum* (DC.) Harms, belonging to the family Fabaceae is endemic to the Western Ghats of India and is commonly used for various ailments, especially by the tribes. *K. pinnatum* is occasionally used as a substitute for *Saraca asoca* in *Asokarishta*, a well-known uterine tonic in Ayurveda. Recent studies revealed a pharmacological similarity between the plants. *S. asoca* is reported to have anti-cancer properties, but there are no reports on *K. pinnatum* except for antioxidant and antimicrobial activities. Therefore, the study is aimed to investigate the anticancer potential of the plant.

Methods Cytotoxicity of methanolic bark extract of the plant was analysed on different cancer cell lines by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Dalton's lymphoma ascites (DLA) cell-induced solid and Ehrlich ascites carcinoma (EAC) cell-induced ascites tumour models in mice were used to study the antitumor potential. Phytochemical screening of the extract was also performed.

Results The extract was found cytotoxic to DLA, EAC, HCT15, MDA-MB-231, T47D and PC3 with inhibitory concentration (IC_{50}) values of 50.09, 74.74, 67.02, 119.22, 149.04 and 194.5 µg/mL, respectively. In the solid tumour model, a significant ($P < 0.001$) reduction in tumour weight of 0.7 ± 0.15 g was observed in 500 mg/kg b.wt. extract treated group compared to the control group (3.6 ± 0.24 g) by oral administration for 30 days. In the ascites tumour model, a high survival rate of 28.2 ± 8.72 days ($P < 0.01$) was found by the extract treatment compared to the control animals. Phytochemicals like alkaloids, flavonoids, phenols, phytosterols, saponins, tannins, steroids and terpenoids were detected in the extract.

Conclusion Results obtained by the cytotoxic and anti-tumour studies revealed the anticancer potential of *K. pinnatum*. The plant exhibits more cytotoxicity towards cancer cell lines of the reproductive system such as the breast and prostate.

Keywords *Kingiodendron pinnatum*, *Saraca asoca*, Asoka, Cytotoxicity, Anti-tumour, Reproductive cancer, Phytoestrogen

Introduction

Cancer is one of the leading causes of death globally and the number of cases is mounting gradually. According to the International Agency for Research on Cancer (IARC), 18.1 million new cancer cases and 9.6 million cancer deaths were accounted for in 2018. The incidence rate of commonly diagnosed cancer types such as lung (2.09 million), breast (2.08 million), colorectal (1.8 million), prostrate (1.3 million) and stomach cancer (1 million) are

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increased [1]. Currently, there are many drugs available on market for different cancers but they are not completely effective and safe. Present treatment modalities like chemotherapy and radiotherapy have serious side effects affecting some vital organs. Hence, the studies are more focused on plant-derived products as they have shown to be effective and have fewer side effects. Many natural products and their analogues including camptothecin, resveratrol, taxol, vinblastine and sulforaphane have been recognised as anti-cancer drugs and various plants with anticancer potential are being identified day by day [2, 3]. Biologically active compounds are often characterized by unique structures, making natural product research an effective method for discovering new compounds with distinct mechanisms of action. Despite the advances that have enabled the use of natural products in the discovery of new therapeutic agents, there are still challenges to be addressed. In most cases, bioactive natural products must be produced at a large scale to meet the manufacturing requirements, which constitutes a major hurdle before they are eventually available for clinical use. For these issues to be resolved, it will be necessary to develop innovative therapeutic concepts and new technologies, thereby advancing the transformation of the field [4].

