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In-Vitro Antiproliferative Analysis of Aqueous Leaf Extract from Cardiospermum Halicacabum in Human Hepatocellular Carcinoma Cell Line

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ABSTRACT

Background: Cancer is one of the multi-factorial, multi-faceted, and multi- mechanistic diseases requiring a multi-dimensional approach for its treatment, control and prevention. Many anticancer drugs in clinical use today are derived from natural sources. Cardiospermum halicacabum (C. halicacabum). is a herbaceous plant, extensively dispersed in tropical and subtropical areas of the world. The present study was designed to determine the anti proliferative activity of C. halicacabum aqueous leaf extract on human hepatocellular carcinoma (HepG-2) cells.

Method: The leaves of C. halicacabum were extracted with ethanol using soxhlet apparatus. The anti-proliferative activity of C. halicacabum was evaluated by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, a yellow tetrazole (MTT) assay and their cell morphological changes was assessed by phase contrast microscope.

Results: Our present study showed the anti proliferative activity of Cardiospermum halicacabum at different concentration (25 µg/mL, 50µg/mL,75 µg/mL, 200 µg/mL and 300 µg/mL). The inhibitory concentration (IC-50) value of aqueous extract of Cardiospermum halicacabum was found to be 50 µg/ml which is considered to be effective dose. We observed significant morphological changes in Cardiospermum halicacabum treated cells.

Conclusion: The aqueous extract of C. halicacabum has confirmed hopeful antiproliferative activity against human hepatocellular carcinoma (HepG-2) cells. In further investigations, to assess its isolates of pure bio active compounds and find out the effectiveness of the C. halicacabum to serve as natural chemotherapeutic agent.

INTRODUCTION

Cancer is a multi-factorial, multi-faceted, and multi- mechanistic disorder that necessitates a multi-faceted care, management and prevention strategy. The most common form of primary liver cancer is Hepatocellular carcinoma [1]. Different factors such as alcohol consumption, nonalcoholic fatty liver, liver cirrhosis, aflatoxin exposure, hepatitis B and C virus infection, gene mutations, and epigenetic modifications all influence the progression of HCC [2]. Most complications which are more common with liver disease include yellow skin (also called jaundice), abdominal swelling due to fluid in the abdominal cavity, rapid bruises from blood clotting defects, reduced appetite, unintentional weight loss, abdominal pain, nausea, vomiting, or feeling exhausted [3]. Several tumour biomarkers, such as Golgi 73 protein (GP73), Glypican-3 (GPC3), and microRNAs, have recently been detected in HCC tests [4]. It is a preventable disease rather than a curable one since there is no well-documented effective treatment modality until now. Treatments for Hepatocellular carcinoma include hepatectomy, liver transplant, radio frequency abortion (RFA), per cutaneous ethanol injection, trans arterial chemoembolization (TACE), anti cancer therapy and radiotherapy [5]. Several types of chemotherapeutic medications have been used to treat hepatic cancer such as cisplatin, doxorubicin, 5-fluorouracil and paclitaxel. There is, however, a low response rate, limited median survival time and toxicity as well as decrease in blood cell production and electrolyte imbalance [6]. At present, these drugs usually have some problems with cancer resistance, due to multidrug resistance and the decrease of apoptotic proteins [7]. As a result, effective therapeutic agents for hepatocellular carcinoma are needed. In recent periods, attention has been reintroduced back to plants as sources of therapeutic agents due to their superior properties. These include low cost, a lower rate of adverse effects and are selective in killing cancer cells but not the normal/physiological healthy host cells compared to current traditional pharmaceuticals and are also ready availability [8].

