ANTI-CANCER ACTIVITY OF CARDIOSPERMUM HALICACABUM LINN. LEAF EXTRACTS AGAINST HEPATOCELLULAR CARCINOMA CELL LINE (Hep-G2)

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ABSTRACT
Cancer is most dangerous diseases in the world. The objective of the research is to explore the phyto-constituents present in the aqueous, chloroform and methanol extracts of C. halicacabum leaves. To investigate the anti-cancer potential of the all three leaf extracts of C. halicacabum against Hep-G2 cell line. To identify the apoptotic effect of leaf extracts against Hep-G2 cell line by propidium iodide staining method. GC-MS study of the leaf extract having higher activity.

KEYWORDS: C. halicacabum, phytochemical screening, MTT assay, propidium iodide staining, GC-MS, Hep G-2 Cell line.

INTRODUCTION
Cancer is manifestation of critical alteration in cell physiology that results in uncontrolled malignant growth. It is a dynamic process that involves many complex factors that causes extensive morbidity and wide mortality in the human population and the costs to society of this dreadful disease are prodigious.[1] Cancer has become an important issue in medicine as it is a major cause of death in both the developed and developing countries and it is now well thought-out as second to myocardial infarction.[2] Hepatocellular carcinoma (HCC), a primary liver cancer, is one of the most frequent tumours responding the fifth commonest malignancy worldwide and the third cause of mortality from cancer.[3-27] The causes of HCC include HBV or HCV infection, alcohol intake, smoking and aflatoxin.[28] HCC is a complex process associated with accumulation of genetic and epigenetic changes, and it is accompanied by
increased expression of several growth factors that enhance cell survival by suppressing apoptosis and increasing elements entering the cell cycle.\[27\]

Crude drugs have been replaced by pure chemical drugs and the developed countries have experienced a decline in popularity of medicinal plant therapy.\[11\-17\] The name Cardiospermum halicacabum Linn is of Greek origin kardia “heart” and sperma “a seed” referring to the shape of the seed or to a heart shaped spot on the seeds and halikkabos.\[11\] C. halicacabum L., known as the Balloon plant or Love in a puff, which is a climbing plant widely distributed in tropical and subtropical Africa and Asia. In rural south India, this plant has been harvested and sold in urban and local market as green vegetable providing a source of revenue for low income families.\[29\] The whole plant has been used for several centuries in the treatment of rheumatisms, stiffness of limbs, snake bite, etc; its roots for nervous diseases, as a diaphoretic, diuretic, emetic, laxative, refrigerant, stomachic and sudorific; its leaves and stalks are used in the treatments of diarrhoea, dysentery, and headache and as a poultice for swellings.\[30\] The leaf juice has been used as treatment of ear ache.\[31\]

The present study aims to explore the phytoconstituents present in the aqueous, chloroform and methanol extracts of leaves C. halicacabum and to investigate the anti-cancer potential of the all three leaf extracts of C. halicacabum against cell line and to identify the apoptotic effect of solvent leaf extracts against cell by propidium iodide staining method.

**MATERIALS AND METHODS**

**Collection and Identification of Cardiospermum halicacabum**

Aerial parts of C. halicacabum were collected from Kannalam village, Gingee Taluk, Vilupuram District, Tamil Nadu, India. Plant material was identified and authenticated by examination of the morphological characteristics by a Botanist Dr. R. Pandikumar, Scientist, Entomology Research Institute (ERI), Loyola College, Chennai-600 034, Tamil Nadu.

**Preparation of the leaf extracts**

**Aqueous extraction**

After collecting, the leaves were separated and shade dried and 20 g of dry leaves were crushed to powder with a mortar and pestle. A suspension of 5\% (w/v) was prepared in a flask by adding hot boiled distilled water and kept in a shaker at 200 rpm for 4 hrs at 37°C. After being shaken, the suspension was brought to room temperature. The suspension was then filtered through four layers of No.1 Whatman filter paper and finally passed through
0.22 µm filter (Millipore, Billerica). The filtered aqueous extract was freeze-dried and powder was stored at -20°C until further use. For cell culture studies, 10 g of the powder was taken and dissolved in DMEM culture medium.

