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Research article

In-vitro cytotoxic studies of *Cordia monoica* (Roxb.) leaves on HeLa E 139 cell lines

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Key words: *Cordia monoica* (Roxb.), Boraginaceae, HeLa cell line E139, MTT Assay.

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Abstract

Aim: The present study was carried out to determine the cytotoxicity of *Cordia monoica* (Roxb.) leaves on cancer cell lines. **Materials and methods:** *Cordia monoica* (Roxb.) belongs to Boraginaceae family and is commonly known as sand paper. The flowers are cream in color and turn brown on drying. The leaves of the plant have a texture of sandpaper. The Leaves of *C. monoica* was extracted with ethanol and subjected for *in-vitro* anti-tumor studies. The *in-vitro* Cytotoxicity screening was carried out with HeLa E 139 cell line using MTT assay. **Result:** The study indicated that the extract is toxic to the cell at higher concentration and was dose dependent. The plant extract has cytotoxic effects on HeLa E 139 cell line as concentration increases. **Conclusion:** Hence, the present study supports *C. monoica* (Roxb.) can be a potent anti-cancer herb if it is exploited.

Introduction

Cancer known as malignant tumor is a dreadful disease that results in an abnormality of cells internal regulation. The growth and division of the cell are under uncontrollable proliferation in an autonomous fashion and thus leads to a progressive increase in the number of dividing cells [1-2]. They can invade into nearby tissues or to distant organs by a process termed as Metastasis [3-4].

Cancer occurs by a single cell in a tissue and is classified based on the type of cell that the tumor cells resemble and are therefore presumed to the origin of tumor [5]. Benign tumors differ from cancer in that it will be localized, self limited and doesn't metastasize. Many diseases such as heart failure may have a worse prognosis than most cases of cancer. Cancer is the subject of widespread fear and taboos around the globe. There are 200 different types of cancer that afflict humans [6]. The cancer cells are produced due to changes in DNA of the cells (Mutation) that are transformed [7]. Cancer is caused by internal factors and external factors [2, 8].

An extremely promising strategy for cancer prevention today is chemoprevention. It is defined as treatment of cancer in humans with the use of synthetic or natural agents (alone/combination) [9]. It is one of the most effective methods of cancer treatment. However, chemotherapeutic agents affect the normal cells severity. Hence the use of natural products has been contemplated of exceptional value in the control of cancer and its eradication program [4, 10]. Drug discovery from medicinal plants have been playing a crucial role in combating cancer over the last half century [11].

There are two main strategies for the selection of anti-cancer agents- random screening and ethno-medical knowledge. In the cancer drug discovery program, a paradigm between ethano-botanical and ethno-pharmacological data would be more economical. The benefit is being for identifying potential anti-cancer molecules than mass screening of plant species [12-13]. The main source of cancer chemoprevention drug discovery and development is the folk and traditional. The usage of a variety of plants, vegetables and herbs has more effects on the disease [10]. In Ayurveda, with the use of nutritional supplements or by use of herbs treatment for chemotherapy is well documented, that has been commonly practiced in India [14]. Plants have been regarded as a potential source of chemoprevention for cancer [15-16]. In recent years plant derived natural products such as flavonoids [17], terpenes [18] and alkaloids [19] have received massive attention due to their diverse medicinal properties including cytotoxic and cancer chemo preventive effects [20].

The literature assessment on the ethano-botanical information revealed that the Boraginaceae family consists of small trees or shrubs that have more medicinal value. These plants have been used to treat various disorders in traditional and folk medicine. Most of the species are yet to be evaluated. In India around 13 species of *Cordia* genus was brought into being. The plant *Cordia monoica* Roxb. belongs to Boraginaceae family. It is a much branched bush, shrub or tree with a height of 6-12m

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[21-22]. It is commonly known as sand paper saucer-berry or Snot berry [23]. The plant is widely distributed in South India [24]. The fruits are orange in color and pulp is edible, which is sweet and gummy [25].