Many researchers have shown that plant extracts and their secondary metabolites, such as polyphenols, terpenes, and alkaloids, have antimutagenic and anticancer effects. Moreover, Vegetables and fruits contain numerous bioactive and potentially anti-carcinogenic components including carotenes, dithiolethiones, flavonoids, indoles, isothiocyanates, phenols, folic acid and vitamins C and E. Cardiospermum halicacabum, a member of the Sapindaceae family, is a tropical and subtropical herbaceous plant with a wide distribution. It has a number of noteworthy characteristics that justify its use in medicine [9]. In comparison to benzene and chloroform, the aqueous extract of Cardiospermum halicacabum was active against gram - positive organisms such as Bacillus cereus, Micrococcus roseus, and Staphylococcus [10]. Among the fungal strains, all the plant extracts inhibited Aspergillus niger but only methanol and water extracts only inhibited Candida tropicalis [11] . Its ethanolic extract increased the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the anti-inflammatory assay in liver tissue, whereas it decreased the levels of nitrite oxide (NO) and malondialdehyde in edematous paw tissue and serum (MDA). These findings indicate that ethanolic extract acts as a natural antioxidant and anti-inflammatory agent [12]. This herb has been used in Chinese medicine for a long time. Cardiospermin, a cyanogenic glucoside found in the plant root, is a well-known agent that mediates anxiolytic action [13]. For the prevention of diarrhoea, local medicinal practitioners in Gulbarga used a formulation of the whole plant. In rodents, this herb has antipyretic action against yeast-induced pyrexia [14]). Furthermore, the plant had anti-ulcer and anti-hyperglycemic properties in rats with streptozotocin-induced diabetes [15]. The entire herb is used to treat urinary tract inflammation, oedema, nephritis, and oliguria in Hong Kong and Korea [16]. We hypothesised antiproliferative activity of cardiospermum halicacabum aqueous leaf extract on human hepatocellular carcinoma cells (HepG-2) based on the above various medicinal properties since there are insufficient studies such as the anti cancer activity of isolated fractions from Cardiospermum halicacabum Methanol leaf extract on human hepatocellular carcinoma cells (HepG-2). Our research and knowledge have resulted in high-quality publications from our team [17-31]

The present study was aimed to demonstrate the antiproliferative activity of aqueous leaf extract of cardiospermum halicacabum on the human hepatocellular carcinoma cell line.

MATERIAL AND METHODS:

CHEMICALS

DMEM medium, 0.25% Trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), low melting agarose, MTT from Sigma Chemicals Co., St. Louis, USA. fetal bovine serum (FBS) and antibiotic/antimycotic solution, DMSO were from Himedia, Sodium phosphate monobasic and dibasic, sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid and methanol were purchased from Sisco Research Laboratories (SRL) India.

Preparation of the Herbal Extract:

Stem powder of Cardiospermum halicacabum obtained from IMPCOPS (Chennai, India) was used for the present study. About 50 g of Cardiospermum halicacabum powder was soaked in 500 mL of aqueous/95% ethanol and kept at room temperature for 3 days in a static condition. Then the solution was filtered with crude filter paper followed by whatman paper. Fine filtrate was subjected to rota evaporation after that 3g of the material was obtained. The total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4℃.

CELL CULTURE REAGENTS

DMEM

Commercially available DMEM contains 7.5% sodium bicarbonate solution. To 500ml of DMEM, 5ml of penicillin/streptomycin solution and 0.5ml of amphotericin B solution was added. Then the medium was sterile filtered (0.22µ) inside the hood. The medium was then dispensed into sterile containers and stored at 4°C.

CELL LINE

Human hepatocellular carcinoma cell line (HepG2) was procured from National Centre for Cell Science (NCCS, Pune), India. The cells were grown in T255 culture flasks containing DMEM medium supplemented with 10% FBS. Upon reaching confluence, the cells were detached using Trypsin-EDTA solution.

TESTING VIABILITY OF CELLS

The viability of HepG-2 cells was assessed by trypan blue exclusion test Perry et al., (1997). 100 ul of trypan blue solution was mixed with 100 ul of cells contained in the medium and incubated for 5 min at 37oC. The cells were then washed thrice with saline and 10 ul of this suspension was placed in a haemocytometer and viewed under a microscope. The unstained cells represented the viable cells whereas the damaged cells were stained. The number of stained and unstained cells was counted and the percentage of viable cells was calculated using the formula:

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

The viability of the cells was found to be between 90-95%.