Kingiodendron pinnatum, the plant belonging to the family Fabaceae is endemic to the Western Ghats of India and is mainly distributed in the evergreen hill and deciduous forests of Karnataka, Kerala and Tamil Nadu states. Traditionally, an oleo-gum-resin extracted from the tree is being used by tribes for gonorrhoea, catarrhal conditions of genitourinary and respiratory tracts and curing sores in elephants [5]. The resin obtained by piercing the trunk has been used for joint pains and to get relief for the fissured foot by *Kanikkar*, a predominant tribal community of Kalakad-Mundanthurai of Western Ghats, Tirunelveli, Tamil Nadu, India [6]. Phytochemicals such as phenols, flavonoids, tannins, glycosides and terpenes were reported from the plant [7]. The plant is reported to have antioxidant, antifungal and antibacterial activities [5]. *K. pinnatum* is occasionally used as a substitute for *Saraca asoca* (Asoka), which is the prime raw material in the preparation of *Asokarishta*, a fermented formulation, commonly used to treat gynaecological ailments especially abnormal uterine bleeding (menorrhagia). The population of the tree is less in wild but is generally used as a substitute due to its massive size and the chance of getting a good amount of bark compared to the Asoka tree [8]. A previous study revealed the pharmacological efficacy of *K. pinnatum* as an alternative for *S. asoca* in *Asokarishta* by demonstrating the inhibitory effect of estrogen-induced uterus endometrial thickening in immature female rats, giving scientific validation for its use in polyherbal formulations [8]. *S. asoca* is reported to have anticancer properties [9], but there are no reports on *K. pinnatum*. Therefore, the present study is intended to analyse the cytotoxic effect of the plant on reproductive cancers such as breast and prostate and its antitumour potential using mouse solid and ascites tumour models. The study is expected to provide insights into the anticancer potential of *K. pinnatum.*

Materials and methods

Chemical and reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) was procured from Thermo Fisher Scientific Inc., USA. Streptomycin, penicillin was purchased from Sigma Aldrich, USA. MTT, trypan blue dye, phosphate buffer saline were procured from Sisco Research Laboratory Pvt. Ltd., India and methanol, isopropanol, HCl, Triton X 100 from Merck, India.

Collection of the plant sample

The stem bark of *K. pinnatum* was collected from the Wayanad region of Western Ghats, Kerala, India. The plant was authenticated by Dr. N. Sasidharan, Taxonomist, Kerala Forest Research Institute (KFRI), Thrissur, Kerala (India). The voucher specimen of *K. pinnatum* (No. KFRI 4725) was deposited in the Herbarium of KFRI. The collected stem bark was shade dried, powdered and stored in air-tight containers until use.

Preparation of the extract

About 20 g of the powder was extracted with 250 mL methanol by stirring overnight. The extract was filtered using Whatman no. 1 filter paper and evaporated to dryness. It was weighed to determine the percentage yield of the soluble constituents using the formula.

$$\% \text{ Yield} = (\text{weight of dry extract/weight taken for extraction}) \times 100$$

The residue thus obtained was stored at 4 °C until use.

Phytochemical analysis

The extract obtained was dissolved in methanol and subjected to various analysis to find out the presence of different phytochemicals. The total phenolic and flavonoid contents of *K. pinnatum* extract was determined by Folin–Ciocalteau colorimetric reagent (FCR) [10] and aluminium chloride colorimetric methods [11], respectively. Dragendorff's, Hagers and Mayer's tests were used to detect the presence of alkaloids. Shinoda's, ferric chloride, Froth formation, Salkowski and Liebermann-Burchard tests, lead acetate and Salkowski

tests were used to detect the presence of flavonoids, phenols, saponins, sterols, tannins and terpenoids [12], respectively.

Animals

Female Swiss albino mice (25–30 g) were purchased from the Small Animal Breeding Station (SABS), College of Veterinary, KVASU, Thrissur, Kerala. The animals were kept in the animal house facility of Amala Cancer Research Centre following standard conditions of 24–28 °C, 60–70% humidity, 12 h dark/light cycle and fed with standard rat feed bought from Sai Durga Feeds, Bangalore, India and water ad libitum. All the animal experiments were carried out with the prior permission of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Ministry of Environment and Forest, Government of India.

Cell lines

Breast cancer cell lines (MDA-MB-231, T47D), colorectal cancer cell line (HCT-15), prostate cancer cell line (PC3) and normal African green monkey kidney epithelial cells (Vero) obtained from National Centre for Cell Science (NCCS), Pune (India) were cultured in DMEM medium supplemented with FBS (10% v/v), streptomycin (100 µg/mL) and penicillin (100 U/mL). All cells were maintained at 37 °C, 5% CO_2, 95% air and 100% relative humidity. Daltons Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cell lines were obtained from Amala Cancer Research Centre's animal house facility. The cells were maintained in the intra-peritoneal cavity of mice.

