ORIGINAL RESEARCH PAPER

Volume-8 | Issue-6 | June-2019 | PRINT ISSN No. 2277 - 8179

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

EVALUATION OF ANTI-TUMOUR POTENTIAL OF *CYNOMETRA TRAVANCORICA* BEDD.

Pharmacology

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ABSTRACT

Cynometra travancorica Bedd. is used as a substitute for *Saraca asoca* in *Asokarishta*, a well-known Ayurvedic polyherbal formulation for various gynaecological disorders. The pharmacological properties of this plant have not been reported so far. Presently, the cytotoxic effect of methanolic extract of *C. travancorica* was analyzed on Daltons Lymphoma Ascites (DLA), colorectal (HCT-15) cancer cells and normal intestinal epithelial (IEC-6) cells. Anti-tumour activity was assessed on DLA induced solid and ascites tumour models in mice. The extract exhibited cytotoxicity towards DLA, HCT-15 cells with IC_{50} values 65±5.6 and 112±6.3 µg/mL, respectively and did not show any toxic effect on IEC-6, implying its specificity on cancer cells. Extract treatment (500 mg/kg b. wt) reduced the solid tumour by 51.1% and increased the life span of ascites tumour bearing mice by 50.3%. Preliminary phytochemical analysis of the extract showed the presence of flavonoids, alkaloids, tannins and phenols. Thus the study reveals the cytotoxic and anti-tumour potential of *C. travancorica*.

KEYWORDS

Cynometra travancorica, cytotoxic, anti-tumour

INTRODUCTION

Cancer, a major public health issue is the second leading cause of death following cardiovascular diseases. According to the latest report released by International Agency for Research on Cancer, the global cancer burden has risen to 18.1 million new cases and 9.6 million deaths in 2018 (Bray et al. 2018). Exposure to harmful radiations, chemicals, viruses, consumption of tobacco, alcohol, lack of physical activity, obesity, hormones etc are few of the several important factors contributing to the rising incidences of cancer. Nevertheless, there have been improvements in cancer survival rates for different types of cancers because of a better understanding of its etiology and availability of new therapeutic modalities, which seems to be continually evolving. Mounting concerns about the unwarranted adverse effects of the treatment strategies such as surgery, radiation and chemotherapy has shifted the focus of research to the natural sources of therapeutics, thereby leading to an upsurge in the use of plant derived products in modern medicine as alternative and complementary therapies. The Western Ghats of India known for its rich biodiversity harbours numerous plant and animal species, many of them being still unexplored.

Saraca asoca (Roxb.) de Wilde is one of the legendary trees of India. Almost all the parts of this tree are considered pharmacologically valuable. *Saraca indica* Linn. leaf and bark extracts demonstrated significant antioxidant and cytotoxic effects (Emran, 2012; Yadav et al. 2015). Flavonoids from the flower extract of *S. asoca* showed chemopreventive effect on 7, 12 dimethylbenz(a)anthracene (DMBA) induced skin cancer in mice models (Cibin et al. 2010). Bark of *S. asoca* is one of the principal ingredients of an Ayurvedic polyherbal formulation known as *Asokarishta* used in the treatment of gynaecological disorders such as mennorhagia, dysfunctional uterine bleeding etc. In a previous study, the phytochemical profiling of *Cynometra travancorica* Bedd., a plant species endemic to Western Ghats of India was found to be more or less similar to *S. asoca* and has been reported to be occasionally used as a substitute for *S. asoca* in *Asokarishta* without any scientific validation. The present study thus evaluates the cytotoxic and anti-tumour properties of *C. travancorica*.

MATERIALS AND METHODS

Collection of plant sample

The plant material was collected from Wayanad District of Kerala and authenticated by Dr. N. Sasidharan, Taxonomist, Kerala Forest Research Institute (KFRI), Peechi, Thrissur, Kerala, India. A voucher specimen (No. 5716) was submitted in the Herbarium of KFRI.

Preparation of extract

The bark of the plant was dried at 45-50°C for one week and powdered using a grinder. About 20 g powder was extracted with 250 mL methanol by overnight stirring. The extract was filtered using Whatman filter paper No. 1 and evaporated to dryness. The residue obtained was used for cytotoxic and anti-tumour studies. The methanolic bark extract of *C. travancorica* has been referred to as CTE in the text.

