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Anticancer Screening of Various Seed Extract of *Cardiospermum halicacabum* on Human Colorectal, Skin and Breast Cancer Cell Lines

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ABSTRACT

Background: In the modern lifestyle, the increase in cancer and related chronic disorders is a major public health problem. In spite of different methods used for the treatment of these conditions, natural medicines have high demands due to their significant effects as immune enhancement and therapeutic agents and fewer side effects in comparison with other treatment methods. Hence, this study was undertaken to evaluate the cytotoxic effect of *cardiospermum halicacabum* Linn. seeds, based on traditional claims.

Methods: A Soxhlet extractor was used to obtain different extracts from seeds of *C. halicacabum* Linn. Sulforhodamine B colorimetric (SRB) assay used for the evaluation of the cytotoxic effect of the various extracts on HT-29, HCT-15 colon carcinoma, SK-MEL-2 skin carcinoma, and MCF-7 breast carcinoma. The results were compared against Doxorubicin as a standard drug.

Results: The results of the present study showed the potent cytotoxic activity of n-hexane extract of seeds of *C. halicacabum* Linn. against the MCF-7 breast cancer cell line with 50% growth inhibition value (GI50) of 12.8 µg/ml but other extracts showed poor activity in other tested cell lines.

Conclusions: The results indicated the potential medicinal value of *C. halicacabum* Linn. seeds oil with the highest extractive yield as an antineoplastic agent. However, further studies are needed for the isolation of the active anticancer compounds and evaluating the mechanism of action of the responsible compound.

Introduction

Since ancient times, cancer has been considered a deathful disease for humans and it has become one of the major leading causes of death worldwide

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in industrialized and developing countries. As different traditional systems of medicine use natural products for improving the health and treatment of various disorders, the use of complementary and alternative medicine is now more of interest. With this concern, many patients demand for natural medicines, especially herbal drugs, for the prevention and treatment of cancer.^{1,2} With this interest in natural medicine, National Cancer Institute (NCI) introduced some anticancer agents from natural sources like herbal and marine products in 1950.³ According to the American Cancer Society,



breast and colorectal cancers are the common types of cancers among women and men worldwide and the skin cancer ratio is higher in the developed countries.¹ Breast cancer is one of the leading cancers in women and colorectal cancer is common in men and women both; thus, the aim of this study was to evaluate the seeds of *C. halicacabum* Linn. to find out the potential natural anticancer agent from this plant based on traditional claims and medicinal value

Cardiospermum halicacabum Linn. is an important medicinal plant used in various traditional systems of medicine such as Ayurveda, the Indian system of medicine, Unani medicine, and Chinese medicine.⁴⁻⁶ In Indian medicine, *C. halicacabum* Linn. is used for the treatment of chronic bronchitis, stiffness of limbs, and snakebite. In Chinese medicine, it is used for the treatment of lumbago, nervous diseases, rheumatism, as a demulcent in orchitis, and in dropsy. In Unani medicine, the seeds are used as a tonic and for the treatment of cancer.⁶⁻⁹ It is also used as marketed herbal products like cream, gel, shampoo, spray, *etc.* and it is helpful in the dry itchy skin and scalp.¹⁰

Methods

Plant materials

The *C. halicacabum* Linn. plant was collected from its natural habitat in Gujarat, India. Herbarium of the plant was prepared and certified by Raw Material, herbarium and museum division CSIR-NISCAIR, Delhi, India. Voucher specimens were lodged in the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, India. Mature seeds were separated, air dried, and subjected to mechanical pulverizer for size reduction and extraction.

Extraction

Two-hundred grams of the coarse powder of seeds was subjected to successive extraction in a Soxhlet apparatus, using different solvent polarities in sequence starting with n-Hexane, chloroform, ethyl acetate, methanol, and hydroalcohol. The extracts of each solvent were concentrated under reduced pressure using a rotary evaporator system and the concentrated extract was evaporated to dry and then stored in an air tight container for further use.

Cell lines and standard drug

Four human cancer cell lines were selected in this study. Two colon carcinomas, namely HT-29 and HCT-15, one breast carcinoma, MCF-7, and SK-MEL-2 skin carcinoma. They were grown in the RPMI1640 medium (Roswell Park Memorial Institute) with contains fetal bovine serum 10% and L-glutamine 2mM. All the cell lines were incubated for 24 hours at 37°C with 5% CO₂, 95% air, and 100% relative humidity before addition of

experimental drugs. Doxorubicin was used as the standard drug.

