EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 10
213-222
Year: 2016

ANTI-CANCER ACTIVITY OF *CARDIOSPERMUM HALICACABUM* LINN. LEAF EXTRACTS AGAINST HUMAN BREAST CANCER CELL LINE (MCF-7)

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Article Received on 27/07/2016 **Article Revised on 16/08/2016** **Article Accepted on 05/09/2016**

ABSTRACT
Objective: The objective of the research to explore the phytoconstituents presents in the aqueous, chloroform and methanol extracts of leaf of *C. halicacabum.* To investigate the anti-cancer potential of the all three leaf extracts of *C. halicacabum* against MCF-7 cell line. GC-MS study of the solvent leaf extract having higher activity. **Methods:** The leaves of *Cardiospermum halicacabum* were extracted using water, chloroform and methanol. The extracts were subjected to preliminary phytochemical analysis; the anticancer activity was evaluated by MTT assay method and GC-MS spectral analysis was carried out for the extract showing best antiproliferative activity. **Results:** Methanol extract gave IC_{50} value of 24.90 µg/ml, whereas aqueous and chloroform extracts gave IC_{50} value of 43.51 µg/ml and 40.34 µg/ml respectively for potential *in vitro* anticancer activity. The GC-MS spectra of methanol extract showed 14 peaks. **Conclusion:** From the results, the present study indicates the anti-cancer potential of*C. halicacabum* leaves and further experiments awaits the characterization of active principle responsible for anti-cancer property.

KEYWORDS: *C. halicacabum*, MCF-7 cell line and GC-MS.

INDRODUCTION
Cancer as a second cause of death after heart disease in the world has posed a great challenge to the field of medicine and immunology.[1] The first description of cancer is found in an Egyptian papyrus which dates back to approximately 1600 BC. It was regarded as an incurable disease until the nineteenth century, before 1950, surgery was most preferred means of treatment. After 1960, radiation therapy started being used to control local disease.[2] Cancer is a multifactorial, multifaceted and multi-mechanistic disease requiring a multidimensional approach for its treatment, control and prevention.[3] The major causes of cancer are smoking, dietary imbalances, hormones and chronic infections leading to chronic inflammation.[4] Age is also a primary risk factor for most cancers, with about 77% of all cancers diagnosed among people aged 55 or older.[5]

Conventional cancer treatments have many modalities, all directed at killing tumour cells or preventing their proliferation. The most common approach is chemotherapy, which is not selective toward tumour cell, but damaging the normal cells too Immunosuppression is a major drawback of chemotherapy.[6-12] Crude drugs have been replaced by pure chemical drugs and the developed countries have experienced a decline in popularity of medicinal plant therapy.[13-19]

The first breast cancer cell line to be established was BT-20 in 1958.[20] It took another 20 years, however, before establishing breast cancer cell lines became more widespread, including the MD Anderson series.[21] and what still remains the most commonly used breast cancer cell line in the world, MCF-7 established in 1973 at the Michigan Cancer Foundation.[22] Breast cancer is the third most common cause of cancer deaths worldwide and is the most common form of cancer in women.[23, 24] The prevalence of breast cancer in Indian women is more at the age of forty.[25] The incidence of breast cancer has been increasing worldwide for many decades[26] with Asian countries attaining highest incidence rate.[27] Some breast tumors stay resistant to conventional treatment [28, 29] and may have many side effects which affect the quality of the treatment.[30] Skirmishing with such a dreadful disease as a treatment must be considered with high importance. Surgical treatments are in need by the specialized proficient surgeons in the area of surgical oncology.[31, 32] Existing radiation oncology infrastructure is not sufficient for the most developing countries.[33] According to the data of World Health Organization, chemotherapy is needed for more than 90% of people affected with breast cancer.

Plants are used medicinally in various countries and are a principle of numerous potential and powerful remedies.

