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Research Article

COMPARATIVE STUDIES OF PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY OF *IN VIVO* PLANT AND *IN VITRO* CALLUS EXTRACT OF *CARDIOSPERMUM HALICACABUM* L.

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ABSTRACT

Objective: The present investigation focuses on the use of *Cardiospermum halicacabum* L. in their phytochemical and biological activities.

Methods: In this study, *in vivo* stem and *in vitro* callus ethanolic extracts of *C. halicacabum* were tested for their phytochemical attributes by qualitative method, Fourier transform infrared (FTIR), antioxidant, antibacterial, and bioactive compound properties. The bactericidal activity of the *in vivo* stem and *in vitro* callus extract has been evaluated in both Gram^{+ve} and Gram^{-ve} microorganisms using the disk diffusion method.

Results: The highest frequency (78%) of well developed, dark green organogenic callus was induced from stem explant on Murashige and Skoog (MS) medium supplemented with 0.7 mg/l 2,4-Dichlorophenoxyacetic acid (2, 4-D) and 0.5 mg/l benzyl adenine (BA). The results of FTIR spectra confirmed the presence of functional groups in wild stem and *in vitro* callus extract of *C. halicacabum* with various peaks. The total phenolic content in ethanolic extract of *in vivo* plant and *in vitro* callus was 80.46 mg gallic acid equivalent (GAE)/g dry weight and 76.4 mg GAE/g dry weight, respectively. The highest percentage of tannins was measured at 78.03 in wild stem ethanol extracts followed by 75.22 in callus extract. The antioxidant activity of 2,2-diphenyl-2-picrylhydrazyl (DPPH) ethanol extract was found to be 206.54 μg/ml. IC50 values of the stem extracts of *C. halicacabum* are 306 μg/ml and 286 μg/ml in callus extract, respectively. Antibacterial activity of the ethanol extract was higher for *Staphylococcus aureus (S. aureus)* with a 17 mm zone of inhibition.

Conclusion: The present investigation recommended that the callus ethanolic extract function as a good source of biologically active compounds and natural antioxidants.

Keywords: *Cardiospermum halicacabum*, Antibacterial activity, Phytochemicals, Antioxidant activity.

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INTRODUCTION

Medicinal plants are natural properties of bioactive phytochemical constituents which, for the physiological activities delivered on the human organism, can be employed against various ailments. Therefore, it is essential for medicinal plants to be evaluated for their phytochemistry so as to determine the ability of these indigenous sources of medicinal products [1]. *Cardiospermum halicacabum* L. (Balloon vine) belonging to the family *Sapindaceae*. *C. halicacabum* belonging a medicinal plant rich in β-sitosterol, D-glucoside, Saponin, and Quebrachitol. *C. halicacabum* is also used to treat diarrhea, dysentery, nephritis, oliguria hemorrhoids, asthma, earaches, muscular pains, nervous disorders, and rheumatism [2]. The entire plant has been used for some centuries in the treatment of atrophic arthritis, stiffness of the limbs, and snakebite; the decoction from its roots is used as a diuretic, emetic, and for laxative; the decoction of its leaves and stems is used in cases of diarrhea, dysentery, and headaches; and an ointment of them as a cure for swelling [3]. The ethanolic extract emphasized the existence of various compounds comprising phenolic acids (P-coumaric acid, 4-hydroxybenzoic acid, hydroquinone, protocatechuic acid, and gallic acid); flavonoids (apigenin, kaempferol, luteolin, and quercetin); tannins; and sterols including, campestrini, stigmasterin, and β-sitosterina. All compounds of *C. halicacabum* have been isolated and evaluated for antioxidant and anti-inflammatory activity [4]. Even though there are several reported publications on *C. halicacabum* phytochemistry in the literature, to date there is no available published report on the comparative phytochemical study of wild (stem) and *in vitro* callus extract. This prompts us to execute the present work to study the phytochemistry of *C. halicacabum*. The novelty of the present investigation is the detection of the main functional groups such as alcohols, phenols and alkanes in the stem derived callus extract, which could further be used for pharmacological study on this plant

METHODS

Plant material

The *C. halicacabum* plant was collected from the spring season in the local area of Karaikudi, Tamil Nadu. Then, the stem was separated and washed under running tap water. The plant material was authenticated at the Department of Botany, Alagappa University, Science Campus, Karaikudi, Tamil Nadu, India. The thoroughly washed stem was allowed for shade drying at room temperature in the laboratory. The dried leaves were ground to a fine powder using a blender.

Surface sterilization

For callus induction experiment, the stem explants were sterilized with 70% ethanol for 30 s followed by 0.1% bavistin treatment for 5 min and finally 0.1% mercuric chloride treatment for 2 min.

Culture conditions

For callus induction experiment Murashige and Skoog (MS) [5] basal medium supplemented with 30 g/l sucrose (HiMedia, India) and gelled with 0.8 % (w/v) agar (HiMedia, India), and the pH of the medium was adjusted to 5.7±0.2 using 0.1 N NaOH or 0.1 N HCl after addition of the plant hormones. The medium was autoclaved at 121°C and 15 psi pressure for 20 min. All the cultures were maintained in the culture room at 25±2°C, under 16/8 h light regime provided by cool white fluorescent light (60 μmol^{-2} s^{-1} light intensity) and with 55–60% relative humidity.