The *Cordia monoica* Roxb. roots were used in treating vomiting and malaria [26]. In siddha medicine, the leaves were used to treat eye diseases [27]. The whole plant is also used for virtual medicine [28]. The stem bark and leaves were also used in the treatment of leprosy [29-31]. The leaves were used in treatment of chest pain [32] and throat infection. The leaves of the plant were used to treat MICH, a febrile disease which has symptoms of fever, headache, sweating [33]. The fruits of the plant are edible and eaten raw [34]. The plant is also used as fodder [35]. The leaves and stem were also used to treat back aches, viral infections [36-37]. In Sri Lanka, people use *C. monoica* leaf and bark for the treatment of ulcer and boils [38]. The bark is also used to treat conjunctivitis [39].

In Pharmacological studies, *C. monoica* root extract were reported to possess significant anti-inflammatory activity and analgesic activity [40]. The methanolic extract of the stem have been reported with potent anti-ulcer activity than chloroform and ethyl acetate extract [41]. As per OECD – 423 guidelines the extracts of both root and stem did not show signs of mortality in rats [40-41]. The stem extract and leaf extract have been studied for anti-microbial and anti- uterine activity. It has been reported that the chloroform extract of *C. monoica* leaves has mild anti-uterine activity [42]. The methanolic extract of *C. monoica* leaves exhibited higher zone of inhibition against *Pseudomonas aeruginosa* and the phytochemical analysis revealed the presence of tannin, saponin and terpenoids [43]. The leaf preparations of several species of *Cordia* are used in traditional medicine as remedies for some tumoral formations [44-45]. Hence, in the present study, the *in-vitro* anti-cancer activity of ethanolic extract of *C. monoica* leaves was evaluated.

Experimental

Materials and methods

Collection of plant material

Cordia monoica Roxb. belonging to Boraginaceae family is a shrub, widely distributed in most districts of Tamil Nadu on rocky hill sides. The plant materials were collected during the month of June. The leaves of the plant were collected from Maruthamalai Hills of Coimbatore, Tamil Nadu, India. Flowering shoots of the plants were also collected for identification. The collected plant material was identified and their authenticity was confirmed by comparing the voucher specimen at the Botanical survey of India, Coimbatore, Tamil Nadu, India (BSI/SRC/5/23/2014-15/Tech/512). The collected specimens were deposited in the Department of Biotechnology, Sri Ramakrishna College of Arts and Science, Coimbatore, Tamil Nadu, India.

Shade drying of the collected leaf materials

The collected leaves were cleaned to remove adhering dust and then dried under shade. Then the dried leaves were powdered in mechanical grinder fine enough to pass through a No.40 sieve for powder analysis. Coarse leaf powder was used for further extraction process and pharmacological studies.

Chemicals used

Analytical/ laboratory reagent grade chemicals were used for the studies, which were purchased from the following manufactures and used without further purification.

Ranbaxy Laboratories Ltd., Chemical division, Punjab.
S.D. fine –Chem Ltd, Bisor.
Fischer inoganies & Aromatics, Madras.
Qualigens Fine Chemicals, Mumbai.
E.Merck (India) Ltd., Mumbai

Extraction process

50gm air dried coarse leaf powder was mixed with 100 ml of ethanol. The extraction was carried out in a closed macerated flask for 24 hours, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, the mixture was filtered rapidly taking precautions against loss of the solvent. 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. The extract is stored and used for further analysis [46-47]. The extractive yield value was 1.98% w/w.

A great number of *in-vitro* methods have been employed to study on antitumor efficacy of plant extract or pure compounds. *In-vitro* methods like LDH (Lactate dehydrogenase) assay, XTT assay, Sulforhodamine B assay, MTT assay, Tryphan Blue dye exclusion assay is most commonly used for estimating anti-cancer properties of natural products. Among all MTT assay is most popular for estimating anti-cancer activity.