CELL PROLIFERATION ASSAY

The proliferation of HepG-2 cells was assessed by MTT assay Safadi et al., (2003). The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt. The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour (Figure-1). Then the cells are lysed using a 20% SDS solution, which releases the formazan crystal. These crystals are solubilized by DMF present in the solubilizer. The colour developed is then determined in an ELISA reader at 570 nm. HepG2 cells were plated in 24 well plates at a concentration of 5 x 104 cells/well 24 hours after plating, cells were washed twice with 500 µl of serum-free medium and starved by incubating the cells in serum-free medium for an hour at 37oC. After starvation, cells were treated with Cardiospermum halicacabum extract of different concentrations for 24 hours. At the end of treatment, the medium from control and Cardiospermum halicacabum extract treated cells were discarded and 500 µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4 h at 37°C in the CO2 incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS (1ml). The crystals were then dissolved by adding 500 µl of solubilization solution and this was mixed properly by pipetting up and down. Spectrophotometric absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm. The OD of each sample was then compared with the control OD and the graph was plotted.

Morphology Study:

Based on the MTT assay we selected the low and high doses of C. halicacabum for further studies. The characterisation of morphological changes in cells treated with C. halicacabum with IC-50 dose compared to their respective controls were observed under phase contrast microscope.

RESULTS:

ASSESSMENT OF ANTI- PROLIFERATIVE ACTIVITY:

Anti-proliferation of the HepG2 Cells was assessed by MTT assay for 24 h in aqueous extracts of C. halicacabum and the data are represented in Figure-1,2. The Graph shows that anti-proliferative activity was seen in the HepG-2 cells when treated with different concentrations (25, 50, 75, 100, 200 and 300 µg/ml) of aqueous extracts of C. halicacabum; the cell anti-proliferation being directly proportional to concentration. Statistical treatment of the data by one-way ANOVA showed that all the values were significant. Percent cell viability of HepG-2 cells was assessed for 24 h in the aqueous extracts of C.halicacabum at varying concentrations. The control cells were 100% viable and the viability decreased significantly with increase in concentration of the extracts. The percent decrease in cell viability was indirectly proportional to the concentration of aqueous extracts of C. halicacabum. ANOVA analysis revealed that all the values were significantly different. The IC50 value of cell viability of HepG-2 cells when treated with various extracts at different concentrations (25, 50, 75, 100, 200 and 300 µg/ml) proved that at the end of 24 h treatment itself, the IC50 values could be obtained. The IC50 value was 50 µg/ml in aqueous extract at 24 h incubation. From the results it is obviously clear that aqueous leaf

extract of C. halicacabum has profound effect in controlling HepG-2 cell proliferation even at a very low concentration.

CELL MORPHOLOGICAL ANALYSIS:

The morphological observations of HepG-2 cells were carried out in 20 X amplification using an inverted Phase contrast microscope (in Figure-3). The control HepG-2 cells showed irregular confluent aggregates with rounded and polygonal cell morphology. On the other hand, treatment of the cells with aqueous extract of Cardiospermum halicacabum (50µg/ml) for 24 h resulted in shrinkage of polygonal cells and appeared spherical in shape as a fiddle. The cell shrinkage expanded logically and it was dosage and time dependent.

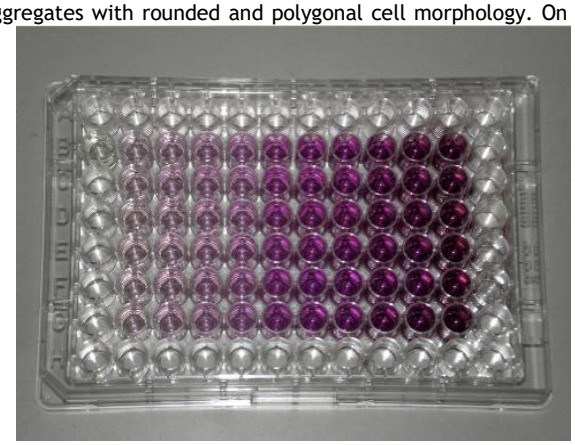

Figure -1: MTT assay in 96 well microtiter plates and colour developed is then determined in an ELISA reader (SpectraMax M5, Molecular Devices, USA) at 570 nm.