In vitro cytotoxicity by trypan blue dye exclusion method

The short-term cytotoxic activity of the extract was evaluated by determining the percentage viability of murine tumour cells like DLA and EAC using the trypan blue exclusion method [13]. Crude methanolic extract of the plant was used for the study. The cells were grown in the peritoneal cavity of female mice (8 weeks old, 25–30 g) by injecting 1×10^6 cells/mL intra-peritoneally. Cells were aspirated aseptically from the cavity of mice after 15 days of inoculation, washed with PBS and centrifuged at 1000 rpm for 5 min. Pellets were resuspended in PBS and the cell count was adjusted to 1×10^6 cells/mL. The cells were pipetted out and added into each tube having PBS with different concentrations of the drug. It was then incubated for 3 h at 37°C. After incubation, trypan blue dye was added and left for 3 min before observation. It was then observed under a light microscope using a haemocytometer. The experiments were performed in triplicate and the percentage of cytotoxicity was determined by counting the number of dead cells to that of live cells and substituting in the equation:

$$\% \text{ of cytotoxicity } = \frac{\text{No. of dead cells}}{\text{Total no. of cells}} \times 100$$

The graph was plotted and the half-maximal inhibitory concentration (IC_{50}) was calculated.

In vitro cytotoxic analysis by MTT assay

MDA-MB-231, T47D, HCT-15, PC3 and Vero cell lines were used to study the cytotoxic activity of crude methanolic extract of *K. pinnatum* using MTT assay [14]. Approximately, 1×10^5 cells were seeded in a 12-well plate containing medium and incubated at 37 °C for 24 h. The cells were incubated with different concentrations of the extract at 37 °C for 24 h. After incubation, 100 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h. The dark blue formazan crystals were dissolved in a 1 mL solubilization solution containing isopropanol, HCl and Triton X 100 by continuous aspiration and resuspension. The absorbance of the coloured product was measured at 570 nm. Three experiments with comparable outcomes were carried out in duplicate and the cytotoxicity was determined by comparing the percentage of death of the treated cell population with the untreated control indicated by their respective absorbance assessed with the MTT assay.

Acute toxicity studies

The extract of *K. pinnatum*, at the concentration 2500 mg/kg b.wt. was administrated to 3 male and 3 female Swiss albino mice (25–30 g) orally according to the Organization of Economic Co-operation and Development (OECD) guideline for testing chemicals [15]. The animals were monitored for 14 successive days for any visible changes in behaviour, body weight, water and food intake, hair loss etc. At the end of the experimental period, the animals were sacrificed and the internal organs were examined for any sign of change by conducting a necropsy. The experiment was repeated with another set of animals.

Anti-tumour analysis in mouse models

For the study, Swiss albino mice were grouped into 5 groups comprising 6 animals. Group I: control-untreated; group II: vehicle control (propylene glycol); group III: KPLD—*K. pinnatum* (KP) low dose (250 mg/kg b.wt.); group IV: KPHD—KP high dose (500 mg/kg b.wt.); group V: standard—cyclophosphamide (10 mg/kg b.wt.). DLA and EAC cells were aspirated from the peritoneal cavity

of the tumour-bearing mice and washed with PBS. DLA cell suspension (100 μL) containing approximately 1×10^6 cells was injected intramuscularly into the right hind limb for the development of solid tumour and EAC cells into the peritoneal cavity of the animals for the ascites tumour development. The extracts were administered 24 h after the induction of the tumour and continued for 10 consecutive days. Solid tumour development was determined by measuring the diameter of the tumour growth in two perpendicular planes using a Vernier calliper. The readings were taken at a 3 days interval basis up to the 30th day [16]. The tumour volume was calculated according to the following formula,

$$V = 4/3\pi\ r_1^2\ r_2$$

where, r_1 is the minor radius and r_2 is the major radius.