India is endowed with luxuriant wealth of medicinal plants which are extensively used by all sections of people either directly as folk drugs or in many indigenous systems of medicine or indirectly in pharmaceutical preparations of novel medicines [34]. According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their main health care demands. Traditional herbal remedies are regarded as safe, cost effective, easily affordable with no adverse side effects and due to this fact worldwide need for herbal drugs is continuously increasing and in India its market is expanding at an annual rate of 20 percentages.[35, 36]

Cardiospermum halicacabum L. is one of the most important medicinal plants used in traditional ayurvedic system of medicine in several parts of India for the treatment of rheumatoid arthritis. It belongs to family-Sapindaceae and widely distributed in tropical and sub-tropical America, Africa, and Asia. Alternatively, it has been used for the treatment of nervous diseases, reduce hardened tumors, asthma, as a demulcent in orchitis and in dropsy. Leaves are emetic, stimulant and their decoctions are given for the treatment of piles, diarrhoea, as an infusion for general sores and to reduce obesity.[37] *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.[38]

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.[39] The leaf juice has been used as treatment of earache.[40]

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 the 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 extractions

Similarly for chloroform and methanol extract preparation, shade dried leaves were crushed to powder with a mortar and pestle. The powdered sample (5 % w/v) was soaked in 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. The chloroform and methanol extracts were dissolved in Dimethyl Sulfoxide (DMSO) to prepare (10 mg/ml) stock solution, and mixed with the DMEM culture media to achieve the desired concentration.

Preliminary Phytochemical Screening

All the three extracts were subjected to preliminary phytochemical screening for its phytoconstituents according to Kokate method.[41]

Quantitative Estimations

Estimation of Carbohydrates

Carbohydrate estimation[42], Protein estimation[43], total flavonoid content (TFC) extracts was determined using Aluminum Chloride Colorimetric Method.

Screening of Drug for Anti-proliferative Effect using MTT Assay

The antiproliferative effect was by assessed by MTT (3-(4, 5-dimethylthiazol- 2yl)-2, 5- diphenyltetrazolium bromide) assay.[44]

Statistical Analysis

Data obtained in the MTT assay were subjected to standard 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.[45] The analytical data are presented in appropriate places in the text.

GC-MS Spectral analysis

Best antitumor activity was observed in methanol extract when compared to other extracts of *C. halicacabum*. Hence the phytoconstituents of methanol extract with its structure was assessed by GC-MS chromatogram.

GC-MS analysis was performed using GC-MS instrument (GC-MS-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.

RESULTS

Preliminary phytochemical qualitative analysis and quantitative estimation of all the three extracts of *C. halicacabum*; the results being published by Rajesh *et al*.[46] For morphological observation of MCF-7 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.

The percent cell viability was also observed for 24 hrs in all the three leaf extracts at varying concentrations (Table 1). The control cells were 100% viable in all the three extracts. In the case of experimental cells, all the three extracts showed a significant decrease in the viability with increase in concentration; the percent decrease being indirectly proportional to the concentration of the extracts. The data altogether indicates that methanol extract showed higher activity leading to decrease in percent cell viability. When the data were subjected to two-way ANOVA, all the values were significantly different among the concentrations and among the extracts.

The percent cell viability was also assessed for 48 hrs in all the three leaf extracts at varying concentrations (Table 1). 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 decrease being indirectly proportional to the concentration of the extracts. The data altogether indicates that aqueous extract showed higher activity leading to decrease in percent cell viability at 125 µg/ml. Two-way ANOVA revealed that all the values were significantly different among the concentrations and among the extracts.

The inhibiting concentration (IC_{50}) value was 123.68 µg/ml of methanol extract, at the end of 24 hrs. On the other hand, in aqueous and chloroform extracts, even at 125 µg/ml, the IC_{50} value could not be achieved. From the results it is obviously known that methanol extract has profound effect in controlling MCF-7 cell proliferation. Likewise, at the end of 48 hrs, the IC_{50} value was observed in all of the three extracts; the trend being methanol extract (24.50 µg/ml) > chloroform extract (40.34 µg/ml) > aqueous extract (43.51 µg/ml) (Table 7). The data altogether depicts that methanol extract is effective in controlling cell proliferation of Mcf-7 cells even at lower concentration. The data are presented in Fig. 2 and Fig. 3.

