Chemical Constituents and Antimicrobial Activity of Indian Green Leafy Vegetable *Cardiospermum halicacabum*

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Received: 27 August 2012 / Accepted: 31 October 2012 / Published online: 10 November 2012
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Abstract The present study was carried out to analyze chemical constituents and antibacterial activity of ethanolic leaf extract of *Cardiospermum halicacabum* (ECH). The FT-IR spectrum confirmed the presence of alcohols, phenols, alkanes, alkynes, aliphatic ester and flavonoids in ECH. The GC-MS analysis revealed that ECH contained about twenty four compounds. The major chemical compounds identified were cyclohexane-1, 4, 5-triol-3-one-1-carboxylic acid, benzene acetic acid, caryophyllene, phytol and neophytadiene. The ECH was screened for its antibacterial activity against different bacterial strains and anti fungal activity against *Candida albicans* by agar well diffusion and minimum inhibitory concentration (MIC) assay. ECH exhibited antibacterial and antifungal activity. All the tested bacterial strains showed MIC values ranging from 80 to 125 µg of extract/ml and *C. albicans* showed 190 µg of extract/ml as a MIC. The maximum activity ECH was observed against human pathogen *Staphylococcus aureus* followed by *Escherichia coli* and the fish pathogen *Aeromonas hydrophila*. ECH exhibited moderate activity against some of the tested multidrug resistant strains.

Keywords *Cardiospermum halicacabum* · FT-IR · GC-MS · Antimicrobial activity · Multidrug resistant strains

Introduction

Natural products from microorganisms have been the primary source of antibiotics. But with the increasing acceptance of herbal medicine as an alternative form of health care, the screening of medicinal plants for active compounds has become very important because these may serve as promising sources of novel antibiotics [1]. The antibiotics used nowadays exhibits certain demerits and side effects, so there has been an increasing interest in natural foods for scavenging the free radicals because of their wide acceptance. About 80 % of people in developing countries still depend on traditional medicine based largely on plants and animals for their primary health care. Most of the people prefer herbal medicines, due to high toxicity and side effects of synthetic drugs. Medicinal plants play an important role in the development of potent therapeutic agents. India is recognized as a land of herbal plants and therefore, any scientific data on such plants could be of clinical importance.

Cardiospermum halicacabum (Sapindaceae) is an herbaceous climber of about 2–4 m long, evergreen, branched with inflated fruits containing white heart-shaped pattern on the seed distributed in tropical and subtropical regions of Africa and Asia, and often consumed as green leafy vegetable in India. *C. halicacabum* is commonly known as Balloon vine, heart pea (England), Parol-paralon (Philippines), Jia hu gua (China) and Mudakathan Keerai (TamilNadu, India). *C. halicacabum* used in Indian traditional medicine system for the treatment of rheumatism, lumbago, cough, hyperthermia, nervous diseases, stiffness of limbs and snake bite [2]. The anti-inflammatory activity of ethanol extract of *C. halicacabum* leaves against carrageenan-induced rat paw edema has already been observed experimentally in rats [3]. Gopalakrishnan et al. [4]

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reported the analgesic and vasodepressive activities of *C. halicacabum*. The ethanol extract of *C. halicacabum* proved to have antipyretic activity against yeast-induced pyrexia in rats [5], anti-ulcer activity [6] and anti hyperglycaemic effect [7]. The ethanol extract of the *C. halicacabum* suppresses the production of TNF-alpha and nitric oxide in human peripheral blood mononuclear cells [8]. The fatty acid profile of seed oil extract of *C. halicacabum* reveals the presence of palmitic acid, oleic acid, stearic acid, linoleic acid, and eicosenoic acid [9]. The phyto-constituent responsible for the anxiolytic activity of *C. halicacabum* was identified as cardiospermin, a cyanogenic glucoside [10]. Authors recently reported the anti-arthritis activity of *C. halicacabum* against adjuvant induced arthritis in Wistar rats [11]. Thus the main objective of the work was to analyze the chemical constituents and to evaluate the antibacterial and antifungal activity of the ethanolic leaf extract of *C. halicacabum* (ECH).

Materials and Methods

Plant Material

Leaves of the plant *C. halicacabum* were collected from Tiruchirappalli district, Tamil Nadu, India and taxonomically identified by Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The leaves were picked and washed with water to remove all unwanted debris.