The percentage inhibition of tumour growth was calculated according to the formula,

$$\%\ \text{inhibition} = [(C - T)/C] \times 100$$

Where C is the tumour volume of control animals on the 30th day and T is the tumour volume of treated animals on the 30th day. The tumours excised from the animal were weighed. In EAC model, the number of survival days of animals was recorded.

Statistical analysis

Data were presented as mean ± standard deviation. Data analysis was performed by one-way ANOVA method followed by Dunnett's multiple comparison test and Kaplan–Meier survival curve by Mantel-Cox test in Graphpad Prism 7. The level of significance was considered as $p < 0.05^*$, $p < 0.01^{**}$ and $p < 0.001^{***}$.

Results

Phytochemicals present in the extract

The total yield of the extract from 20 g of powdered bark using 250 mL methanol was 5.6022 g. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenols, phytosterols, saponins, tannins and terpenoids in the methanolic extract (Tab. 1). The total phenol and flavonoid contents were found to be 2.297 mg GAE/g extract and 0.246 mg QE/g extract, respectively.

Table 1 Phytochemicals present in *K. pinnatum*

Phytochemicals	Presence
Alkaloids	+
Flavonoids	+
Phenols	+
Phytosterols	+
Saponins	+
Tannins	+
Terpenoids	+

Cytotoxic effect of the extract on cancer cells

The extract was shown to have cytotoxicity against murine tumour cells like DLA and EAC cells in a dose-dependent manner with IC_{50} values of 50.09 and 74.74 µg/mL, respectively (Fig. 1). In the MTT assay, the extract was found to be cytotoxic on breast cancer cell lines MDA-MB-231 and T47D with IC_{50} of 70.22 and 149.04 µg/mL, respectively. It also showed cytotoxicity towards a colorectal cancer cell line, HCT-15 in a dose-dependent manner with an IC_{50} value of 67.02 µg/mL and prostate cancer cell line, PC3 with an IC_{50} of 198 µg/mL. The extract shows less cytotoxicity in the normal African green monkey kidney epithelial cell line, Vero even at the concentration of 280 µg/mL (Figs. 2 and 3).

Acute toxicity

The animals treated with the extract appeared healthy and no mortality was observed. There was no significant change in the behaviour of animals including, breathing, skin effects, water and food consumption, body weight etc. No changes in colour, texture and relative organ weights of the liver, heart, spleen, kidney, uterus and ovary were observed. Therefore, the extract seems to be safe up to a dose level of 2500 mg/kg. The parameters observed after the administration of the extract are represented in (Table 2).

Anti-tumour potential of the extract

The methanolic extract of *K. pinnatum* was found to inhibit the DLA-induced solid tumour in mice. The tumour weight in the control and vehicle group of animals was found to be 3.6 ± 0.24 and 3.1 ± 0.3 g, respectively on day 30th of tumour inoculation. High dose of *K. pinnatum* treated animals shows a significant reduction in tumour weight (0.7 ± 0.15 g, $P < 0.001$) compared to the control group animals (3.6 ± 0.24 g). The high dose of the extract reduced the tumour volume to 65.69 ± 18.1 cm^3 from 426.25 ± 36.61 cm^3 in the control group animals (Fig. 4). The tumour size and weight of control and treatment group animals are represented in Fig. 5.

K. pinnatum also shows a significant anti-tumour effect in the ascites tumour model. Animals in the control and vehicle control groups show almost the same mean survival rate with values of 20.4 ± 4.33 and 20.4 ± 4.77 days, respectively. The animals administrated with 500 mg/kg. b.wt. dose *K. pinnatum* extract showed a high survival