Cell line

The human cervical cancer cell lines (HeLa E139) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% foetal bovine serum (FBS), 1% non-essential amino acids, sodium pyruvate, sodium bicarbonate and 2mM glutamine. The cells were maintained at 37ºC, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin- ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5%

FBS to give final density of $1x10^5$ cells/ml. 100 microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºC, 5% CO_2, 95% air and 100% relative humidity. After 24 h, the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethyl sulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100µl of these different sample dilutions were added to the appropriate wells already containing 100µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 hr at 37ºC, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations [48].

MTT assay

After 48 hr of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37ºC for 4h. The medium with MTT was then flicked off and the formed formazon crystals were solubilised in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader [49]. The percentage cell inhibition was determined using the following formula.

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Morphological changes

The cell morphology was observed under light microscope to spot the percentage of cell shrinkage and signs of apoptosis [50].

Statistical analysis

Non-linear regression graph was plotted between % Cell inhibition and Log concentration and IC_{50} was determined using Graph Pad Prism software.

Results and discussion

Results

Table 1 and 2 shows the Cytotoxicity properties of *C. monoica* ethanolic leaf extract on HeLa E 139 cell line by using MTT assay. The ethanolic extract of *C. monoica* leaves showed potent cytotoxicity against the cancer cell line HeLa E139 and the percentage activity was measured using MTT assay. The percentage inhibition was found to be increasing with increasing concentration of the *C. monoica* leaf extract. The extract exhibited a dose dependent activity (Figure 1). The death rate of HeLa E139 cell line was most prominent at a dose of 200µg/ml ethanolic extract of *C. monoica* leaves. The inhibition was about 100% with a regression of R^2 0.997. The IC_{50} value was found to be 67.19µg/ml. Figure 2 describes the microscopic examination of treated cell lines at different concentrations. This showed that *C. monoica* leaves possess strong cytotoxicity against the cancerous cell lines. The morphology of HeLa E 139 cell lines on treatment with *C. monoica* extract showed cell shrinkage, apoptosis and clumping of cells. Macrophage differentiation, cell adhesion and the growth of cells was inhibited at high concentration of 200µg/ml.

Table 1. *In-vitro* cytotoxic activity of *Cordia monoica* Ethanolic Leaf Extract on HeLa E 139 cell line

	CML Concentration (µg/ml)					
	Control	**12.5 µg**	**25 µg**	**50 µg**	**100 µg**	**200 µg**
Absorbance 1	0.578	0.575	0.552	0.444	0.111	0
Absorbance 2	0.577	0.577	0.559	0.429	0.105	0
Absorbance 3	0.573	0.568	0.569	0.436	0.095	0
Average of Absorbance	**0.576**	**0.57**	**0.56**	**0.436**	**0.10**	**0**

The values are mean ± Standard Error Mean (n=3).CML-*Cordia monoica* ethanolic leaf extract

Table 2. Percentage of Cell Inhibition and IC_{50} value of *Cordia monoica* Ethanolic Leaf Extract

Concentration of CML Extract (µg/ml)	% Cell Inhibition	IC_{50}	R^2
12.5	0.46	67.19 µg/ml	0.9997
25	2.78		
50	24.25		
100	82.00		
200	100		

CML-*Cordia monoica* ethanolic leaf extract. The values are mean ± Standard Error Mean (n=3)

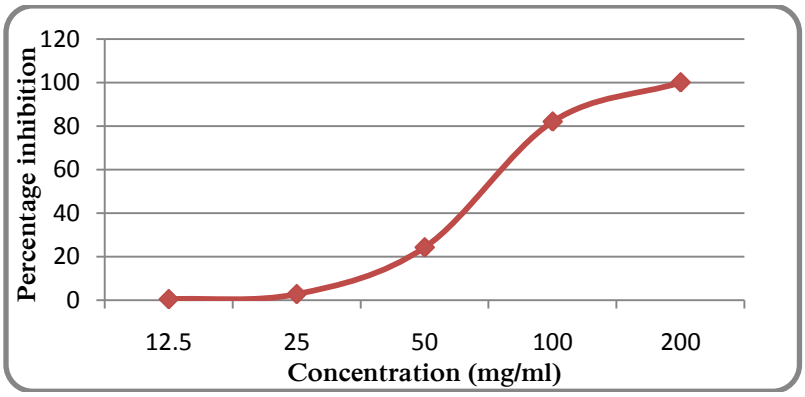


Figure 1. Percentage of Cell inhibition of *Cordia monoica* Ethanolic leaf extract.

