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Promising anticancer activities of *Justicia simplex D. Don*. in cellular and animal models

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ABSTRACT

Ethnopharmacological relevance: *Justicia simplex* D. Don. belonging to the family of Acanthaceae has been traditionally used for treatment of rheumatism, inflammation and bronchitis. The plant is traditionally considered as an anticancer medicine and is used by healers of Karnataka to treat various types of cancers.
Aim of the study: The present study aims at the elucidation of anticancer activity of various extracts of *J. simplex*, isolation of its active constituents and assessment of the role in growth inhibition and angiogenesis both *in vitro* and *in vivo*.
Materials and methods: Extracts of *J. simplex* was evaluated for the *in vitro* cytotoxic effect by Brine Shrimp Lethality assay, Trypan Blue dye exclusion assay and antiproliferative assay. *In vivo* cytotoxicity of the extracts were determined by liquid tumor model in *Swiss albino* mice. Tumor prognosis, metastasis and angiogenesis were assessed by VEGF expression of the solid tumor. Phytochemical analysis afforded the isolation of a compound, the chemical structure of which was established using IR, NMR and TOF-MS spectral method. The compound was also evaluated for the growth inhibitory and angiogenic effects.
Results and conclusion: The petroleum ether extract revealed potent anticancer activity in *in vitro* and *in vivo* studies. The anti-angiogenic effect is due to the down regulation of VEGF expression. The growth inhibitory assay revealed that the isolated compound namely triacontanoic ester of 5″-hydroxyjustisolin is responsible for the anticancer activity.

1. Introduction

Cancer is one of the most dreaded diseases of this century. Over the years, the most important cancer treatment modalities are chemotherapy, surgery and radiotherapy. Advances in these techniques have led to an improved survival time for many cancer patients. The toxicity of currently available anticancer drugs, the inefficiency of chemotherapeutic treatments, especially for advanced stages of the disease, the resistance of the malignant cells to the drug and the inability of antineoplastic agents to target tumor cells selectively have limited the effective chemotherapeutic protocols (Liang et al., 2010).

Metastasis is the major cause of death of patients. A five-year survival study reveals that the survival rate is 100% for breast cancer when it localized, whereas it drops to 25% when the cancer has colonized in distant sites (Douglas and Weinberg, 2000). Angiogenesis is also critical in tumor metastasis. Tumor cells can hardly extravasate into the circulation system significantly until angiogenesis has been induced. In addition, metastatic cell seeding at new organs must attract new vessels before they can grow to a clinically significant size.

Review of natural products over the 30 years from 1981 to 2010 revealed that approximately 40% of the therapeutic agents approved by FDA are natural products (NPs) and their derivatives or synthetic mimetic related to NPs (Newman and Cragg, 2012). In this scenario, intervention to decelerate, arrest or reverse the process of carcinogenesis using natural products has emerged as a promising and pragmatic medical approach to reduce cancer risk. Of the 121 prescription drugs in use today for cancer treatment, 90 are derived from plants. Almost 74% of these, including taxol, were discovered by investigating the folklore claims (Pan et al., 2012).

Chemotherapy is an important option in modern cancer treatment and plant derived chemotherapeutic agents have contributed greatly to the progress of oncology chemotherapy. Thus it is evident that small molecules of plant origin continue to be valuable as a source of potential lead compounds in anticancer drug discovery. *Justicia simplex* D. Don belonging to the family of Acanthaceae has been used

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for the treatment of rheumatism, inflammation and bronchitis. The plant is traditionally considered as an anticancer medicine and is used by healers of Karnataka to treat various types of cancers (Corrêa and Alcântara, 2012)

2. Materials and methods

2.1. Materials used

2.1.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), DMSO and Tris-EDTA were purchased from Sigma-Aldrich, USA; fetal bovine serum (FBS) and TRIzol® reagent (15596018) were obtained from Invitrogen Bio Services India Pvt. Ltd., Bangalore, India. Tissue culture flasks and other accessories were procured (Tarsons Products Pvt. Ltd. Bangalore, India). Verso One step RT-PCR kit was brought from Thermo-scientific, USA, while Brine shrimp eggs (Cochin Marine and Fisheries Research Institute) were got from Cochin, India.

2.1.2. Cell lines and maintenance

MDA MB-231 (human breast adenocarcinoma cell line, ER, tumorigenic and invasive), MCF 7 (Human breast adenocarcinoma cell line, ER+, tumorigenic and non-invasive), C6 (Rat glioma brain tumor cell), T47D (Human ductal breast epithelial tumor cell), HeLa (Human cervical carcinoma, tumorigenic and invasive), HaCat (Human Keratinocyte Immortalised) and L929 (Immortalised Fibroblast) were initially procured from NCCS, Pune. The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37 °C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). Ehrlich Ascites Carcinoma (EAC) and Daltons Lymphoma Ascites (DLA) cell lines were obtained through the courtesy of Amala Cancer Research Centre, Trissur, Kerala.