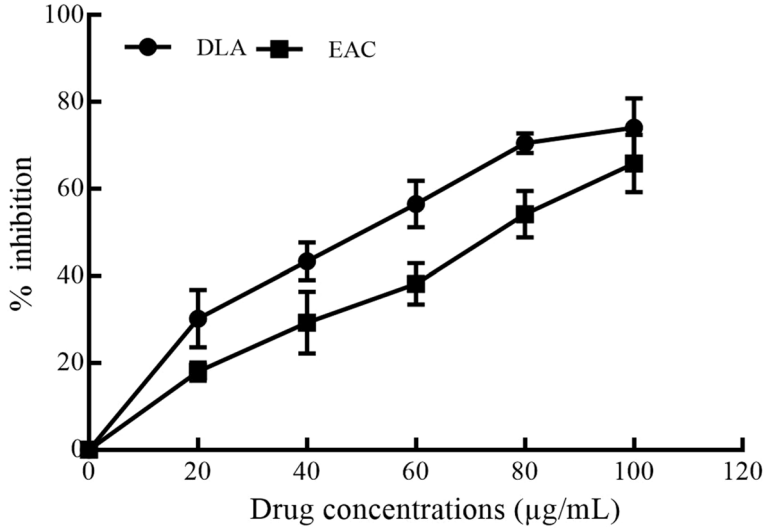

Fig. 1 Short-term cytotoxicity of *K. pinnatum* on murine tumour cells using trypan blue dye exclusion method

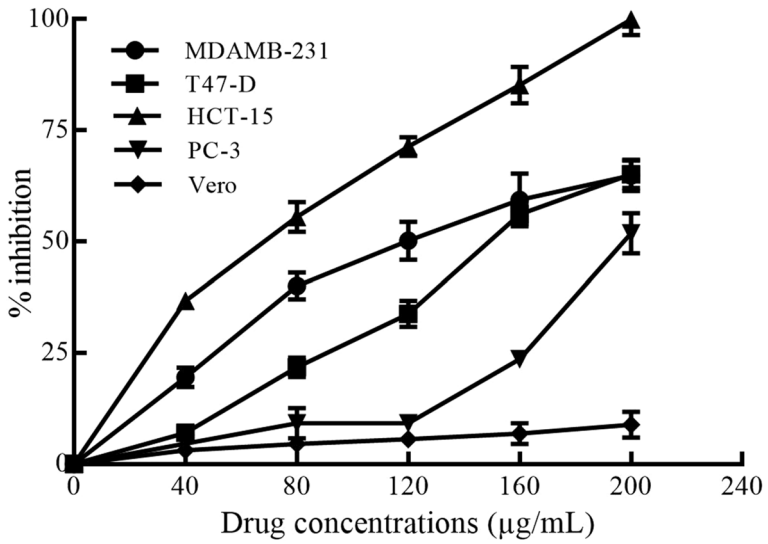

Fig. 2 Cytotoxicity of *K. pinnatum* on different cancer cell lines by MTT assay

rate of 28.2 ± 8.72 days ($P < 0.01$) compared to the control group animals (20.4 ± 4.33 days). Animals treated with the standard drug cyclophosphamide survived up to 30.8 ± 7.52 days, which was close to animals administrated with a high dose of *K. pinnatum* extract (Fig. 6).

Discussion

It has been reported that *Kingiodendron pinnatum* has a pharmacological similarity to that of *S. asoca* and can be used as a substitute in Ayurvedic preparations [8]. There are reports of cytotoxic and anticancer activities of *S. asoca* against different cancer cell lines [9, 17] and mouse tumour models [18], but no reports on *K. pinnatum* even though the plant is reported to have antioxidant, antifungal and antibacterial activities [5]. In our study, the plant *K. pinnatum* shows a cytotoxic and anti-proliferative effect on different types of cancer cell lines such as breast, colorectal, prostate and murine tumour cells and inhibits the development of mouse solid and ascites tumours suggesting its anticancer potential. *K. pinnatum* exhibited considerable in vitro cytotoxicity towards reproductive cancers such as breast and prostate cancer cell lines. In DLA induced solid tumour model, the extract treated group showed a decrease of the tumour growth. The mean tumour weight of the control was comparable to other groups except for the standard group. In the EAC