Callus induction

After sterilization treatment, the sterilized stem explants were cultured on MS medium supplemented with auxins (α-Naphthalene acetic acid [NAA], Indole-3 acetic acid [IAA] and 2,4-Dichlorophenoxyacetic acid (2, 4-D) 0.5 mg/l to 2.0 mg/l) (HiMedia, India) and cytokinin 6-benzyl

adenine (BA) (0.5 mg/l) for callus induction. Callus percentage was recorded after 4 weeks of culture.

Preparation of extracts

The *in vivo* grown plant was collected and shade dried for 2–3 weeks. The dried plant materials were then ground into a coarse powder in a mechanical blender and kept in an airtight container. The coarsely powdered sample was weighed (50 g) and the extract was prepared and was kept on a rotator shaker with 100 ml of ethanol, acetone, petroleum ether, and water solvents each alone for 2 days. The stem extracts were filtered through Whatman No.1 filter paper and collected in a 1 l beaker and they were covered with aluminum foil to avert evaporation. The stem extract of *C. halicacabum* was obtained by using three types of solvents (Petroleum ether, Ethanol, and Aqueous) and stored in an airtight container at 4°C. Further, the dried residue was preserved in an airtight container or glassware for further analysis. The *in vitro* grown callus was dried at room temperature for 3 days. The dried callus was macerated using a mortar and pestle. Ten grams of coarsely macerated callus were subjected separately for the extraction using 100 ml distilled water and solvent such as petroleum ether, chloroform, and ethanol.

Preliminary phytochemical studies

The ethanol extract was evaluated for the following phytochemical components such as reducing sugars, terpenoids, alkaloids, tannins, steroids, flavonoids, saponins, phenolic compounds, amino acids, and anthraquinones.

Test for alkaloids

Mayer's test

Two milliliters plant sample extract, two drops of Mayer's reagent are included in a test tube. The form of white creamy precipitate specified the existence of alkaloids [6].

Test for phenols

Ferric chloride test

Fifty milligrams extract was dissolved with distilled water and added few drops of neutral 5% ferric chloride solution. The form of a dark green color specified the existence of the phenolic compound [7].

Test for flavonoids

Lead acetate test

One milliliter of the crude extract added with two to three drops of dilute sodium hydroxide. A strong yellow color exhibited in the crude extract, which turns colorless with the inclusion of a few drops of dilute acid which signify the existence of flavonoid [8].

Test for reducing sugars

Plant extract (1 ml) was added with 1 ml of each Fehling's solution A and B and boiled in a water bath. The presence of red precipitate specified the existence of reducing sugars [9].

Test for tannin

Gelatin test

One milliliter of plant extract was added with 5 ml of distilled water and 1% of gelatine was mixed into the test tube. Afterward, 10% Sodium chloride (NaCl) solution was added. The appearance of a white precipitate displayed the existence of tannin [10].

Test for anthraquinones

Ammonium hydroxide test

The plant extract (10 mg) was dissolved in isopropyl alcohol and a few drops of concentrated ammonium hydroxide solution were added. After 2 min a form of red color indicated the existence of anthraquinones [11].

Test for terpenoids

The plant extract (5 ml) added with chloroform (1 ml), and then placed in a water bath and 3 ml of concentrated Sulphuric acid (H_2SO_4) was included ultimately boiled on a water bath. The presence of grey color specified the existence of terpenoids [11].

Test for amino acids

Biuret test

The plant extract (2 ml) was added with one drop of 2% copper sulfate solution. Afterward, 1 ml of 95% ethanol and few Potassium hydroxide (KOH) pellets were added. The presence of a pink color formation confirms the existence of amino acids [12].

Test for steroids

Salkowski test

Plant extract (2 ml) was added with 2 ml of chloroform and 2 ml of H_2SO_4. The presence of a reddish-brown ring junction confirms the existence of steroids [13].

Determination of total phenolic content

Folin-Ciocalteu assay method was used for the determination of the total phenol content [14]. Briefly, in a volumetric flask (25 ml), 1 ml of the extract was added with 9 ml of distilled water then 1 ml of Folin-Ciocalteu phenol reagent was added and shaken well. Ten milliliter of 7% sodium carbonate (Na_2CO_3) was added to the reaction mixture after 10 min. Afterward, the solution was incubated for 60 min at room temperature and the absorbance was measured at 550 nm with a UV visible spectrophotometer. Gallic acid at different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml) was used as a standard. In the same manner, as delineated earlier and the results were expressed as mg of gallic acid equivalent (GAE)/g of extract.

Determination of tannin content

The total tannin content was firm by Folin-Ciocalteu method with slight variations [15]. Approximately, 0.1 ml of the plant extract was added to a volumetric flask (10 ml) comprising 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent. One milliliter of 35% Na_2CO_3 solution diluted with 10 ml of distilled water. The reaction mixture was stirred well and kept at room temperature for 15 min. The different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0mg/ml) of gallic acid standard solutions were prepared in the same manner as described earlier. The absorbance was determined against the blank at 725 nm. The content of tannin was calculated as mg of tannic acid equivalents (TAE)/g of plant extract.