Cytotoxicity assay¹¹

The anticancer activities of extracts were evaluated at Advanced Centre for Treatment, Research and Education in Cancer, Mumbai. Sulforhodamine B colorimetric (SRB) assay was used in the present study. All cells were inoculated into 96-well microtiter plates, depending on the doubling time of each cell line. Following the incubation for 24 hours, the cell lines were fixed in-situ with trichloroacetic acid (TCA) for count the population of the cells when that drug was loaded (Tz). All the extracts were dissolved in dimethyl sulfoxide (DMS) and further diluted with cell culture medium. Afterwards, different concentrations of each extract were prepared in final drug concentrations of 10, 20, 40, and 80 µg/ml. The known anticancer drug Adriamycin (Doxorubicin) was used as a positive control for each test drug on cell lines.

After adding all the compounds, the plate was kept for 48 hours for incubation and the assay ended by adding cold TCA. Cells were fixed in-situ by the slow addition of 50 µl of cold TCA 30% (w/v) (final concentration, 10% TCA) and incubated at 4°C for 1 hour. The supernatant was discarded and the plates were washed with water for 5 times and then dried. To each well, the SRB solution was added and incubated at room temperature for 20 minutes, and then stained. The unbound dye was recovered, and washed 5 times with 1% acetic acid to remove the residual dye. After that, the plates were air dried. In the next step, 10 mM Trizma base was used to subsequently elute the bound stain. The compound was then used for absorbance reading by ELISA plate reader at 540nm wavelength and 690 nm reference wavelength.

The percentage of growth was calculated on a plate-by-plate basis for test wells compared to control wells. The percentage of growth was calculated by dividing the average of the absorbance of test wells to the average of the absorbance of control wells.

The six absorbance measurements, (Time zero (Tz), Control growth (C), Test growth with drug at four concentration level (Ti)) used for calculating the percentage growth of each drug concentration. Percentage growth inhibition was calculated as follows:

$$\left[\frac{(Ti - Tz)}{(C - Tz)} \right] \times 100$$
 for concentrations for which $Ti \geq Tz$ (Ti-Tz) positive or zero
$$\left[\frac{(Ti - Tz)}{Tz} \right] \times 100$$
 for concentrations for which $Ti < Tz$. (Ti-Tz) negative.

All the calculated parameters with GI50, TGI and LC50, were in a dose-response manner for the samples. The GI50 is defined as 50% growth inhibition (the concentration that results in a 50% reduction in the



net protein increase in control cells during the incubation of the drug). TGI is the concentration of the drug that results in total growth inhibition, and LC50 indicates a net loss of cells (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning). The following formula was used for the calculations:

GI50 was calculated as $[(Ti-Tz)/(C-Tz)] \times 100 = 50$, TGI was calculated as $Ti = Tz$, and LC50 was calculated as $[(Ti-Tz)/Tz] \times 100 = -50$. Values were calculated for parameters if the level of activity reached. However, if the effect was not reached or was surpassed, the values for that parameter indicated as larger or less than the maximum or minimum concentration tested.

Statistical analysis

Data was used from three independent experiments. GI50, TGI, LC50 values were

calculated from dose-response control growth curves by the mean graph.

Results

The results indicated that the n-hexane extract from extractions of *C. halicacabum* Linn. seeds, was the most active extract with highest extractive yield. The yield percentage of each extract was calculated and shown in table 1. The anticancer activity of *C. halicacabum* Linn. seeds extracts was assessed with SRB assay method against four human cancer cell lines (HT-29, HCT-15, SK-MEL-2 and MCF-7) and GI50, TGI, LC50 were calculated which are summarized in table 1. N-hexane extract of the seeds had the highest yield in form of a light greenish oil with an aromatic odor and had potent anti-proliferative effects on the breast cancer cell line, with a GI50 of 12.8 which is $<20 \mu\text{g/ml}$ indicating activity. However, other extracts did not show any significant cytotoxic effects on other cell lines.

Table 1. Yield of different extracts of *C. halicacabum* and their cytotoxic activity against different human cancers cell lines.