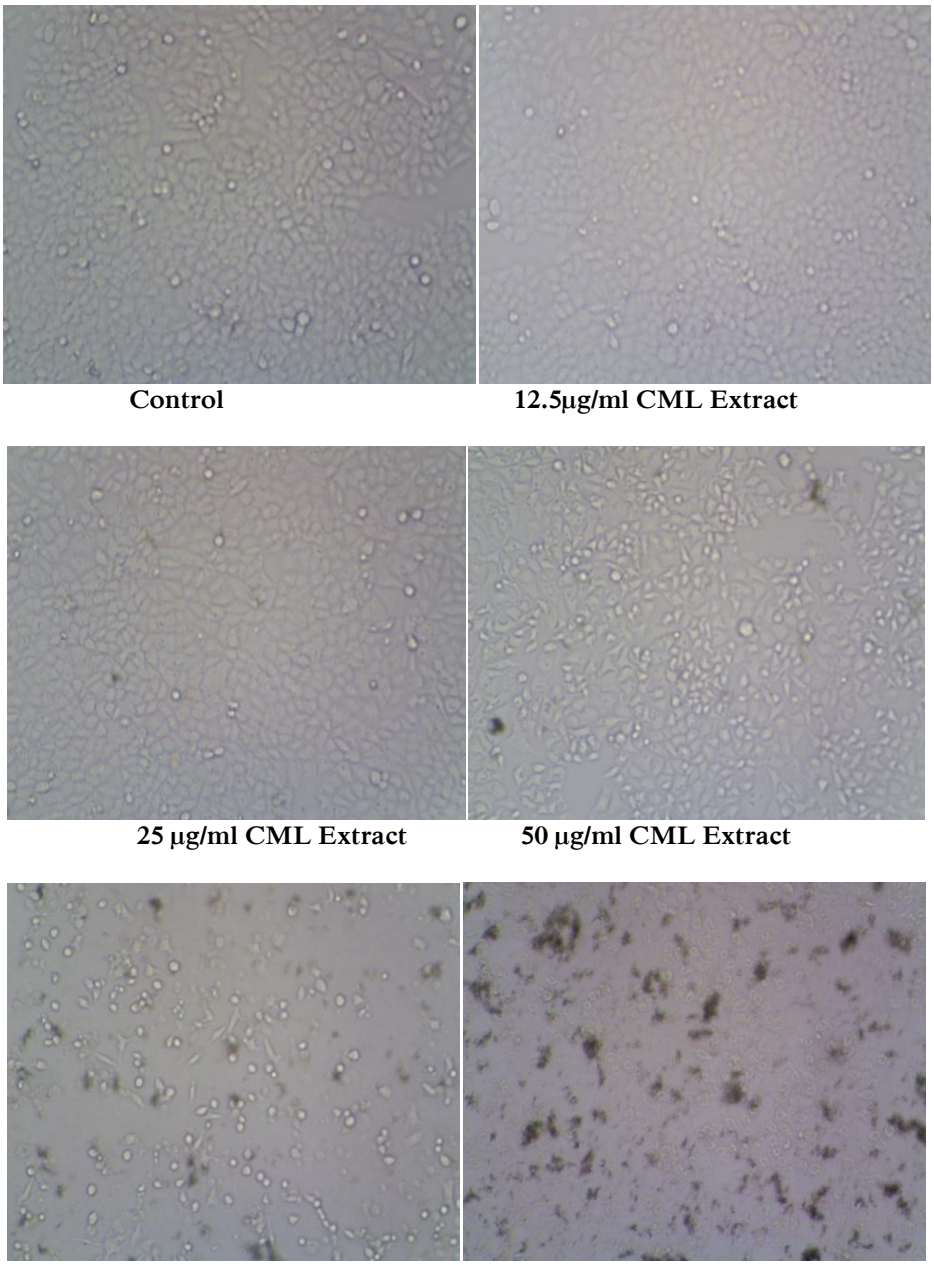


Figure 2. Microscopic examination of control and treated HeLa E-139 Cell lines at different concentrations of CML (*C. monoica* ethanolic leaf extract).