Animals

Female Swiss albino mice (25-30 g) were obtained from Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala. They were maintained under standardized environmental conditions of temperature and humidity (22-28°C, 60-79 % relative humidity, 12 h dark/light cycle) and fed with standard rat feed (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All the animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and conducted strictly according to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

Cell lines

IEC-6 (normal intestinal epithelial cells from rat) and HCT-15 (human colorectal adenocarcinoma) cell lines procured from National Centre for Cell Sciences, Pune, India were cultured in RPMI-1640 medium supplemented with Fetal Bovine Serum (10% v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml) and incubated at 37°C in an incubator with 5% CO_2. Daltons Lymphoma Ascites (DLA) cells maintained in the intraperitoneal cavity of mice were used for the study.

Determination of cytotoxicity by trypan blue exclusion method

Cytotoxic activity of methanolic bark extract of *C. travancorica* (CTE) was assayed by determining the percentage viability of DLA cells using trypan blue dye exclusion method. The cells were grown in the peritoneal cavity of mice by injecting $1x10^6$ cells, intraperitoneally. The cells were aspirated aseptically from the cavity of mice after two weeks of tumour induction, washed with phosphate buffered saline (PBS) and centrifuged at 1000 rpm for 5 min. The pellet was re-suspended in PBS and the count was adjusted to $1x10^6$ cells/mL. The cells were then added to the test tubes containing different concentrations of the extract in 1 mL PBS and incubated at 37°C for 3 hr. Percentage viability and IC_{50} value was calculated.

Determination of cytotoxicity by MTT assay

IEC-6 and HCT-15 cell lines were used for the study. Approximately, $1x10^5$ cells of each cell line were seeded in 12 well-plate containing 1 mL medium and incubated at 37°C for 24 hr. Cells were then incubated with different concentrations of CTE at 37°C for the next 48 hr. The test also included a blank containing complete culture medium without cells. After incubation 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 hr. The dark blue formazan crystals were dissolved in 1 mL solubilization solution containing isopropanol, concentrated HCl

and triton X-100 by continuous aspiration and re-suspension. Absorbance of the coloured product was measured at 570 nm. The cytotoxicity was determined by comparing the percentage death of treated cell population with the untreated control, indicated by their respective absorbance at 570 nm.

Solid tumour model

Swiss albino mice (Females, 5-6 weeks old) weighing 25-30 g were divided into four groups, comprising of six animals in each group. Tumour was induced by injecting DLA cells ($1x10^6$ cells/mouse) on to the right hind limb of all the animals. Group I was kept as control without drug treatment. Group II and III were treated with CTE at the doses of 250 and 500 mg/kg b. wt, respectively. Cyclophosphamide (10 mg/kg b. wt) used as the standard drug was administered to group IV animals. CTE as well as cyclophosphamide was given orally from the first day of tumour induction for ten consecutive days. The tumour development in mice was determined by measuring the diameter of tumour growth in two perpendicular planes using a digital Vernier caliper at 3 days interval for one month. The tumour volume was calculated using the formula $V=4/3\ \pi\ r_1^2 r_2$, where r_1 and r_2 represent the minor and major radii, respectively.

Ascites tumour model

Swiss albino mice (Females, 5-6 weeks old) weighing 25-30 g were divided into four groups with six animals in each group. Ascites tumour was induced by injecting DLA cells ($1x10^6$ cells/mouse) into the peritoneal cavity of the mice. Group I was kept as control without drug treatment. Mice in group II and III were administered orally with 250 and 500 mg/kg b. wt extract, respectively. Group IV was treated with the standard drug, cyclophosphamide (10 mg/kg b. wt). Twenty-four hours post the injection of DLA cells, the mice were treated with the extracts and standard drug for a period of 10 days. The effect of the extracts on ascites tumour was monitored by recording the mortality of mice daily for 30 days which was then used to calculate the percentage increase in life span (%ILS). %ILS was calculated using the formula, % ILS = {(MST of drug treated group/ MST of control group)-1} x 100, where MST represents mean survival time.

Phytochemical screening of CTE

CTE was screened qualitatively for the phytochemical constituents according to the standard procedures (Trease and Evans, 2002).

Statistical analysis

The values are expressed as mean ± SD. The statistical significance was evaluated by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnet multiple comparison test) using Graphpad Instat 3 software.

RESULTS

Cytotoxicity of CTE on DLA cells

CTE exhibited remarkable cytotoxicity towards DLA cells (Figure 1) marked by the increase in the number of dead cells that took the trypan blue dye (Figure 2). Concentration of the extract required for 50 % death of cells (IC_{50}) was estimated to be 65 ± 5.6 µg/mL.