Preparation of Extract

The leaves were cut into small pieces, frozen in liquid nitrogen, lyophilized using a vacuum freeze dryer (Christ alpha 1-2/LD plus, Germany) and the lyophilized sample was coarsely ground to powder stored at -20°C . Ten grams of powder was extracted with 200 ml of 99 % ethanol using a soxhlet apparatus. The solvent was evaporated under reduced pressure at 45°C using rotary evaporator (Buchi R-210, Switzerland) to produce a yield of 9 % of dry extract. The dried extract obtained was stored at -20°C until further use. Then the extract was resuspended in sterile distilled water to produce the desired concentration and used for further analysis.

FT-IR Analysis

FT-IR is the most powerful tool for identifying types of chemical bonds (functional groups). The FT-IR spectra of ECH were recorded on a Perkin Elmer 2000 model FT-IR spectrometer (Thermo Fisher Scientific Inc., MA, and

USA). The dried sample was ground with potassium bromide powder and pressed into pellet for spectrometric measurement in the frequency range of $4,000\text{--}400\text{ cm}^{-1}$.

GC–MS Analysis

GC/MS analysis of the ethanol extracts was performed using a Shimadzu QP-2010 Plus with Thermal Desorption System TD 20. For MS detection, the electron ionization mode with ionization energy of 70 eV was used, with a mass range at m/z 50–550. An Omega Wax-30 $m \times 0.25\text{ mm}$ i.d., film thickness of $0.25\text{ }\mu\text{m}$ was used for GC/MS. The column temperature was programmed from 70 to 220°C at the rate of $4^{\circ}\text{C}/\text{min}$ with the lower and upper temperatures being held for 3 and 20 min, respectively. The GC injector and MS transfer line temperatures were set at 230 and 280°C , respectively. GC was performed in the normal mode. Helium was used as a carrier gas and the flow rate was maintained at 1 ml/min. An injection volume of 1 μl of ECH was injected into the column. Chemical composition of ECH were identified by their retention time and mass fragmentation pattern using data of standards at Wiley and Nist library-mass spectral databases [12, 13]. The percentage of composition of ECH was calculated by peak area.

Microbial Pathogens

A set of reference gram negative bacterial strains viz. *Escherichia coli* (MTCC 739 and MTCC 2939), *Aeromonas hydrophila* sub sps. (MTCC 1739), *Pseudomonas aeruginosa* (MTCC 2453 and MTCC 1934), *Enterobacter faecalis* and a gram positive bacterial strain *Staphylococcus aureus* sub sps. *aureus* (MTCC 2940) and a fungal strain *Candida albicans* (MTCC 227) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. In addition to that clinically isolated multidrug resistant strains (*Shigella* sp., *Klebsiella pneumoniae* and *Citrobacter freundii*) were obtained from Government hospital, Thanjore, Tamilnadu, India. The bacterial strains were maintained on nutrient agar slants, subcultured regularly and stored at 4°C for further use.

Inoculum Preparation

One loopful of overnight grown bacterial culture was inoculated in 5 ml peptone water, incubated at 37°C for 6 h. This actively growing culture suspension was adjusted with peptone water so as to obtain a turbidity that could be visually comparable with 0.5 MacFarland standard which has been prepared by adding 0.5 ml of 0.048 barium chloride with 99.5 ml of 0.36 N sulphuric acid. The turbidity is approximately equal to $1\text{--}2 \times 10^8\text{ CFU/ml}$.

Fig. 1 FT-IR spectra of ethanol leaf extract of *C. halicacabum*

Fig. 2 Gas chromatogram of ethanol leaf extract of *C. halicacabum*

Determination of Antimicrobial Activity

Sensitivity of test bacterial strains to ethanolic extract of *C. halicacabum* was measured by means of zone of inhibition using well diffusion assay. 20 ml of sterilized Muller Hinton Agar (MHA) were poured into petriplates and allowed to solidify. A sterile swab was dipped in broth suspension of 0.5 MacFarland standard. Excess suspension was removed by rotating the swab against the wall of the tube. The entire surfaces of the MHA plates were spread

uniformly. Wells were cut out on agar plates with sterile well borer and it was filled with 100 µl plant extract. The inoculated plates were incubated at 37 °C for 24 h and the resulting zone of inhibition was measured and incubated for another 24 h to find any bacteriostatic effect. The experiment was repeated thrice to ensure the sensitivity of pathogens to the plant extract. The pathogens which showed clear zone of inhibition more than 10 mm were considered to be sensitive. Chloramphenicol (10 µg) and nystatin (20 µg) were used as positive reference standards