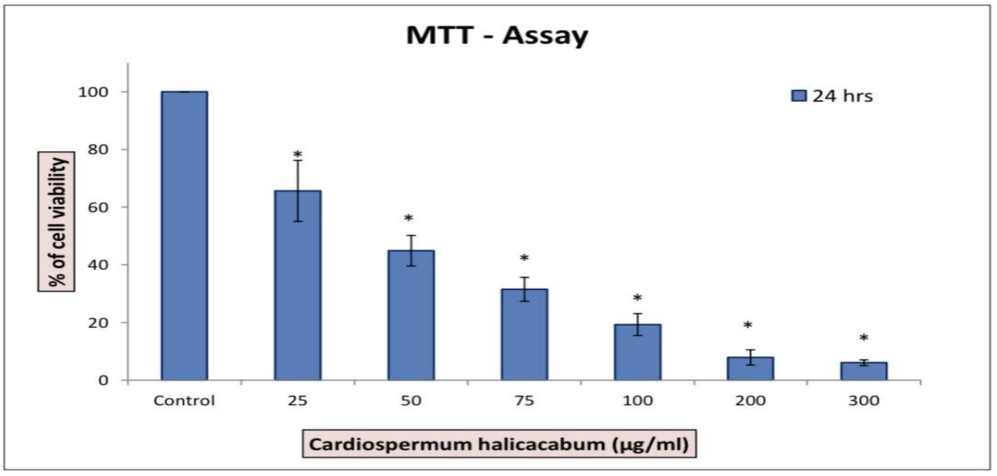

Figure-2: The anti proliferative activity of Cardiospermum halicacabum on HepG2 cells. Cells were treated with C. Halicacabum (25, 50, 75, 100, 200 and 300 µg/ml) for 24 h, and cell viability was evaluated by MTT assay. Data are shown as means ± SD (n = 3). * compared with the control-blank group, * represents statistical significance between control versus treatment group was $p<0.04$ at 50µg/ml, with $p<0.05$ level using Student's - Newman-Keuls test, statistically significant.

Control

Cardiospermum halicacabum

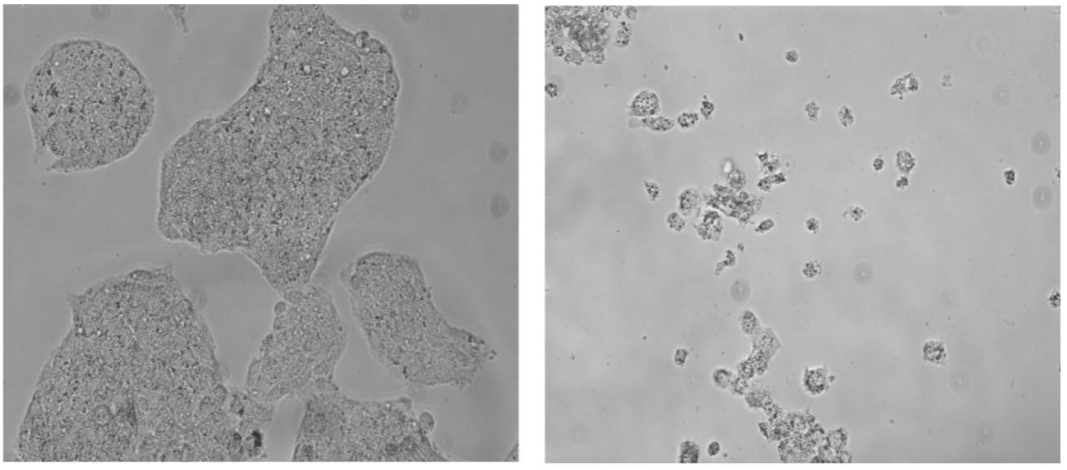

Figure-3: Assessment of cell morphology of HepG2 cells treated with and without C. halicacabum. Cells were treated with Cardiospermum halicacabum (50µg/ ml) for 24 h along with the control group. Images were obtained using an inverted phase contrast microscope.