**Chloroform and Methanol Extraction**

Similarly for chloroform and methanol extracts preparation, shade dried leaves were crushed to powder with a mortar and pestle. The powdered sample (5% w/v) was soaked in their respective solvents and kept for 4 hrs on a shaker, filtered and evaporated at room temperature in petri dishes. The dried material was retrieved and stored in tubes at -20°C until further experiments. Then each extracts were dissolved separately in Dimethyl Sulfoxide (DMSO) to prepare (10 mg/ml) stock solution, and mixed with the DMEM culture Media to achieve the desired concentration.

**Qualitative Phytochemical Screening**

All the three extracts were subjected to preliminary phytochemical screening for its qualitative phytoconstituents according to Kokate method.[32]

**Quantitative Estimations**

Carbohydrate[33], Protein[34] and total flavonoid content (TFC) were determined (Aluminum Chloride Colorimetric Method) quantitatively in all the three leaf extracts.

**Screening of Drug for Antiproliferation Activity using MTT method**

The antiproliferative effect was by assessed by MTT (3-(4,5-dimethylthiazol- 2yl)-2, 5-diphenyltetrazolium bromide) method.[35]

**Propidium Iodide Staining**

Hep-G2 cells were plated at 5 × 10⁴ cells/well into a six chamber plate. At >90% confluence, the cells were treated with extracts for 24 and 48 hrs. The cells were washed with PBS fixed in methanol : acetic acid (3:1, v/v) for 10 min and stained with 50 µg/ml propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined under confocal microscope[37]

**GC-MS Spectral analysis**

Best antitumor activity was observed in methanol extract when compared to other extracts of C. halicacabum and finally the phytoconstituents of methanol extract with its structure was assessed by GC-MS chromatogram.
GC-MS analysis of methanolic extract of *C. halicacabum* was performed using GC-MS instrument (GCMS-QP-2010) equipped with glass column SGE BPX5 and capillary dimension 30 m x 0.25 mm x 0.25 μ. The oven temperature was programmed from 80-260°C. Inlet and interface temperature were 250°C and 200°C, respectively. Carrier gas was helium at a flow rate of 1.0 ml/min. Ion source temperatures were maintained at 200°C and spectra were measured. GC-MS spectral analysis was done at Sophisticated Analytical Instrument Facility, Indian Institute of Technology Madras, Chennai, India.

**Statistical Analysis**
Data obtained in the MTT assay were subjected to statistical analysis and the mean value along with the standard error for five individual observations were calculated for each parameter and presented in appropriate tables in the text. The significance of the sample mean between various extracts and the concentrations of each extract was tested using Two Way ANOVA. The analytical data together with Tables and bar diagrams are presented in appropriate places in the text.

**RESULTS**
Traditionally *C. halicacabum* is highly useful in Ayurvedha, Siddha, Homeopathic and Unani system of Indian medicines to treat rheumatoid arthritis, gastrointestinal diseases, respiratory diseases, urinogenital diseases, inflammatory diseases in India and China. The use of this plant is enormous, where each and every part of this plant is useful, and people are using this plant in day-to-day life as medicine or as vegetable all over the world. Aqueous, chloroform and methanol extracts of *C. halicacabum* leaves, resembled a dark brown coloured paste and the powder is highly soluble in water, chloroform and methanol. Active metabolites of plants contribute for the widely varying physiological functions inhibited by them.