Antioxidant activity

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of the extracts was examined as stated by the earlier described technique [16]. The reaction mixture enclosed 1 ml of various concentration of extracts (2–10 mg/ml), 1.0 ml of iron-ethylenediamine tetra acetic acid (EDTA) solution (1.5 mM ferrous ammonium sulfate and 0.1 mM EDTA), 0.018% EDTA 0.5 ml, DMSO 1.0 ml (0.85% in 0.1 mol/l phosphate buffer pH 7.4), and 0.22% ascorbic acid 0.5 ml. The firmly enclosed reaction mixture contained test tubes were heated in a water bath at 70–80°C for 20 min, this process was ended by the addition of ice-cold trichloroacetic acid (TCA) (1.0 ml) (17.5%). Eventually, Nash reagent 3.0 ml (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2.0 ml of acetylacetone and distilled water was added to a total volume of 1 l) was added and kept room temperature for 15 min for color change. The formation of yellow color developed was measured at 412 nm and gallic acid was used as a standard. The IC50 value for the extract and standard preparation was assessed using the following formula,

% scavenging/Inhibition = [Absorbance of control-Absorbance of test sample/Absorbance of control] × 100

Ferric reducing antioxidant potential (FRAP) assay

The reducing power capability of the extracts was investigated as stated by the earlier described method [17]. In brief, 1.0 ml of different concentrations of extracts (20 µg/ml–100 µg/ml), 2.5 ml of 1% potassium ferricyanide, and 2.5 ml of 0.2 mol/L sodium phosphate buffer were mixed well and incubated at 60°C for 20 min. Afterward, the reaction was ended by the addition of 2.5 mL of 10% trichloroacetic acid, followed by centrifugation at 2000 rpm/min for 15 min. Ultimately, the upper layer (2.5 ml) was mixed with deionized water (2.5 ml) and 0.5 ml of 0.1% ferric chloride. Ascorbic acid was used as standard and the absorbance was measured at 700 nm.

2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The capability of the ethanolic extract of *C. halicacabum* to scavenge the DPPH free radical was determined according to the previously described method [18] with minor modification. The extract at different concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml) was prepared. Each sample (0.5 ml) was prepared and mixed with 0.5 ml of 1 mM DPPH solution in ethanol. The reaction mixture was kept at room temperature for 20 min. The reduction ability of DPPH radicals was measured by decreased induction of antioxidants in their absorbance at 517 nm. The ascorbic acid was used as a standard and similar concentrations were prepared as the test solutions. The difference in absorbance between the test and the control (DPPH in ethanol) was determined and expressed as % scavenging of DPPH radical. The ability to scavenge the DPPH radical was measured using the following equation.

$$\% \text{ DPPH radical scavenging activity} = \{(A0 - A1)/A0\} \times 100$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. Afterward, the % of inhibition was calculated against concentration, and from the graph IC_{50} was determined.

Fourier transform infrared (FT-IR) analysis

FTIR spectrophotometer is possibly the most efficient tool to find the types of chemical bonds and functional groups existing in compounds. Dried powder of *C. halicacabum* ethanolic extract was used for FTIR analysis. The powdered ethanolic extract (10 mg) was placed in 100 mg of potassium bromide (KBr) pellet, to prepare a translucent sample disc. The powdered sample was loaded on to FTIR spectroscope and the spectroscopy results were documented on an FTIR spectrometer alpha II Compact Bruker, with a scan range from 400 to 4000 cm^{-1}.

Antibacterial activity

Test microorganism

The antibacterial properties of *C. halicacabum* extracts were examined against Gram-positive bacteria: *Staphylococcus aureus, Enterococcus faecalis,* and Gram-Negative bacteria: *Moraxella* sp., *Escherichia coli,* and *Proteus mirabilis*. The bacterial strains were obtained from the Department of microbiology, Alagappa University, Karaikudi.

Antibacterial assay

The disk diffusion method was used to examine the antibacterial activity of the *C. halicacabum* extract. The bacterial strains were inoculated in the nutrient broth under aseptic conditions and incubated at 37°C for 24 h. After the incubation period, the test bacteria were inoculated on the nutrient agar (HiMedia) plate using a sterile cotton swab. The different concentrations of petroleum ether, chloroform, acetone, ethanol, and aqueous extracts (200, 400, 600, and 800 µg/disc) were loaded on the 6 mm sterilized discs (Hi Media,) to measure the dose-dependent activity of the extracts. Cefepime (30 mcg/disc) was used as a positive control and the individual solvents were used as a negative control. Afterward, the Petri plates were kept at incubator (37°C) for 18–24 h. The zones of inhibition and the mean diameter were documented.

Statistical analysis

Each experiment of quantitative phytochemical analysis and antibacterial activity was conducted in three replicates. Statistical analysis was performed by SPSS version 20.0 (SPSS Inc. Chicago, IL). Differences between means were verified using one-way ANOVA and the least significant difference test. The significance levels were considered at p≤0.05.