DISCUSSION

The word "cancer" refers to the uncontrollable, engineered development of irregular cells in the body. It can happen anywhere in the body and disrupt the normal functioning of a cell [32]. Surgery, chemotherapy and radiation are available therapies for cancer. Chemotherapy and medication currently in use provide numbers of debilitating side effects. Medicinal plants both from land and sea, as well as bioactive compounds isolated from them, have been shown to be effective in the treatment of a variety of illnesses, including cancer [33] [34] [35] [36] [37] [38] [39] [40] [41] [42] [43] [44] [45] [46] [47]. Similar anti-proliferative activities of C. halicacabum have been reported on a variety of cancer cells with favourable results. According to Zhi-Zhong et al. (2016), the petroleum ether fraction, CHCl3 fraction, EtOAc fraction, n-BuOH fraction, and water fraction of P. niruri 75 percent EtOH extract proved antitumor activity against Chang-liver (HeLa) cells [48]. In a related analysis, the IC50 values of the methanol fraction of C. pentandra against HepG-2 cells were 14.895 g/mL [49]. Thus the reports of the above authors support the findings of this work. Recently, the preliminary phytochemical screening of various extracts of the Cardiospermum halicacabum L. was analyzed. Anticancer activity has been identified in a variety of active compounds, including flavonoids, diterpenoids, triterpenoids, and alkaloids. Due to the extreme nature of coumarin, quinones, saponins, steroids, and tannins, chloroform extracts of Cardiospermum halicacabum L. is selective against Ehrlich Ascites Carcinoma (EAC) cell line at high doses (mean IC50 = 31,384 g/ml) [50]. For prospective in vitro anticancer action, methanol extract had an IC50 value of 24.90 g/ml, while aqueous and chloroform extracts had IC50 values of 43.51 g/ml and 40.34 g/ml, respectively. Overall, methanol extracts are more active in MCF-7 breast cancer cells than aqueous and chloroform extracts [51]. This clearly indicates that the secondary metabolites from Methanol extract of C. halicacabum have contributed to the anticancer activity. In contrast to previous literature, chloroform and ethanolic extracts has potent cytotoxicity and maximum cytotoxicity 65.11% and 59.56%was found at the concentration of 200µg/ml and the IC50 values were found to be 140µg/ml and 150µg/ml respectively [52], when compared plant drugs to the positive control, the test compound's cytotoxicity levels were as ineffective as cyclophosphamide, but the compound concentrations had strong cytotoxicity ability [53]. Moreover, the American National Cancer Institute defines a potent cytotoxic extract as an anticancer agent if the IC50 value is less than 30 mg/mL [54]. Data from our MTT assay showed that aqueous extracts of C. halicacabum have better IC50 values. Therefore, our study revealed that the aqueous extract can be categorized to have strong cytotoxic activity. Hence furthermore investigations with higher concentrations and use of this product as an adjuvant with other compounds as adjuvant can yield better results.

CONCLUSION

Based on the results, we conclude that aqueous extract of C. halicacabum has confirmed hopeful antiproliferative activity against human hepatocellular carcinoma (HepG-2) cells. Thus the current study supports the ethnobotanical claim of C. halicacabum for the treatment of cancer. The leaves of C. halicacabum prove that they have dose dependent antiproliferative activity. In further investigations, to assess its isolates of pure bio active compounds and find out the effectiveness of the C. halicacabum to serve as natural chemotherapeutic agent.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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