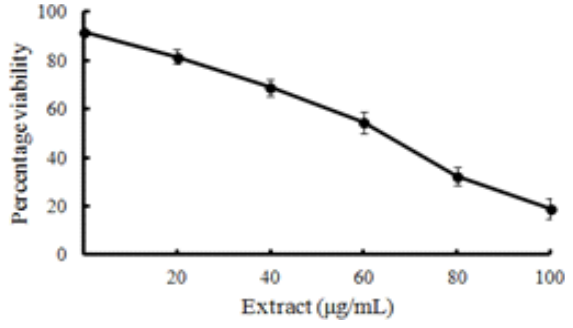

Figure 1 - Cytotoxic effect of CTE on DLA cells

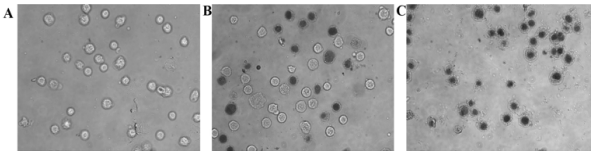

Figure 2 - Effect of CTE on morphology of DLA cells (200x). A. Untreated control, B. CTE 50 µg/mL, C. CTE 100 µg/mL

Cytotoxicity of CTE on IEC-6 and HCT-15 cells

CTE did not show a cytotoxic effect on IEC-6 even up to a concentration of 200 µg/mL (Figure 3A) whereas it showed a concentration dependent inhibition of HCT-15 cells (Figure 3B) with an IC_{50} value of 112 ± 6.3 µg/mL. Morphological changes upon treatment with CTE were observed using a phase contrast microscope (Figure 4).

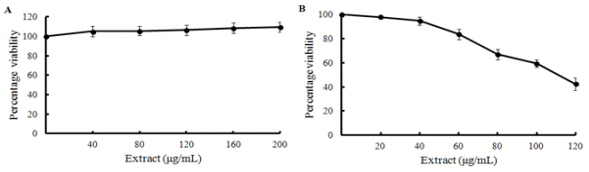

Figure 3 - Cytotoxic effect of CTE on IEC-6 (A) and HCT-15 (B)

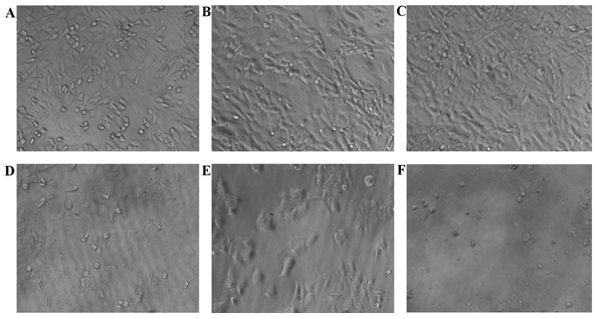

Figure 4 - Effect of CTE on morphology of IEC-6 cells (A, B, C) and HCT-15 (D, E, F) (200x). A and D. Untreated control, B and E. CTE 80 µg/mL, C and F. CTE 200 µg/mL

Effect of CTE on solid tumour

CTE treatment showed a dose-dependent reduction in the tumour volume when compared to the untreated control group. Tumour volume in the untreated control group was found to be 1.59 ± 0.23 cm^3 on 30th day of the experimental study whereas in CTE treated groups at 250 and 500 mg/kg b. wt, the tumour volume reduced to 0.97 ± 0.14 (39 % reduction) and 0.77 ± 0.15 cm^3 (51.1 % reduction) , respectively. Tumour volume in cyclophosphamide treated group was reduced to 0.51 ± 0.13 cm^3 (67.8 % reduction) (Figure 5).

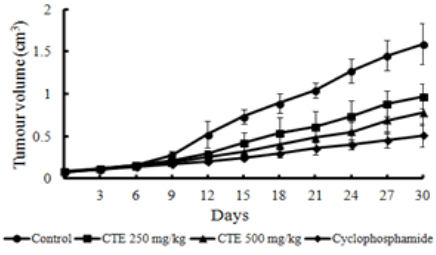

Figure 5 - Effect of CTE on DLA induced solid tumour in mice

Effect of CTE on ascites tumour

Percentage increase in life span (%ILS) of CTE treated mice provided a strong evidence of its anti-tumour potential. In CTE treated groups at 250 and 500 mg/kg b. wt, the mean survival time (MST) of tumour bearing mice was increased to 20.8 ± 3.54 (35.8 % ILS) and 23 ± 2.68 days (50.3 % ILS) at the doses of 250 and 500 mg/kg b. wt, respectively when compared to the untreated control group with MST of 15.3 ± 3.98 days. MST in case of cyclophosphamide treated group was increased to 24.6 ± 3.66 days (60.8 % ILS) (Figure 6).