RESULTS AND DISCUSSION

Callus induction

Callus induction was detected from cut margins of stem explants of *C. halicacabum* after 2 weeks of incubation, cultured on MS medium supplemented with auxins, namely, 2, 4-D, IAA, indolebutyric acid (IBA), and NAA (0.5–2.0 mg/l) along with BA (0.5 mg/l), under a partial incubation in dark (Table 1). Initial responses such as stem expand subsequently swelling of explant were observed from the 6th day of the culture period (Fig. 1a-d). Among various treatments, 2, 4-D along with BA gave the best callus initiation and proliferation, followed by NAA, IAA, and IBA (Table 1, Fig. 1b and e). Contingent on the concentration and combination of plant growth regulators (PGR) benefitted, an extensive range of variation in the frequency of callus formation and nature of callus was observed. The fresh weight and dry weight of callus biomass for stem explants of *C. halicacabum* are shown in Table 1. The highest fresh weight was achieved on 0.5 mg/l BA and 0.7 mg/l 2, 4-D. NAA at lower and higher concentrations produced light yellow to brown friable or nodular callus from stem explants, which were failed to respond further (Table 1). In contrast, NAA was reported to develop higher callus induction [19]. Medium containing 0.7 mg/l IAA and 0.5 mg/l BA produced yellowish green compact callus was obtained from stem explants, whereas IBA (0.7 mg/l) with BA (0.5 mg/l) produced dark brown callus with rooting. Likewise, Thaniarasu *et al*. [20] proposed that the modified callus did not form adventitious roots on medium with auxins but only with cytokinins; therefore, it is suggested that cytokinin has a stimulating effect on root formation from callus. The highest frequency (78%) of well developed,

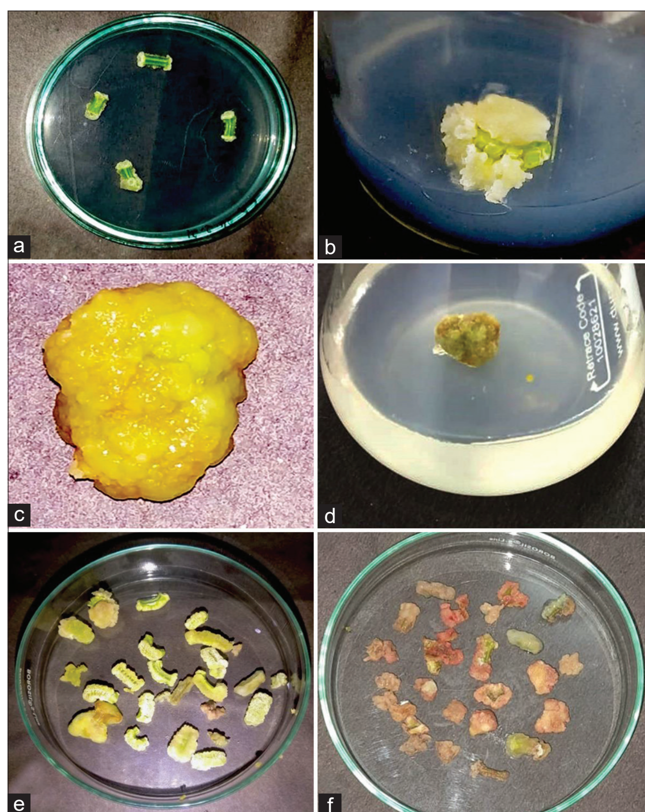

Fig. 1: Callus induction and proliferation of *Cardiospermum halicacabum* L. (a) Stem explant, (b–d) Callus induction on MS + 2,4-D (0.7 mg/l) + BA (0.5 mg/l), (e) Callus fresh weight (407 mg), (f) Callus dry weight (99 mg)

Table 1: Callus induction from leaf and internode explants of *C. halicacabum* cultured on MS medium supplemented with different concentrations of auxins with BA, after 4 weeks of culture

PGR's (mg/l)	% of response	Callus fresh weight mg/explant	Callus dry weight mg/explant	Texture of callus
Control	00.0±0.0	00.0±0.0	00.0±0.0	00.0±0.0
2,4-D+BA (0.5)				
0.5	63.0±1.5	335.4±2.45	84.5±0.59	GC
0.7	78.0±1.3	407.6±1.20	99.0±1.30	GC
1.0	58.0±1.3	326.6±0.90	79.0±0.53	GC
2.0	44.0±1.6	278.2±1.40	63.0±0.56	GF
NAA+BA (0.5)				
0.5	35.0±1.6	268.1±0.64	59.1±0.54	YF
0.7	55.0±2.2	348.3±1.33	70.6±.40	GYF
1.0	43.0±1.5	308.9±0.38	66.8±0.53	YGC
2.0	32.0±1.3	230.2±0.41	54.6±0.90	YF
IAA+BA (0.5)				
0.5	32.0±1.3	160.7±0.68	46.4±0.71	YF
0.7	34.0±1.6	228.4±0.49	47.5±0.52	YGC
1.0	–	–	–	–
2.0	–	–	–	–
IBA+BA (0.5)				
0.5	33.0±1.5	140.4±0.21	34.0±0.68	BCC
0.7	35.0±1.6	124.4±0.39	34.2±0.76	BCC
1.0	–	–	–	–
2.0	–	–	–	–

Values are mean±S.E. from seven replicates per treatment and all the experiments were repeated thrice. Means followed by the same letters in each column are not significantly different (p=0.05) using Duncan's multiple range test. GC: Green Compact, BCC: Brownish Compact, GYF: Greenish Yellow Friable, YF: Yellow friable, GF: Green friable, YGC: Yellowish Green Compact,-: no response, PGR: Plant growth regulators, NAA: Naphthalene acetic acid, IAA: Indole-3 acetic acid, BA: Benzyl adenine, IBA: Indolebutyric acid.

dark green organogenic callus was induced from stem explant on MS medium supplemented with 0.7 mg/l 2, 4-D, and 0.5 mg/l BA (Fig. 1b and e). Lower concentrations of 2, 4-D (0.5–1.0 mg/l) along with BA (0.5 mg/l) formed profuse amount of callus in contrast to higher concentrations (Table 1). Among the different concentrations of auxin tested, 2, 4-D with BA proved to be the best for the high frequency of greenish compact callus induction. Previous investigations proved the same [21-23]. All the callus cultures were sub-cultured every 15 days onto a fresh medium containing the same PGR composition. Maximum calli were induced and developed as light yellow in color and become green compact organogenic callus in 4 weeks.