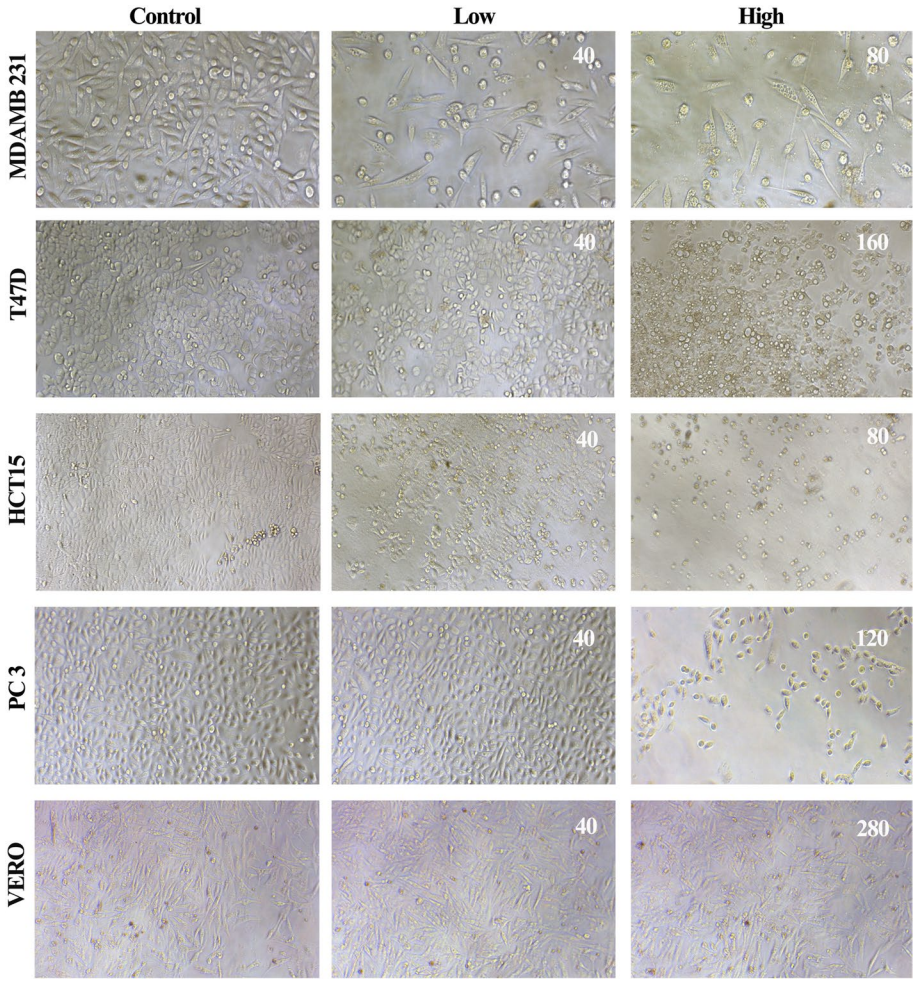

Fig. 3 Morphology of different cancer cell lines treated with *K. pinnatum* at different concentrations

Table 2 General appearance and behavioral observations of control and treated animals

Observations	Normal group	Treated groups
Food intake	Normal	Normal
Water intake	Normal	Normal
Body weight	Normal	No change
Fatigue	Not present	Not present
Changes in skin	No change	No change
Diarrhoea	Not present	Not present
Sedation	No effect	No effect
General physique	Normal	Normal
Death	Alive	Alive

ascites model, the survival curves were statistically significant for the high extract treated group and standard, compared to other groups.

S. asoca is reported to have phytoestrogens such as quercetin, kaempferol, β-sitosterol and luteolin [18] with anticancer potential. The mechanisms of action of these are suggested to be the modulation of estrogen receptors. Many studies have stated a connection between phytoestrogens and their possible role in cancer therapy or prevention [17, 19]. Phytoestrogens are reported to induce apoptosis in breast cancer cells and inhibit prostate and ovarian cancer growth [20–22]. They can interact and modulate different growth factors and activate/inhibit cytokine signalling pathways. genistein, a phytoestrogen, induced apoptosis in MCF-7 breast cancer cells through the down-regulation of the Akt signalling pathway [23]. Also, it inhibits triple-negative breast cancer cell, MDA-MB-231 growth by inhibiting NF-kB activity *via* the Notch-1 pathway [24]. In prostate cancer cells, it inhibits the activation of NF kB *via* the Akt signalling pathway [25].