Ext. Yield in % w/w	Skin / SK-MEL-2			Colon / HT-29			Colon / HCT-15			Breast / MCF-7			
	LC50	TGI	GI50	LC50	TGI	GI50	LC50	TGI	GI50	LC50	TGI	GI50	
N-hexane	24.18	NE	NE	NE	>80	>80	>80	>80	>80	>80	>80	>80	12.8
Chloroform	0.96	NE	NE	NE	>80	>80	>80	>80	>80	>80	>80	>80	>80
Ethyl Acetate	0.71	NE	NE	NE	>80	>80	>80	>80	>80	>80	>80	>80	66.9
Methanol	2.72	NE	NE	NE	>80	>80	>80	>80	>80	>80	>80	>80	68.0
Hydroalcohol	1.15	NE	NE	NE	>80	>80	>80	>80	>80	>80	>80	>80	53.0
Doxorubicin	-	<10	<10	<10	45.4	<10	<10	>80	34.0	<10	85.26	38.22	<10

* Abbreviations; GI50: Median Growth Inhibition values ($\mu\text{g/ml}$); TGI: Total growth Inhibition values ($\mu\text{g/ml}$); LC50: Lethal concentration values ($\mu\text{g/ml}$); NE: Non Evaluable Activity.

Discussion

For cytotoxic screening, different values were considered as indication of cytotoxicity effect, as described procedures by the National Cancer Institute (NCI).¹² NCI replaced IC50, which is the concentration that causes 50% growth inhibition, with GI50 to emphasize the correction for time zero count of the cells, and measure the growth inhibitory power of the tested agent, as in TGI and LC50, as described in materials and methods. These parameters are interpolated values and can use the concentrations giving GI50PRCNT values above and below the reference values to make interpolations on the concentration axis. If the level of activity reaches the level that inhibits the growth, the values are then calculated for these parameters; otherwise, they are considered to be not evaluable (NE), as seen in the SK-MEL-2 cell line in this study. If the effect does not reach the level or

exceeds, the value for the parameters is shown as more or less than the maximum or minimum tested concentration, which was >80 and <10 in our study, respectively.^{11,13}

In the present study, five different extracts from *C. halicacabum* Linn. seeds were obtained and used in four concentrations of 20, 40, 60, 80 $\mu\text{g/ml}$ to test against four human cancer cell lines HT-29, HCT-15, SK-MEL-2, and MCF-7 and to compare with Adriamycin (Doxorubicin), a known anticancer drug, in the same concentrations to examine their anti-proliferative effects. The cytotoxic potential of plants is usually related to their different phytochemicals. A review of the literature on *C. halicacabum* Linn. shows the anticancer effect of the methanolic extract of the plant stem on the breast cancer cell line.¹⁴ In the present study, the seeds were selected for the investigation according to their traditional use and high medicinal value of seeds in



plants. The results revealed the n-hexane extract of seeds had the highest yield among all extracts with 24.18 percent yield, which was the most active extract on the breast cancer cell line as shown in Table 1. The extract is in the form of oil and seeds are important in this regard and considered as a good source of nutrition and essential oils; they contain fat that is composed of both saturated and unsaturated (monosaturated and polyunsaturated) fatty acids.^{15,16} Earlier studies have shown the presence of secondary metabolites and various chemical constituents like amino acids, glycoside, phenolics, and flavonoids in the *C. halicacabum* Linn. seeds. A review of the literature also shows the presence of erucic acid, eicosonic acid, oleic acid, tetradecanoic acid, octanoic and n-hexadecanoic acid as fatty acids in the seeds.¹⁷ Different secondary metabolites like phenolics and flavonoids compounds, essential fatty acids such as omega-3,6,9 and other fatty acids that are available in many plants are fundamental for humans in the diet and help to lower the risk of heart disease, inflammation, and healing the lipid barriers. They play a role in oxidative stress conditions, cancer prevention and treatment of cancerous cells. However, many actions like inflammatory responses, hormonal changes, cell cycle, apoptosis, *etc.* can be responsible for these activities and multiple cancer related processes that may account for the ability of plants to inhibit the cancer.^{18,19} Based on the results of this study, the n-hexane extract of *C. halicacabum* Linn. seeds had desirable cytotoxic effects on the MCF-7 cell line in human breast cancer. However, further studies are needed for separation and purification of the active components to evaluate their effects on different breast cancer cell lines in order to identify and characterize the bioactive compounds.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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