Table 1 Chemical constituents of ethanol leaf extract of *C. halicacabum*

S. no.	Compound name	Retention time (min)	Amount (%)
1	Cyclohexane-1,4,5-triol-3-one-1-carboxylic acid	5.336	0.67
2	1-hydroxytetradecane	5.799	0.89
3	11-trimethyl-8-methylene	7.735	1.79
4	Caryophyllene	8.408	1.21
5	N-methyl tomatidine	9.011	0.37
6	3-methylbutanamide	10.496	0.14
7	Phenylethyl alcohol	10.592	0.21
8	Alpha-octadecene	10.772	0.83
9	Neophytadiene	11.537	11.11
10	Hexadecene	11.843	1.65
11	Nonadecene	12.130	3.06
12	Heptadecane	13.498	1.07
13	1,2-benzenedi carboxylic acid	15.085	3.39
14	Benzene acetic acid	16.052	5.16
15	14-methyl-8-hexadecyne	16.558	0.98
16	Benzaldehyde	16.796	0.51
17	1-tert-butyl-2-methoxy-4-methyl-3,5-dinitrobenzene	17.154	6.03
18	Phytol	17.514	5.06
19	Beta-phenethylphenyl acetate	18.168	3.12
20	2-phenylethyl ester	18.786	2.85
21	Hexadeconoic acid	21.224	13.67
22	Octadeconoic acid	26.187	4.90
23	2-nitro-2-(3'-hydroxybutyl) cyclododecanone	26.994	8.06
24	9-octadecenoic acid	28.703	6.25

for bacteria and fungi respectively to determine the sensitivity of the tested strains.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the plant extract was carried out using broth dilution method [14]. For bacteria, nutrient broth containing different concentrations (20–200 µg/ml) of ethanol extract were prepared and inoculated with 0.1 ml of inoculum. For fungi, sabouraud dextrose broth containing different concentrations (20–200 µg/ml) of the ECH were prepared and inoculated with 0.1 ml of inoculum. The inoculated cultures were incubated at 37 °C for 24 h. The least concentration of the extract causing complete inhibition of the growth was taken as MIC after 24 h of incubation. The results were compared with standard antibiotic chloramphenicol and nystatin for bacteria and fungi respectively. All the tests were carried out thrice.

Table 2 Antimicrobial activity of ethanol leaf extract of *C. halicacabum*

Test strains	Mean zone of inhibition (in mm)		MIC of ECH (µg/ml)
	ECH (1 mg/ml)	Chloramphenicol (10 µg)	
<i>E. coli</i> (MTCC 739)	16.3	13.7	90
<i>P. aeruginosa</i> (MTCC 2453)	11	18	120
<i>S. aureus</i> sub sps. <i>aureus</i>	18	20.8	80
<i>Shigella</i> sp.	11.5	15	100
<i>C. freundii</i>	NA	10	ND
<i>P. aeruginosa</i> (MTCC 1934)	10	12.5	120
<i>A. hydrophila</i>	17	14	125
<i>E. faecalis</i>	12	11.2	90
<i>E. coli</i> (MTCC 2939)	12	13	110
<i>K. pneumoniae</i> *	NA	NA	ND
<i>C. albicans</i> (MTCC 227)	15	20**	190

MIC minimum inhibitory concentration, ND not detected, NA no activity

* Multidrug resistant strain

** Nystatin (20 µg)

Results and Discussion

Cardiospermum halicacabum is an uncultivated food crop, widely distributed in India and consumed as green leafy vegetable. The FT-IR spectra of crude ECH are shown in Fig. 1. Strong and broad absorption peak at 3,356.49 cm⁻¹ for OH stretching vibrations, peaks at 2,364.53 and 2,135.63 cm⁻¹ for CH stretching vibrations, and a strong extensive absorption in the region of 1,649.08 for flavonoid carbonyl (C=O) stretching and 1,248.94 cm⁻¹ for alkane or aliphatic ester vibrations were observed in the spectra. Furthermore, characteristic peaks at 2,927.17 cm⁻¹ (for CH₂ stretching), 1,420.73 cm⁻¹ (for CH₃ stretching) and 616.6 cm⁻¹ (alkyne) were also observed in Fig. 1. The FT-IR spectrum of ECH confirms the presence of functional groups for phenolics and flavonoids, which are widely reported for their antioxidant potential. Flavonoids and phenolic acids have antibacterial, antifungal, antiviral, hepatoprotective, immunomodulating, and anti-inflammatory properties [15].