Table 1 represents the preliminary phytochemical analysis of all the three extracts of *C. halicacabum*. The analysis showed the presence of carbohydrates, quinone, saponin and tannin in methanol extract, triterpenoids, carbohydrates, coumarin and saponin in chloroform extract and triterpenoids, flavonoids and acids in aqueous extract. Likewise the quantitative estimation of protein, carbohydrate and flavonoid content are given in Table 2. The data reveals that protein and carbohydrate contents were high in chloroform extract, while flavonoid content was high in aqueous extract.
For morphological observation of Hep-G2 cells were photographed. The control cells showed irregular confluent aggregates with rounded and polygonal cell morphology. But in the cells treated with aqueous, chloroform and methanol extracts of *C. halicacabum*, after 24 and 48 hrs of incubation, the appearance of polygonal cells began to shrink to spherical in shape (Fig 1. A-D) and the cell shrinkage increased progressively; the increase being dose and time dependent. The shrinkage was high in methanol extract than that of the other extracts.

Per cent cell viability was also observed for 24 hrs in all the three leaf extracts at varying concentrations (Table 3). The control cells were 100% viable in all the three extracts. In the case of aqueous, chloroform and methanol extract, the viability decreased significantly with increase in concentration; the per cent decrease being indirectly proportional to the concentration of the extracts. The data altogether indicates that methanol extract showed higher activity leading to decrease in per cent cell viability. When the data were subjected to two-way ANOVA, all the values were significantly different among the concentrations and among the extracts (Table 4).

Per cent cell viability was also assessed for 48 hrs in all the three leaf extracts at varying concentrations (Table 5). The control cells were 100% viable in all the three extracts. In the case of aqueous, chloroform and methanol extracts, the viability decreased significantly with increase in concentration; the per cent decrease being indirectly proportional to the concentration of the extracts. The data altogether indicates that aqueous extract showed higher activity leading to decrease in per cent cell viability at 125 µg/ml. Two-way ANOVA revealed that all the values were significantly different among the concentrations and among the extracts (Table 6).

The inhibiting concentration (IC₅₀) value was 50% of viable cells in 121.25 µg/ml of methanol extract, at the end of 24 hrs. On the other hand, in aqueous and chloroform extracts, even at 125µg/ml, the IC₅₀ value could not be achieved. From the results it is obviously known that methanol extract has profound effect in controlling Hep-G2 cell proliferation. Likewise, at the end of 48 hrs, the IC₅₀ value was observed in all of the three extracts; the trend being methanol extract (23.42 µg/ml) > aqueous extract (24.1 µg/ml) > chloroform extract (31.96 µg/ml) (Table 7). The data altogether depicts that methanol extract is significantly controlling cell proliferation of Hep-G2 cells even at lower concentration. The data are presented in Fig. 2 and Fig. 3.
To confirm whether the cytotoxic effect induced by leaf extracts of C. halicacabum involves apoptotic changes, the nuclear condensation was studied by the propidium iodide staining method. The photos are presented in Fig. 4. In the case of control cells, a very negligible number of propidium iodide positive cells were present. In the case of cells treated with 20µg/ml of leaf extracts for 48 hrs, a progressive increase in the number of propidium iodide positive cells were observed.

GC-MS Spectra of methanol extract of C. halicacabum contains many active compounds such as 1,2,4- Trioxolane-2-octanic acid, 5-octyl-methyl ester (RT-15.05), Ethanol, 2-[9-octadecenyl]oxy], 1,2,4- Trioxolane -2-octanic acid, 5-octyl methyl ester (RT-17.23), Ricinolenic acid (RT-18.67), [1,1-Bicyclopropyl]-2-octanic acid, 2-hexyl-methyl ester (RT-18.90), 11-octadecenoic acid, methyl ester (RT-18.97), 7-methyl-7-tetradecan-1-ol acetate (RT-19.12), Oleic acid (RT-19.75), 9-Octadecenoic acid,1,2,3-propanetriyl ester, [E,E,E]- (RT-25.88). The peaks are given in Fig. 5(A-K).

Table 1: Phytochemical screening of aqueous, chloroform and methanol leaf extracts of C. halicacabum.