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-octadecenyloxyl], 1,2,4-Trioxolane -2-octanic acid, 5-octyl methyl ester (RT-17.23), Ricinolenic acid (RT-18.67), [1,1-Bicylopropyl]-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 presented in Rajesh *et al*.[46]

Table 1: Per cent cell viability of Mcf-7 cells for 24 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

Concentration of Extract	Aqueous	Chloroform	Methanol
Control (0µg/ml)	100.00 ± 0.000	100.00 ± 0.000	100.00 ± 0.000
25 µg/ml	91.66 ± 0.64* (-8.35)	86.51 ± 0.74* (-13.50)	86.13 ± 0.86* (-13.88)
50 µg/ml	87.63 ± 0.86* (-12.38)	80.89 ± 1.15* (-19.13)	76.74 ± 0.44* (-23.26)
75 µg/ml	82.01 ± 0.97* (-17.99)	73.25± 0.94* (-26.75)	68.59 ± 1.10* (-31.41)
100 µg/ml	73.84 ± 0.93* (-26.16)	69.10 ± 0.97* (-30.91)	56.61 ± 0.49* (-43.40)
125 µg/ml	67.71 ± 0.63* (-32.30)	66.36 ± 1.21* (-33.64)	49.63 ± 0.94* (-50.37)

Values are mean ± S.E. of five individual observations.
Values in parentheses are per cent change over control.
- Denotes per cent decrease over control.
Values are significant at F<0.05

Table 2: Per cent cell viability of Mcf-7 cells for 48 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

Concentration of Extract	Aqueous	Chloroform	Methanol
Control (0µg/ml)	100.00 ± 0.000	100.00 ± 0.000	100.00 ± 0.000
25 µg/ml	60.65 ± 1.73 (-39.35)	56.59 ± 1.43 (-43.41)	49.78 ± 0.54 (-50.22)
50 µg/ml	46.26 ± 0.86 (-53.74)	45.85 ± 0.95 (-54.15)	39.18 ± 1.01 (-60.82)
75 µg/ml	41.09 ± 0.85 (-58.91)	36.62 ± 0.72 (-63.38)	30.99 ± 0.53 (-69.01)
100 µg/ml	36.98 ± 0.75 (-63.02)	30.84 ± 0.92 (-69.16)	21.90 ± 0.76 (-78.10)
125 µg/ml	26.65 ± 0.58 (-73.35)	25.48 ± 0.74 (-74.52)	15.06 ± 0.57 (-84.94)

Values are mean ± S.E. of five individual observations.
Values in parentheses are per cent change over control.
- Denotes per cent decrease over control.
Values are significant at F<0.05

Table 3: IC_{50} Value of Mcf-7 cells when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

Time Taken	Aqueous	Chloroform	Methanol
24hrs	197.07 µg/ml	274.82 µg/ml	123.68 µg/ml
48hrs	43.51 µg/ml	40.34 µg/ml	24.90 µg/ml