Screening and qualitative comparison of phytochemicals

Both the crude and callus extracts were subjected to preliminary chemical tests 18 to detect the presence and absence of various phytochemicals such as alkaloids, flavonoids, steroids, triterpenes, saponins, phenolics, and tannins (Table 2). The phytochemical constituents greatly differed among the tested solvents in both wild stem extract and in vitro callus extract. This is a sign of solvents varied extracting abilities for phytochemicals [24].

FT-IR analysis

The results of FTIR spectra confirmed the presence of functional groups in wild stem extract of *C. halicacabum* with peaks at 3329.72 cm^{-1} (alcohols, phenols), 2973.28 cm^{-1} (alkanes), 2927.64 cm^{-1} (amine salts), 1655.88 cm^{-1} (alkanes, amines), 1450.96 cm^{-1} (Alkanes), 1417.42 cm^{-1} (alcohol), 1379.87 cm^{-1} (phenols, nitrogen groups, alcohols, carboxylic acids, esters, and ethers), 1275.39 cm^{-1} (alkyl esters), 1086.75 cm^{-1} (anhydride), and 661.42 cm^{-1} (acid chlorides and alkanes) (Fig. 2 and Table 3), followed by callus ethanolic extracts with peaks at 3388.01 cm^{-1} (alcohols, phenols), 2974.38 cm^{-1} (Carboxylic acid), 2924.01 cm^{-1} (carboxylic acid and amine salts), 2138.50 cm^{-1} (alkanes and amines), 1706.78 cm^{-1} (alkanes and amides), 1362.76 cm^{-1} (alcohol), 1274.01 cm^{-1}(amines, phenols, nitrogen groups, alcohols, carboxylic acids, esters, and ethers), 1087.08 cm^{-1} (alkyl esters), 880.01 cm^{-1} (anhydride), and 670.66 cm^{-1} (acid chlorides and alkanes) (Fig. 3 and Table 4). The FT-IR spectrum of wild stem and callus ethanolic extracts confirms the presence of functional groups for phenolics and flavonoids, which are widely reported for their antioxidant potential. Major groups

Table 2: Qualitative analysis of ethanolic extracts of stem and callus of *C. halicacabum*

Phytochemicals	Callus extract	Plant extract
Steroids	–	+
Terpenoids	+	+
Reducing sugar	–	–
Alkaloids	+	+
Tannins	+	+
Flavonoids	+	+
Saponins	–	+
Phenolic compounds	+	+
Amino acids	–	+
Anthraquinones	–	–

(+): Present, (-): Absent.

are present in the callus extract. Flavonoids and phenolic acids have antibacterial, antifungal, antiviral, hepatoprotective, immunomodulating, and anti-inflammatory properties [25]. Major peaks were observed at 3388.01 cm^{-1} that possibly designated to the O-H stretching vibrations of O-H Alcohol. Consequently, the present investigation results show that the primary functional group presents in *C. halicacabum* is O-H Alcohol. O-H, group possibly specifies the higher potential toward inhibitory activity against microorganisms. Like a higher antimicrobial activity of ethanol extracts of the leaf has been previously established by some authors [26]. Through the FT-IR spectrum, we identified the functional groups from the given extract. Many researchers employed the FT-IR spectrum as a tool for observing closely associated plants and other organisms. The outcomes of the present investigation improved an innovative phytochemical marker to find the medicinally significant plant.

Determination of total phenol

The total phenolic content for *C. halicacabum* (1.0 mg/ml) was calculated from the standard graph of gallic acid with the standard curve equation, Y=0.97×–0.176, R^2=0.999 (Fig. 4). The total phenolic content in aqueous extract of *in vivo* plant and *in vitro* callus was 80.46 mg GAE/g dry weight and 76.4 mg GAE/g dry weight, respectively (Fig. 5). At present, plant materials rich in phenolics are used in the