<table>
<thead>
<tr>
<th>Primary Phytochemical test</th>
<th>Methanol Extract</th>
<th>Chloroform Extract</th>
<th>Aqueous Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triterpenoid</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Coumarin</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Quinone</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Protein</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Present     - Absent

Table 2: Quantitative estimation of phyto-constituents of aqueous, chloroform and methanol leaf extracts of C. halicacabum.

<table>
<thead>
<tr>
<th>Test</th>
<th>Methanol Extract (mg/g)</th>
<th>Chloroform Extract (mg/g)</th>
<th>Aqueous Extract (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>12.0</td>
<td>18.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>21.0</td>
<td>33.0</td>
<td>15.5</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>19.4</td>
<td>24.2</td>
<td>32.5</td>
</tr>
</tbody>
</table>
Table 3: Per cent cell viability of Hep-G2 cells for 24 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

<table>
<thead>
<tr>
<th>Concentration of Extract</th>
<th>Aqueous</th>
<th>Chloroform</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0µg/ml)</td>
<td>100.00 ± 0.000</td>
<td>100.00 ± 0.000</td>
<td>100.00 ± 0.000</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>96.198 ± 0.640</td>
<td>87.924 ± 0.742</td>
<td>88.854 ± 0.435</td>
</tr>
<tr>
<td></td>
<td>(-3.81)</td>
<td>(-12.08)</td>
<td>(-11.15)</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>90.404 ± 0.619</td>
<td>82.032 ± 0.405</td>
<td>78.430 ± 0.531</td>
</tr>
<tr>
<td></td>
<td>(-9.60)</td>
<td>(-17.97)</td>
<td>(-21.57)</td>
</tr>
<tr>
<td>75 µg/ml</td>
<td>84.246 ± 1.993</td>
<td>77.566 ± 0.523</td>
<td>69.280 ± 1.463</td>
</tr>
<tr>
<td></td>
<td>(-15.76)</td>
<td>(-22.44)</td>
<td>(-30.72)</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>77.622 ± 1.081</td>
<td>74.050 ± 0.882</td>
<td>56.390 ± 0.568</td>
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<tr>
<td></td>
<td>(-22.38)</td>
<td>(-25.97)</td>
<td>(-43.61)</td>
</tr>
<tr>
<td>125 µg/ml</td>
<td>67.848 ± 0.788</td>
<td>71.124 ± 0.244</td>
<td>48.870 ± 0.708</td>
</tr>
<tr>
<td></td>
<td>(-32.16)</td>
<td>(-28.88)</td>
<td>(-51.13)</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of five individual observations.

Values in parentheses are per cent change over control.

- Denotes per cent decrease over control.

Table 4: Two-way ANOVA for per cent cell viability of Hep-G2 cells for 24 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

<table>
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<tr>
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<tr>
<td></td>
<td><strong>(3.81)</strong></td>
<td><strong>(12.08)</strong></td>
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</tbody>
</table>

Values are significant at F<0.05

Table 5: Per cent cell viability of Hep-G2 cells for 48 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

<table>
<thead>
<tr>
<th>Concentration of Extract</th>
<th>Aqueous</th>
<th>Chloroform</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
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<td>100.00 ± 0.000</td>
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<td>100.00 ± 0.000</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>48.132 ± 6.285</td>
<td>51.492 ± 0.742</td>
<td>46.184 ± 0.410</td>
</tr>
<tr>
<td></td>
<td>(-51.87)</td>
<td>(-48.51)</td>
<td>(-53.39)</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>42.852 ± 0.217</td>
<td>46.166 ± 2.203</td>
<td>34.366 ± 0.666</td>
</tr>
<tr>
<td></td>
<td>(-57.15)</td>
<td>(-53.84)</td>
<td>(-65.64)</td>
</tr>
<tr>
<td>75 µg/ml</td>
<td>33.290 ± 0.715</td>
<td>33.876 ± 0.746</td>
<td>30.034 ± 0.997</td>
</tr>
<tr>
<td></td>
<td>(-66.71)</td>
<td>(-66.13)</td>
<td>(-69.97)</td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>28.446 ± 0.463</td>
<td>31.238 ± 0.766</td>
<td>21.284 ± 0.190</td>
</tr>
<tr>
<td></td>
<td>(-71.56)</td>
<td>(-68.77)</td>
<td>(-78.72)</td>
</tr>
<tr>
<td>125 µg/ml</td>
<td>24.888 ± 0.451</td>
<td>27.040 ± 1.154</td>
<td>15.964 ± 1.176</td>
</tr>
<tr>
<td></td>
<td>(-75.12)</td>
<td>(-72.96)</td>
<td>(-84.04)</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of five individual observations.
Values in parentheses are per cent change over control.
- Denotes per cent decrease over control.