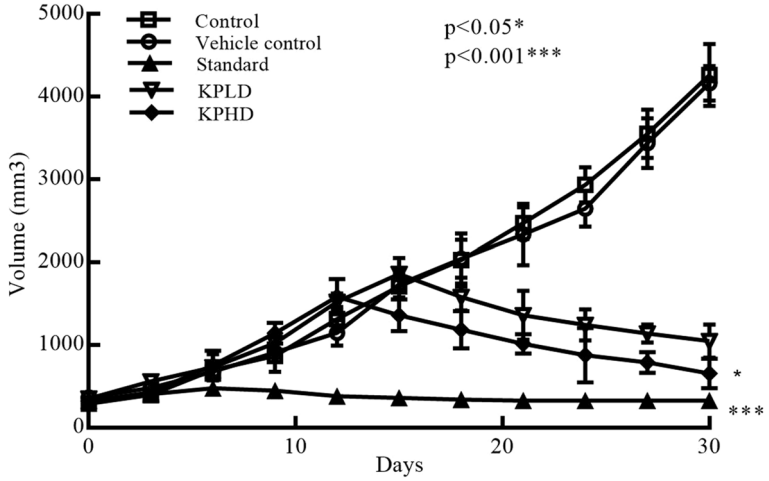

Fig. 4 Effect of *K. pinnatum* extract on DLA induced mouse solid tumor. Results are presented as mean ± SD, n = 5. One-way ANOVA was used to determine the statistical comparison followed by Dunnett's multiple comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, statistically significant as compared to the control group

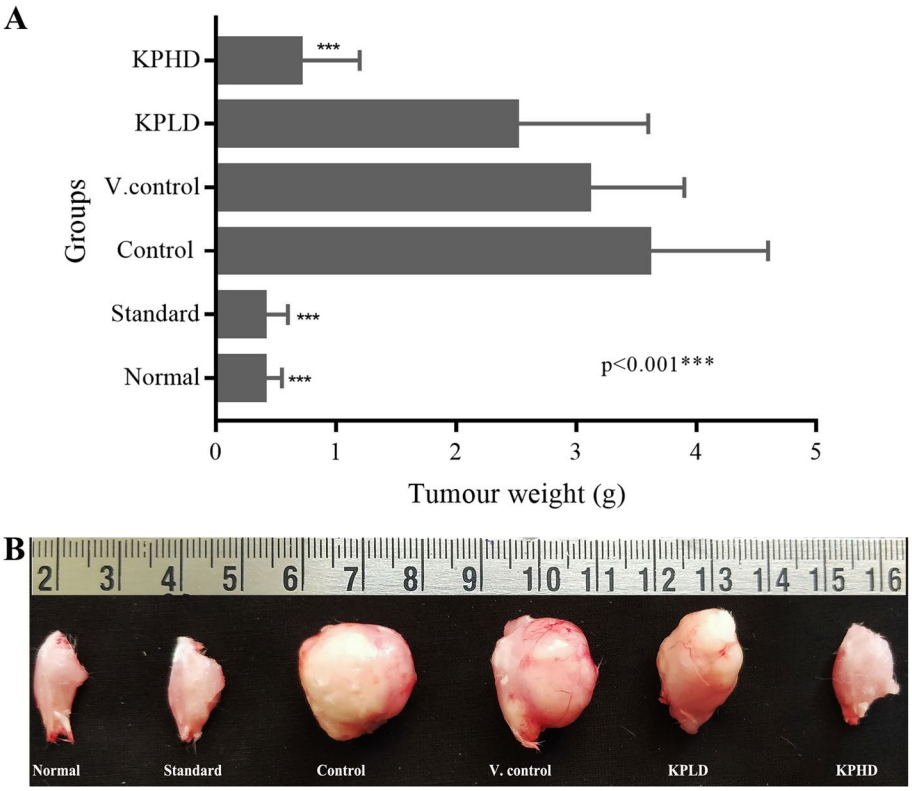

Fig. 5 **A** Comparison of tumour weight (in grams) (**B**) tumour size. Results are presented as mean ± SD, *n* = 5. One-way ANOVA was used to determine the statistical comparison followed by Dunnett's multiple comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, statistically significant as compared to the control group