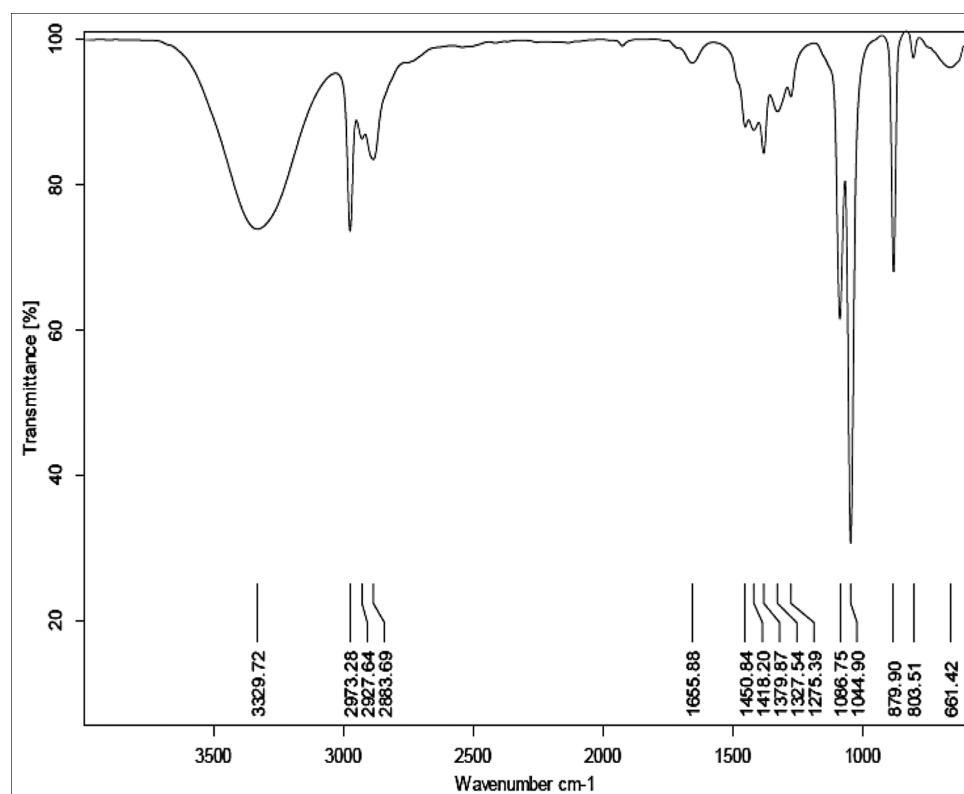

Fig. 2: Fourier transform infrared spectrum of the ethanolic extract of *Cardiospermum halicacabum*

Table 3: FT-IR analysis of *in vivo* stem ethanolic extract of *C. halicacabum* L

Frequency	Frequency range	Bond	Functional group name
3329.72	~3400–3300	O-H Stretch	Aromatics
2973.28	~2950–2800	C-H Stretch	Alkanes
2927.64	~3400–2400	O-H Stretch	Carboxylic acids
2883.69	~2950–2800	C-H Stretch	Alkanes
1655.88	1690–1640	$R_2C=N-R$ Stretch	Imines
1450.84	1550–1490	$-NO_2$ (Aromatic)	Nitro groups
1418.20	1440–1400	O-H Bend	Carboxylic acids
1379.87	1400–1000	C-F Stretch	Alkyl halides
1327.54	1360–1250	C-N Stretch (Aryl)	Amines
1275.39	1300–1100	C-C Stretch	Ketones
1086.75	1090–810	PH Bend	Phosphines
1044.90	~1400–1000	C-F Stretch	Alkyl halides
879.90	~880	C-H Bend(meta)	Aromatics
803.51	850–800	C-H Bend(para)	Aromatics
661.42	730–550	C-Cl Stretch	Acid chlorides

FT-IR: Fourier transform infrared.

Table 4: FT-IR analysis of ethanolic callus extract of C. *halicacabum* L.

Frequency	Frequency range	Bond	Functional Group Name
3388.01	~3400–3300	O-H stretch	Alcohols
2974.38	~3400–2400	O-H stretch	Carboxylic acid
2924.01	~3400–2400	O-H stretch	Carboxylic acid
2138.50	~2150–1800	C, C triple bond stretch	Alkynes
1706.78	1730–1700	C=O stretch	Carboxylic acid
1653.34	1680–1630	C=O stretch	Amides
1420.63	1440–1400	O=H bend	Carboxylic acid
1362.76	1390–1300	$-NO_2$ (aliphatic)	Nitro group
1274.01	1360–1250	C-N stretch (aryl)	Amines
1225.35	1260–1000	C=O stretch	Alcohols
1087.08	1260–1000	C=O stretch	Alcohols
1046.28	1260–1000	C=O stretch	Alcohols
880.01	880	C-H bend (meta)	Aromatics
803.58	850–800	C-H bend (para)	Aromatics
670.66	715–685	C-H bend (mono)	Aromatics

FT-IR: Fourier transform infrared.

food industry because they decrease the oxidative degradation of lipids and maintain the quality and nutritional value of food. We determined that the total phenolic content was slightly more in the aqueous extract compared to the methanol and ethyl acetate extract [27].

Determination of total tannin content

The concentration of total tannin in *C. halicacabum* samples was derived from standard curve of tannic acid ranging from 0.2 to 1.0 mg/ml (y=0.97×–0.086; R=0.9932) (Fig. 6). The obtained values for the concentration of tannin contents are measured as mg of TAE/g of extract. The maximum percentage of tannins was determined at 78.03 in wild stem ethanol extracts followed by 75.22 in callus extract (Fig. 7). Tannins possess high radical scavenging capability can be extensively found in plants and have several positive results on human health.