Table 6: Two-way ANOVA for per cent cell viability of Hep-G2 cells for 48 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

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<th>Methanol</th>
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</tr>
<tr>
<td>25 µg/ml</td>
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<td>51.492 ± 0.755***</td>
<td>46.618 ± 0.410***</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>42.852 ± 0.217***</td>
<td>46.166 ± 2.203***</td>
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<td>24.888 ± 0.451***</td>
<td>27.040 ± 1.154***</td>
<td>15.964 ± 1.176***</td>
</tr>
</tbody>
</table>

Values are significant at F<0.05

Table 7: IC$_{50}$ Value of Hep-G2 cells when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

<table>
<thead>
<tr>
<th>Time Taken</th>
<th>Aqueous</th>
<th>Chloroform</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>24hrs</td>
<td>170.61 µg/ml</td>
<td>305.24 µg/ml</td>
<td>121.25 µg/ml</td>
</tr>
<tr>
<td>48hrs</td>
<td>24.10 µg/ml</td>
<td>31.96 µg/ml</td>
<td>23.42 µg/ml</td>
</tr>
</tbody>
</table>

Fig. 1: Photomicrographs of Hep-G2 cells
A: Control cells  
B: Aqueous extract treated cells  
C: Chloroform extract treated cells  
D: Methanol extract treated cells

Fig. 2: Bar diagram showing decrease in per cent cell viability of Hep-G2 cells for 24 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

Fig. 3: Bar diagram showing decrease in per cent cell viability of Hep-G2 cells for 48 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.
Fig. 4: Propidium iodide staining of Hep-G2 cells in both control and treated with C. halicacabum leaf extracts.
A – Control                     B – Aqueous
C – Chloroform                 D – Methanol

Fig. 5: GC-MS Chromatogram of C. halicacabum methanol extract
Mass peak: 15.05

Fig. 5A: GC-MS spectra of methanol extract of *C. halicacabum*

Mass peak: 16.25

Fig. 5B: GC-MS spectra of methanol extract of *C. halicacabum*
Mass peak: 17.23

Fig. 5C: GC-MS spectra of methanol extract of *C. halicacabum*

Mass peak: 18.67

Fig. 5D: GC-MS spectra of methanol extract of *C. halicacabum*
Mass peak: 18.90

**Fig. 5E**: GC-MS spectra of methanol extract of *C. halicacabum*

Mass peak: 18.97

**Fig. 5F**: GC-MS spectra of methanol extract of *C. halicacabum*
Mass peak: 19.12

Fig. 5G: GC-MS spectra of methanol extract of *C. halicacabum*

Mass peak: 19.75

Fig. 5H: GC-MS spectra of methanol extract of *C. halicacabum*
Mass peak: 20.8

Fig. 5I: GC-MS spectra of methanol extract of *C. halicacabum*

Mass peak: 25.88

Fig. 5J: GC-MS spectra of methanol extract of *C. halicacabum*
Mass peak: 28.13

Fig. 5K: GC-MS spectra of methanol extract of *C. halicacabum*

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display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci USA, 2009; 106: 13820-13825.