Gas Chromatogram of ECH is presented in Fig. 2. Table 1 shows the chemical constituents identified in the ECH by GC–MS analysis and listed in the order of retention time. Twenty five compounds were identified in the ethanol extract of *C. halicacabum* leaves. Cyclohexane-1,

Fig. 3 Inhibition effect of ethanol extract of *C. halicacabum* (ECH) and positive control chloramphenicol (C) against the microorganisms in well diffusion assay. **a** *A. hydrophila*, **b** *S. aureus* and bacteriostatic effect on **(c)** *E. coli* (MTCC 739)

Fig. 4 Zone of antifungal inhibition of ethanol extract of *C. halicacabum* (ECH) and positive control nystatin (N) against *C. albicans*

4, 5-triol-3-one-1-carboxylic acid (0.67 %), 1-hydroxytetradecane (29.6 %) followed by caryophyllene (8.4 %), neophytadiene (11.11 %), 1, 2-benzenedi carboxylic acid (3.39 %), benzaldehyde, phytol, hexadecanoic acid (13.67 %), and octadecanoic acid (4.90 %) were identified as the major chemical constituents.

The ethanolic extract resulted in varying zone of inhibition (11–18 mm) for all the tested microbial pathogens

except *C. freundii* and *K. pneumoniae* (Table 2). Of all the tested organisms, *S. aureus* and *A. hydrophila* were found to be the most sensitive among the bacteria used in the present study (Fig. 3a, b). Interestingly ECH exhibited inhibition zone of about 16.3 mm against *E. coli* (MTCC 739) but it was found to be bacteriostatic after 48 h (Fig. 3c). The MIC of ECH for bacteria ranges between 80 and 125 µg/ml. ECH showed maximum activity against *S. aureus* with the zone of inhibition about 18 mm followed by *A. hydrophila* about 17 mm and *E. coli* about 16.3 mm. The ECH showed minimum activity against *Pseudomonas* spp., *Enterococcus faecalis* (Table 2). Moreover, ECH showed zone of inhibition of about 11 mm diameter against the multidrug resistant *Shigella* sp. In addition to the antibacterial activity, ECH also exhibited anticandidal activity against the tested *C. albicans* (MTCC 227) by showing a zone of inhibition of 15 mm (Fig. 4). The phytochemical analysis revealed the detection of various functional groups for alcohols, phenolics, alkanes, alkynes and flavonoid compounds in ECH and that could be responsible for their antibacterial activity. Phytochemicals have been already reported for their antibacterial activity and that could be attributed to their ability to form complex with extracellular, soluble proteins and bacterial cell wall [16]. Previous reports demonstrated the antibacterial activity of phenolics and flavonoids [17, 18].

Moreover, it was also suggested that compounds in lower quantities might be involved in some type of synergism with the active compound. Phytochemicals exhibit antimicrobial activity through formation of a complex with the bacterial cell wall. The present study evaluated the antimicrobial activity of the medicinal plant *C. halicacabum*. The results are considered important since all the organisms tested are important pathogens in humans and animals and were resistance to other drugs is reported in many literatures [19–21]. Antibiotics of plant origin have enormous therapeutic potential and have been proven effective in the treatment of infectious diseases simultaneously reduces the side effects which are often associated with synthetic antibiotics [22]. Plants products remain as a reservoir of useful chemical compounds not only as drugs but also serve as unique templates for the manufacturing of synthetic analogs. The leaf extract of *C. halicacabum* possess appreciable antibacterial activity, so this plant could potentially be used for the treatment of infectious diseases caused by microorganisms which show resistance to currently available antibiotics.

The present study reports the antimicrobial activity of *C. halicacabum*. The GC–MS analysis of ethanolic extract of *C. halicacabum* reveals the presence of antimicrobial phytochemicals. The observed antimicrobial activity confirms the effectiveness of traditional use of this herbal drug against microbes. We hope the presented results will contribute to knowledge about the chemical composition of *C. halicacabum* in general, and as a source of natural products with potential use in the pharmaceutical industry.

Acknowledgments We thank University Grants Commission, New Delhi, India for the financial support. A. Ilavarasi acknowledges the Department of Science and Technology (DST) for INSPIRE-JRF fellowship. We also thank Mrs. K. Kanagalakshmi and Mrs. B. Vikneswari for their assistance in interpretation of FT-IR result.

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