Antioxidant activity

Hydrogen peroxide radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in the biological organization. It is as main active oxygen gathering radicals developed from the reaction of several hydroperoxides with transition metal ions and it is proficient in damaging about every molecule found in the living system causing lipid peroxidation and harmful to the biological system [28]. The higher hydroxyl radical scavenging activity was delivered at the concentration of 1000 µg/ml. The *C. halicacabum* showed higher scavenging activity shown in Fig. 8**.** Each concentration of the extracts display hydroxyl radical scavenging activity was increased with the increasing concentration of the extracts. Significantly lower IC50 values were observed in comparison to *in vivo* grown stem and *in vitro* raised callus. It is found that IC50 values of the stem extracts of *C. halicacabum* are 306 µg/ml and 286 µg/ml in callus extract respectively (Fig. 8).

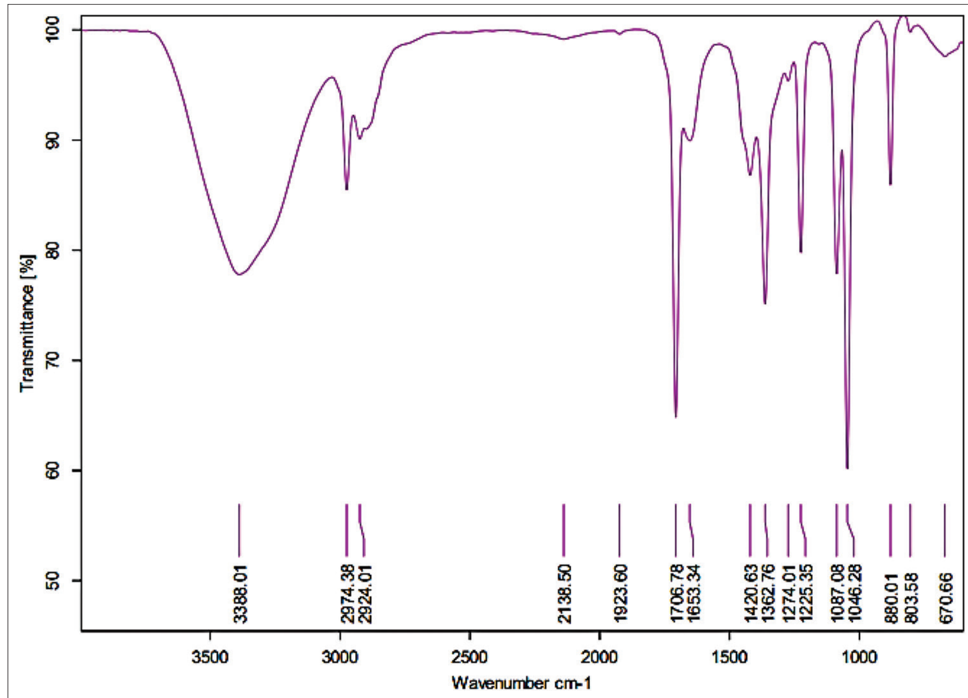

Fig. 3: Fourier transform infrared Spectrum of ethanolic callus extract of *Cardiospermum halicacabum*

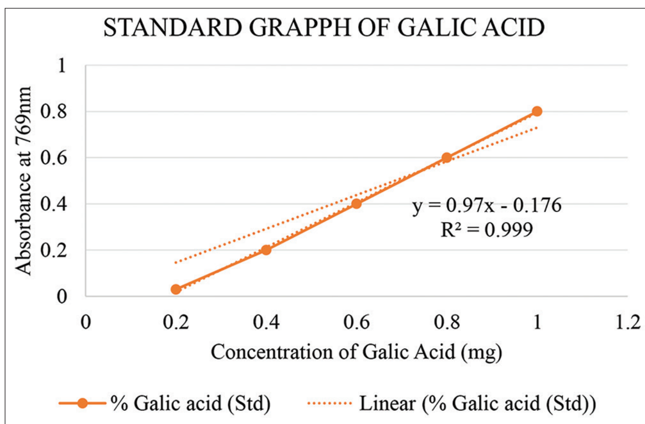

Fig. 4: Total phenolic content standard graph.