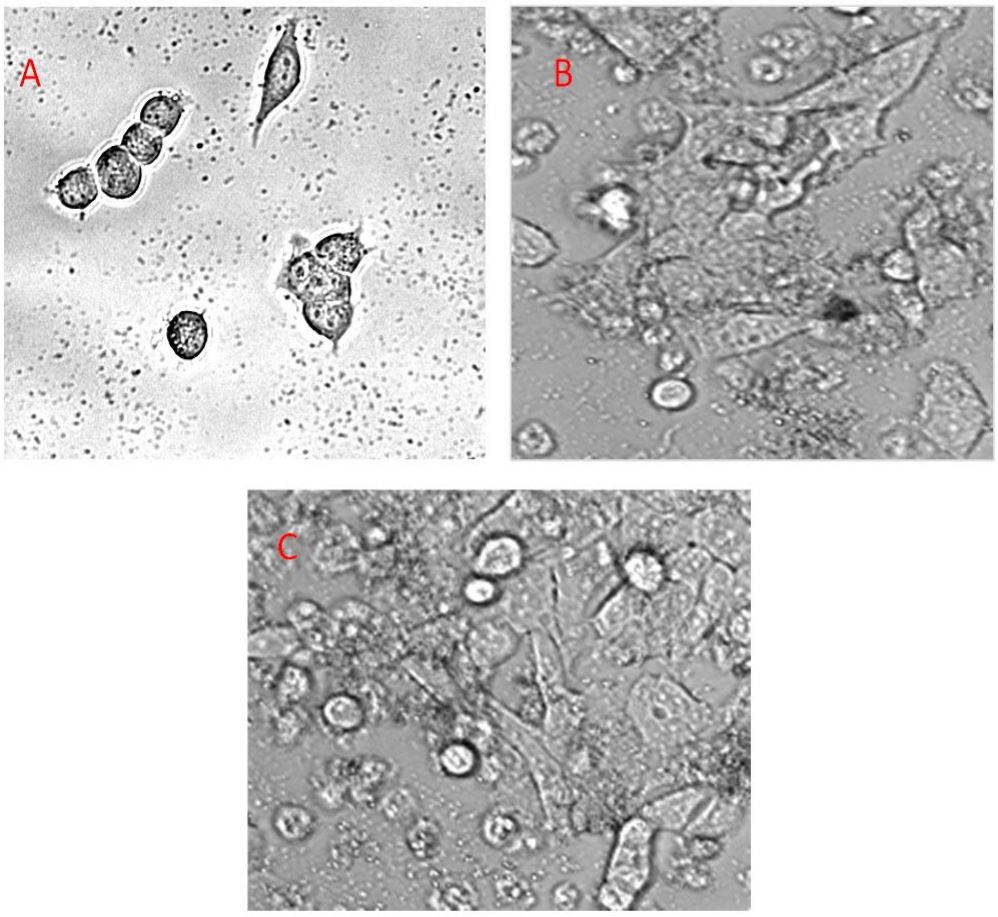

Fig. 1: Photomicrographs of MCF-7 cells

A: Control MCF-7 cells
B: Aqueous extract treated cells (IC_{50} concentration)
C: Chloroform extract treated cells (IC_{50} concentration)
D: Methanol extract treated cells (IC_{50} concentration)

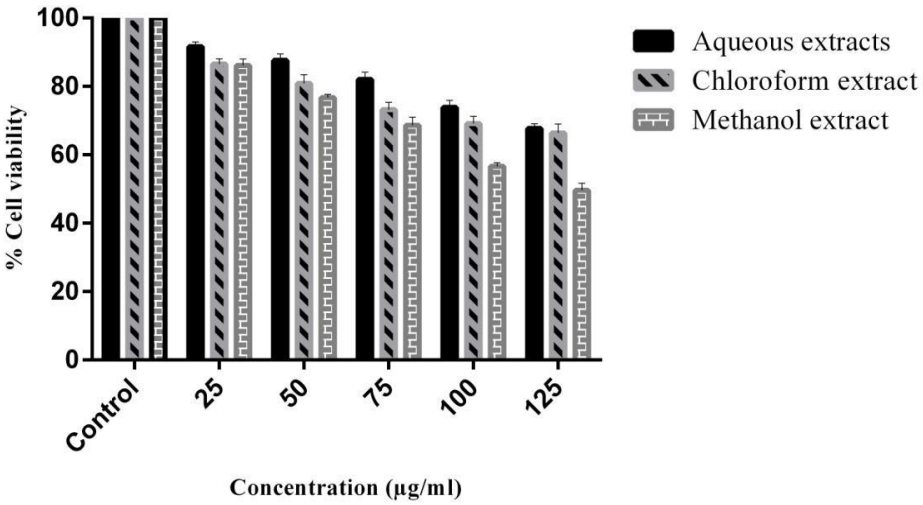

Fig. 2: Bar diagram showing decrease in per cent cell viability of MCF-7 cells for 24 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