The phytochemical screening of *K. pinnatum* has shown the presence of important phytoestrogens and revealed a similar phytochemical profile as that of *S. asoca*. In a pharmacological study on estradiol-induced keratinization, *K. pinnatum* was found to reduce cornification in immature rat uterus. The elevated level of

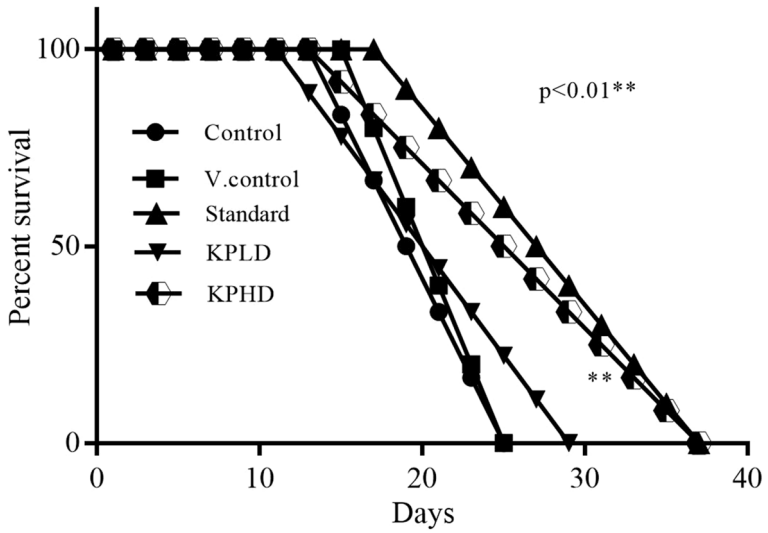

Fig. 6 Effect of *K. pinnatum* extract on the survival of ascites bearing animals. Results are presented as mean ± SD, $n = 5$. Kaplan–Meier survival curve was used to analyse the statistical comparison followed by Mantel-Cox test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, statistically significant as compared to the control group

estrogen in estradiol-administered animals has reduced and inhibited acute as well as chronic inflammations in mice [8]. This study gives a scientific validation for the use of *K. pinnatum* in polyherbal uterine tonic, *Asokarishta* as a substitute for *S. asoca*. In the present study, *K. pinnatum* exhibits more inhibitory effects toward reproductive cancers such as breast and prostate cancers. Thus, the activity of *K. pinnatum* may be due to the presence of similar phytoestrogens seen in *S. asoca* as they are pharmacologically related.

Conclusion

The results obtained by the cytotoxic and antitumour studies indicate the anticancer potential of the plant *K. pinnatum,* especially on reproductive cancers. The study also suggests the presence of phytoestrogens which are reported to have a variety of biological activities. The phytoestrogens can bind to reproductive cancer cells with estrogen receptors and can be a possible target of some phytoestrogens. This modulation of estrogen receptors may be the reason for the cytotoxic properties of some phytoestrogens against breast cancer cells. Thus, to comprehend the subsequent mechanism, it is necessary to analyse how phytoestrogens of *K. pinnatum* interact with estrogen receptors.

Abbreviations

BWT	Body weight
DLA	Dalton's lymphoma ascites
EAC	Ehlich ascites carcinoma
IC_{50}	Half maximal inhibitory concentration
KP	*Kingiodendron pinnatum*
KPLD	*Kingiodendron pinnatum* Low dose
KPHD	*Kingiodendron pinnatum* High dose
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Authors' contributions
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Availability of data and materials
Data supporting the findings of this study are available on request from the corresponding author

Declarations

Ethics of approval and consent to participate
All the animal experiments were carried out with the prior permission of the Institutional Animal Ethics Committee (IAEC) (Approval No: ACRC/IAEC/17(I)/P-05 dt: 22–07-2017) and were conducted strictly according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by Ministry of Environment and Forest, Government India.

Consent for publication
All authors agreed to the publication of the research.

Competing interests
The authors declare that they have no competing interest.

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