Discussion

The *in-vitro* anti-cancer activities of ethanolic *C. monoica* leaf extract was tested using MTT assay on HeLa E139 cell line. It induced the apoptosis of cancer cells by destroying the mitochondrial membrane [51]. Succinate dehydrogenase, a mitochondrial enzyme in living cells cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazon. The amount of formazon produced is directly proportional to the number of viable cells [52]. The resulting intracellular purple formazon can be solubilised and quantified by spectroscopic methods [53]. The activity might be dependent upon the morphology of cell lines and mechanism of action of the plant extract. Many anti-cancer drugs are effective against HeLa E139 Cells by causing apoptosis through expression of caspase-3 generating reactive oxygen species and damaging DNA [54, 55]. HeLa cell lines are also reported to contain Human papilloma Virus 18 (HPV 18) sequences, normal expression of pRB (retinoblastoma suppressor) and a low expression of p53. The p53 gene appears to trigger apoptosis as a way of regulating uncontrolled cellular proliferation in the setting of aberrant growth signals [56].

The results were in accordance with a recent study by Endalkachew and Michael [57] who reported the cytotoxic effect of methanol and chloroform extract of *C. monoica* Roxb. leaves. The assay was carried out with HL-60 cell lines. The IC_{50} value of methanol extract was found to be 53.2µg/ml and with chloroform extract the value was 219.9µg/ml. The result was in comparison with standard drug Diminazene aceturate. This study strongly correlates with the present *in-vitro* assays. However, it is also important to perform many other studies, both *in-vitro* and *in-vivo* to determine their true potential for development of medicines. The Cell's ability to overcome a toxic insult has been the source of most cytotoxic assays. Morphological changes like shrinkage of cells, adhesion of cell lines to surface and inhibition of cell growth when treated with *C. monoica* leaf ethanolic extract is a sign for anticancer property [50].

Preliminary phytochemical analysis of *C. monoica* Roxb. leaves were carried out with ethanolic extract and the bioactive compounds such as flavanoid, phenol, tannin and steroid were determined. Further, the ethanolic extract of *C. monoica* Roxb. leaves also exhibited potent anti-oxidant property [58]. The Gas Chromatography-Mass Spectroscopic analysis concluded the presence of bioactive phytochemical compounds in the plant. It was reported that the concentrated ethanol extract contains a variety of bioactive compounds such as 2,7,12,17-tetrabrom-(allàs) cyclotetrathiophen (2,7,12,17-tetrabromcycloocta[1,2-b:4,3-b':5,6-b":8,7-b"'] tetrathiophen., Nonacosane, carotene, neophytadiene, Lycopene 7,n- Hexadecanoic acid, Octadecanoic acid, Phenol 3-pentadecyl, Heptacosane, Tetracosahexane hexamethyl (CAS), Benzofuran and Carotene[59]. [58]. Hence, the reported cytotoxic property may be due to phenolic content and flavonoid content [60-61]. The study justifies the use of *C. monoica* leaves as an anti-oxidant, anti-inflammatory and anticancer agent in herbal medicine.

Conclusion

The present study indicates the therapeutic potential of *C. monoica* leaf extract and justifies the use of *C. monoica* leaves in traditional medicine. The GC-MS studies also reveal the presence of various bioactive constituents which may be responsible for pharmacological activities. Further studies may be needed to separate and characterize these bioactive compounds which in turn lead to the production of a novel anti-cancer drug.

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