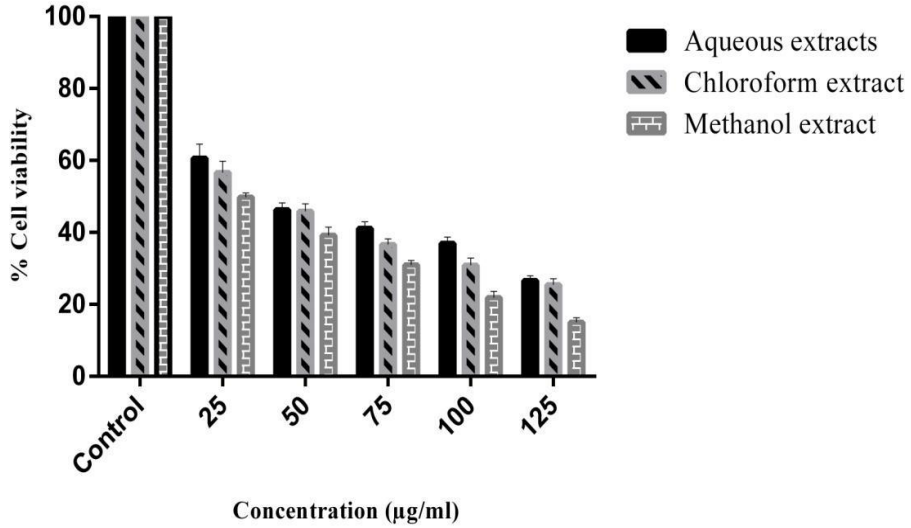

Fig. 3: Bar diagram showing decrease in per cent cell viability of MCF-7 cells for 48 hrs when treated with aqueous, chloroform and methanol leaf extracts of *C. halicacabum*.

DISCUSSION

Plants have been used as medicines for thousands of years and are used today in their genuine as well as processed from several medicinal plants which have been forgotten by modern man as a result of his dependence on the speedy results of allopathic awareness and are being rediscovered because of growing awareness of unwanted side effects and other aspects of the later.[47] Plants have always the source of drugs and have several uses to mankind. According to some previous researchers[48-53] plants have been used in traditional medicine for many thousand years.[54] They are also used as food supplements by nutraceuticals, pharmaceutical industries and chemical entities for preparing synthetic drugs from these plants.[55] Modern medicine has evolved from folk medicine and traditional system, only after thorough chemical and pharmaceutical screening.[56, 57] Herbal medicines have become more popular in the treatment of many diseases due to popular belief that green medicine is safe, easily available and with fewer side effects. Indeed, the market and public demand has been increasing, so that there is a great risk that many

medicinal plants today, face either extinction or loss of genetic diversity.[58]

Medicinal plants are a source of great economic value in the Indian subcontinent. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country.[59] Crude drugs have been replaced by pure chemical drugs and the developed countries have experienced a decline in popularity of medicinal plant therapy. The modern medicinal system has grown phenomenally as manifested by global pharmaceutical scales which have increased to 7% in 2006 fuelled by strong international demand for cancer treatments and robust growth in US market.

In the current study, *Cardiospermum* genus from Sapindaceae family is considered. *C. halicacabum* used in the treatment of rheumatism, cough, hyperthermia, lumbago, nervous illness and amenorrhea.[16] The whole plant is diaphoretic, diuretic[60], emmenagogic[17] and laxative.[19] The leaf juice has been used for treatment of earache.[40] It is also diuretic, stomachic and rubefacient.[61-63] Although traditional knowledge is available on this vine for its medicinal usage[64], very little attention has been paid by scientists and pharmacologists towards understanding the bioactive principle present in this weed.[65-68] The whole plant of *C. halicacabum* contains saponins, traces of alkaloids, flavonoids, proanthocyanidin, apigenin and phytosterols.[69] The leaves of plant contain β- sitosterol, D- glucose, oxalic acid, (+) pinitol, 7-o-glucuronides of apigenin, chrysoeriol and luteolin. The root portion contains β- sitosterol, phlobaphene, phloba-tanin and pro anthocyanidine. The seed portion contains fixed oil, fatty acid. Extract of this plant have been reported to contain different triterpenoids, glycosides, and a range of fatty acids.[70-72]