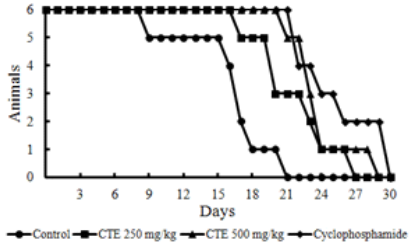

Figure 6 - Effect of CTE on DLA induced ascites tumour in mice

Phytochemical analysis

The phytochemical analysis of CTE revealed the presence of flavonoids, alkaloids, tannins and phenols, compounds which are proficient of causing diverse pharmacological effects.

DISCUSSION

Incidence rates of cancer are increasing at an alarming rate across the world. Due to undesirable effects caused by the cancer treatment strategies, people are now more inclined towards Ayurveda, traditional medicinal system of India that utilizes natural elements to eliminate the root cause of a disease (Parasuraman et al. 2014). Owing to their availability, minimal toxicity and potential effectiveness, natural products have received considerable attention as chemopreventive as well as chemotherapeutic agents. There has been an upsurge in the field of screening natural products, either in the form of isolated compounds or crude extracts, for apoptotic abilities to uncover potential anticancer compounds (Samarghandian et al. 2016).

Medicinal plants provide an array of secondary metabolites with relevant therapeutic potential. *Saraca asoca*, a universal panacea in Ayurveda is well-known for its medicinal values. In this study, *Cynometra travancorica*, an occasionally used substitute for *Saraca asoca* in *Asokarishta* exhibited remarkable cytotoxicity towards cancer cells. CTE caused a reduction in the cellular density and also induced distinctive morphological changes in cancer cells such as membrane blebbing, cell shrinkage etc when compared to their respective untreated controls. Treatment with the extract did not inhibit the growth of normal intestinal epithelial cells and neither did induce any morphological changes thus indicating its specificity towards cancer cells. The potent cytotoxic activity of the extract provided the base to evaluate its anti-tumour activity.

The parameters considered in selecting an animal model to study the cytotoxic effect of a drug are genetic stability and heterogeneity of transplanted cell line, its immunogenicity within the host animal and a suitable biological endpoint. In this regard, Daltons lymphoma model, a transplantable T-cell lymphoma of spontaneous origin in the thymus of murine host serves as an appropriate model. Several parameters such as tumour volume, survival time, changes in tumour immunogenicity etc are used to evaluate the activity of drug on tumour regression in this model (Koiri et al. 2017). This study demonstrated the efficacy of extract to impede the progressive development of tumour cells in mice thereby indicating towards its anti-tumour potential.

Phytochemical screening revealed the presence of phyto-constituents such as flavonoids, alkaloids, phenols and tannins in the extract. Flavonoids are polyphenolic compounds exhibiting a broad spectrum of pharmacological activities and are known to influence the series of immunological events associated with development and progression of cancer (Batra and Sharma, 2013). Alkaloids target DNA replication or protein synthesis in the mechanism of action on cancer cells thus resulting in their apoptosis (Hsiang et al. 1985). Tannins and phenolic acids from medicinal plants are responsible for their chemopreventive properties and also play important roles in the induction of apoptosis by arresting cell cycle, inhibiting DNA binding, cell adhesion, migration, differentiation etc (Huang et al. 2010). Hence, the cytotoxic and anti-tumour potential demonstrated by *C. travancorica* may be attributed to the presence of these biologically active secondary metabolites.

CONCLUSION

This study demonstrated the cytotoxic potential of methanolic bark extract of *C. travancorica* on murine tumour cell, DLA and human colorectal cancer cell, HCT-15. Extract did not exhibit a cytotoxic effect on normal rat intestinal epithelial cells, IEC-6 thus indicating its specificity towards neoplastic cells. Administration of extract reduced the tumour volume in solid tumour model and increased the life span of tumour bearing mice in ascites tumour model, thereby signifying its anti-tumour potential. This study also provided the basis for further investigation into characterization and mechanism of cytotoxic compounds present in the extract. Hence, *C. travancorica* could be an important source of novel drug candidates in the prevention and treatment of cancer.

ACKNOWLEDGEMENT

The authors are thankful to KSCSTE (Kerala State Council for Science, Technology and Environment) for providing the research fellowship to undertake this work (No. 012-10/FSHP/2012/CSTE dated 28.12.2012).

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