2.1.3. Plant material

Aerial parts of *Justicia simplex* D. Don (Hooker, 1885; Gamble, 1989) was collected in the month of October from Kottayam (Dist.) in Kerala. A voucher specimen (voucher number 1505 dated 07.01.2011) of the same was deposited in the herbarium of Dept. of Pharmaceutical Sciences, Mahatma Gandhi University, Kottayam, India after authentication by Dr. Jomy Augustine, Post Graduate and Research Department of Botany, St. Thomas College, Kerala, India.

2.1.4. Extraction of plant material

Shade dried powder of the aerial parts of *J. simplex* was defatted with petroleum ether (60–80 °C) and then subjected to exhaustive extraction with ethanol in a soxhlet. The extracts were dried over sodium sulphate (anhydrous) and distilled under reduced pressure until the solvent was completely removed to get a semi-solid consistency. It was then kept in a desiccator for two days (Harborne, 1998).

The above two extracts were subjected to phytochemical studies and cytotoxicity assays.

2.1.5. Qualitative determination of the phytochemical constituents

The presence of phytoconstituents like alkaloids (Mayer's, Dragendorff's, Hager's and Wagner's), phenolics ($FeCl_3$ test, Lead acetate test and Pot. dichromate test), saponins (froth test), sterols (Salkowski's test and Liebermann Burchard's test) and carbohydrates in the extracts were tested (Trease and Evans, 1989).

2.2. Establishment of cytotoxicity by in vitro toxicological assay

2.2.1. Brine shrimp lethality bioassay (BSLB assay)

A stock solution of 1 mg/mL concentration was prepared by dissolving the test samples in 2% DMSO. Brine shrimp eggs (*Artemia salina* Leach) were permitted to hatch and mature as nauplii (Larvae) in artificial sea water for 48 h at 25–28 °C, under a continuous light regimen. The experiment was carried out by following the method described by Meyer (Meyer et al., 1982).

The percentage of mortality (% M)

$$= \frac{(Average\ of\ survival\ in\ control - Average\ of\ survival\ in\ treatment)}{Average\ survival\ of\ control}$$

2.2.2. Trypan blue dye exclusion method

Cell populations were typically sampled at various time points, mixed with dye and live versus dead cells counted in Neubauer's chamber by light microscopy (Vishnupriya et al., 2011).

$$Percentageviability = \frac{Number\ of\ viable\ cells}{Total\ number\ of\ cells} \times 100$$

2.2.3. Antiproliferative assay

Ten milligrams of the extracts were added to 10 mL of DMEM and dissolved completely by mixing in a cyclomixer (Amkette, India). The solution was filtered through 0.22 µm Millipore syringe filter to ensure sterility. MTT assay which measures mitochondrial dehydrogenase activity as a reflection of cell viability was performed as described by Mossman (1983) using MDA MB-231, MCF 7, C6, T47D, HeLa and HaCat cell lines (Mossman.,1983).

2.3. In vivo studies

2.3.1. Experimental animals

Healthy female *Swiss albino mice* (90–95 days old), weighing 22–28 g, maintained in the animal house of Department of Pharmaceutical Sciences, M G University, Kottayam were used in the study. Animals were maintained as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experiments were performed in accordance with the ethical norms approved by the Institutional Animal Ethics Committee (No: 023/ PHD/ UCP/ CVR/12).

2.3.2. Acute toxicity studies (OECD 425)

Acute oral toxicity studies were performed according to the OECD 425 guidelines. A limiting dose of 2000 mg/kg of the extract was given orally to fasting female mouse. Animals were observed for any toxicity signs (behavior, neurological and morphological profiles) for the first 4 h continuously and thereafter daily for 14 days. No mortality or toxicities were observed in any of the treatments.

2.3.3. Induction of experimental ascitic tumor

EAC cells were aspirated aseptically from the tumor bearing mice using 18 G needle. The cells were counted and phosphate-buffered-saline (pH 7.2) was used to adjust the volume and the cell viability was checked. Cell suspensions containing 2.5×10^6 cells were injected intraperitoneally to obtain ascitic tumor in mice (Kuttan et al., 1985).

2.3.4. Experimental design

The animals were divided into nine groups, each containing 12 animals.

Group I: Normal control mice received vehicle 0.25% carboxy methylcellulose (CMC) *p.o.*

Group II: Positive control mice with EAC induced ascites tumor on day zero.

Group III, IV and V: Mice with EAC induced tumor on day zero treated with JSPE at doses of 50, 100 and 200 mg/kg respectively (suspended in 0.25% CMC) on day 3, 5, 7, 10, 12 and 14.

Group VI, VII and VIII: Mice with EAC induced tumor on day zero treated with JSOH at doses of 50, 100 and 200 mg/kg respectively (suspended in 0.25% CMC) on day 3, 5, 7, 10, 12 and 14.