In the present investigation, the photochemical and quantitative estimation of the leaf extract 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, thus finding support from the above authors. India is the birthplace of renewed system of indigenous medicine such as Siddha, Ayurvedha and Unani. Traditional systems of medicines are prepared from a single plant or combinations of number of plants.[57] The efficacy depends on the use of proper plant part and its biological potency, which in turn depends upon the presence of required quantity and nature of secondary metabolites in the raw drug.[57, 73, 74] The results of the present study indicate that the presence of the above mentioned phytochemical constituents has the efficacy in curing various ailments. The efficacy in turn might be due to the biological potency of the phyto-chemcial constituents and secondary metabolites, either alone or in combination, thus finding support from the above authors.

Studies conducted on the plants antibacterial[14, 75, 76], antipyretic[14, 77, 78], analgesic[14], anti-parasitic[79], anti-diarrheal[69], antioxidant[18, 80, 81], suppression of TNF production[82], larvicidal and ovicidal activity[83] and anti-cancer activity[63] revealed its therapeutic potential. Various pharmacological actions of *C. halicacabum* have also been investigated in animal models for anti-inflammatory[84], analgesic and vasodepressor[66], anti-malarial[85] and anti-ulcer activities.[63] Anti-hyperglycemic effect of *C. halicacabum* leaf extract on STZ- induced diabetic rats.[86-88] Several authors have worked on various aspects of *C. halicacabum* such as antiviral activity of the plant extract against HIV and HBV virus.[79, 89-96]

C. halicacabum extract incorporated in Chinese traditional herbal medicine was reported to inhibit histamine release and nitric oxide production, a potential inhibitory activity in the treatment of inflammatory and chronic immune conditions.[97] Further, the ethanol extract of the plant suppressed the production of TNF and nitric oxide in the human peripheral blood mononuclear cells[83], exhibited potent *in vitro* hydroxyl radical scavenging and inhibition of lipid per oxidation, and was devoid of any conspicuous acute and short-term toxicity in rats.[59]

Therefore, the present study was undertaken to determine the anti-tumor effect of the aqueous, chloroform and methanol leaf extracts against human breast cancer (MCF-7) cells. The morphological analysis of the cells showed shrinkage in all the three extracts; the higher shrinkage level being reported in methanol extract. This shrinkage may due to the growth inhibitory effect of the phytoconstituents present in the leaves of *C. halicacabum*. In our experiments, we could not attribute reason for the differences in biological effects observed between the methanol, chloroform and aqueous leaf extracts of *C. halicacabum*, since our study investigated the anti-cancer activity of *C. halicacabum* leaf extract. Our data suggests that these extracts may show the greater effects of rather than the individual constituents present in plant extracts. On the basis of the findings of this study, we are currently undertaking extensive experiments to characterize and identify compounds from *C. halicacabum* and their anti-cancer effects on cancer cells MCF-7 cells *in vitro*. This might lead to the possibility that synergetic combination of biologically active agents might exist naturally in plants and whether such combinations might be chemically useful for anti-cancer agents.

CONCLUSION

Since human breast cancer is recognized as one of the most dangerous disease in the world, the research focus was put on it. This effort led us to the findings of the anticancer activity of *Cardiospermum halicacabum* leaf extract. This will be helpful in developing pharmaceutical standards for global acceptance. Finally, it is to be said that herbal medicine has been used up to

80% of the population in developing countries. Now it is the right time to start consuming safe herbal products for benefit of the recipient so that the context "Herbal plants preserves human life" will be justified.

ACKNOWLEDGMENT

The authors are thankful to V CLIN BIO LAB Pvt. Ltd, Sri Ramachandra Medical University, Porur, Chennai, Captain Srinivasa Murti Research Institute of Ayurveda and Siddha Drug Development, Arumbakkam, Chennai, Entomology Research Institute, Loyola College, Chennai and SAIF, IIT Madras, Chennai for their technical support.

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