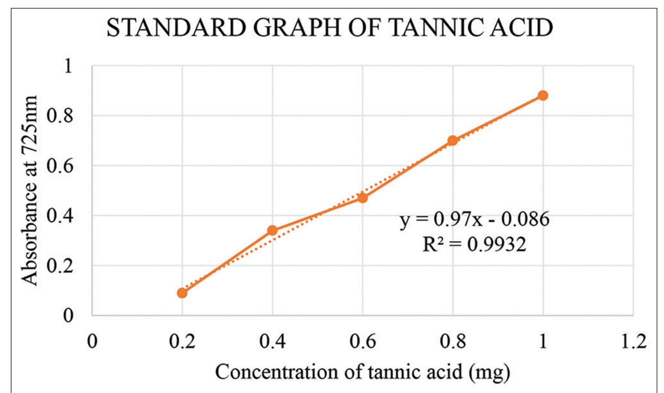

Fig. 6: Total tannin content standard graph

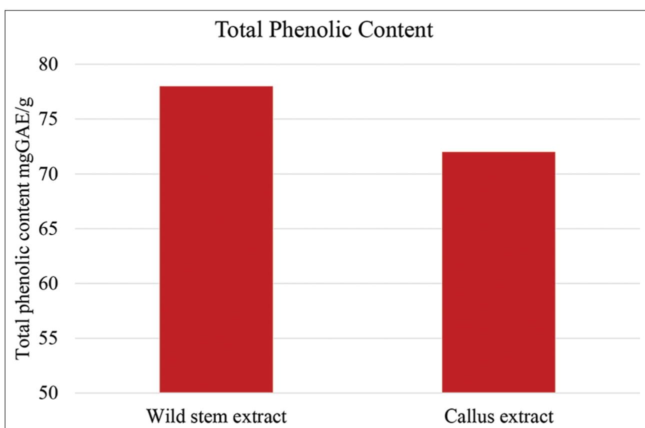

Fig. 5: Total phenolic content of *Cardiospermum halicacabum*

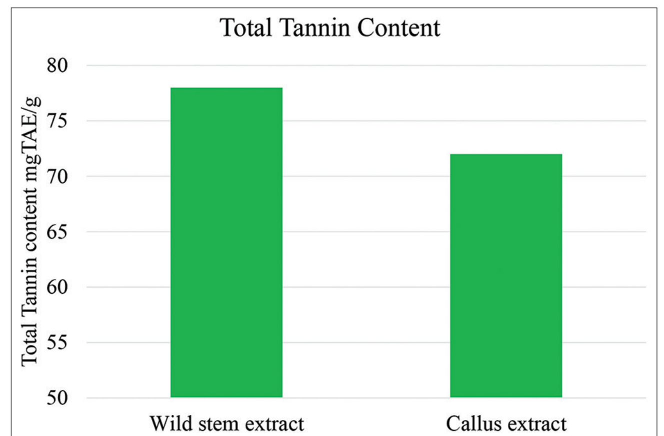

Fig. 7: Total tannin content of *Cardiospermum halicacabum*

FRAP Assay

The reducing potential of the ethanolic extract of *C. halicacabum* wild stem and callus extracts was examined by the FRAP method to determine the direct electron transfer capability of the extract. The FRAP results were measured from a calibration graph which was linear over the calibration range with an R^2 value of 0.9794 (Fig. 9) and the antioxidant ability of callus extract was found to be 225.29 µmoles/mg followed by wild stem extract 424 µmoles/mg. The reducing capacity of the *C. halicacabum* callus extract was similar to earlier studies on medicinal plants such as *Albizia odoratissima* [29] and *Salvia officinalis* [30].

DPPH radical scavenging activity

DPPH radical scavenging assay is a simple and extensively employed method to determine the quenching potential of antioxidants. DPPH is an unfirm radical forming purple in color and gets reduced in the presence of antioxidant to a non-radical firm form. The absorbance is measured at 517 nm by a UV spectrophotometer [31]. Ascorbic acid was used as a standard. It was noted, that the DPPH radical scavenging activity was increased with the increasing concentration of the extract. The IC50 value was estimated with the help of a graph and the lower IC50 gives better radical scavenging capability [32]. The IC50 value was calculated from a formerly developed standard curve (Fig. 10) and was found to be 206.54 µg/ml. This least value of IC50 of the current study suggests that *C. halicacabum* callus extract has strong hydrogen donating capability. The examines performed on ethanolic plant extracts disclose that *C. halicacabum* displays DPPH radical scavenging capability in dose-dependent manner. The standard ascorbic acid exhibits higher radical scavenging compare to plant extract. The results were measured and expressed as ascorbic acid equivalent.

Antibacterial activity

The antibacterial activity of the various solvent extracts of *C. halicacabum in vivo* stem and *in vitro* callus against human pathogenic bacteria such as, *S. aureus*, *E. faecalis*, *Moraxella* sp., *P. mirabilis*, and *E. coli* was examined and assessed by the zone of inhibition

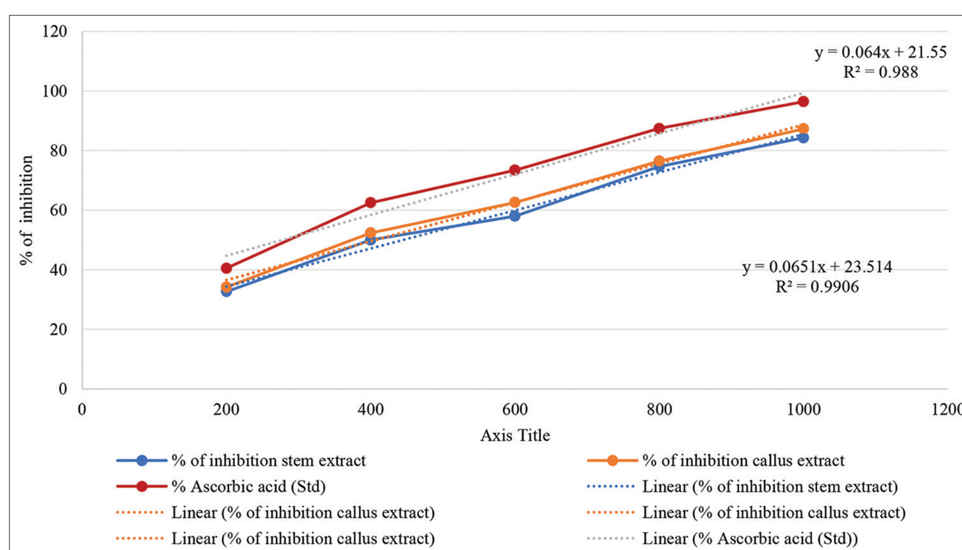

Fig. 8: Percentage of hydrogen peroxide inhibition *in vivo* stem and *in vitro* callus extract with respect to ascorbic acid standard

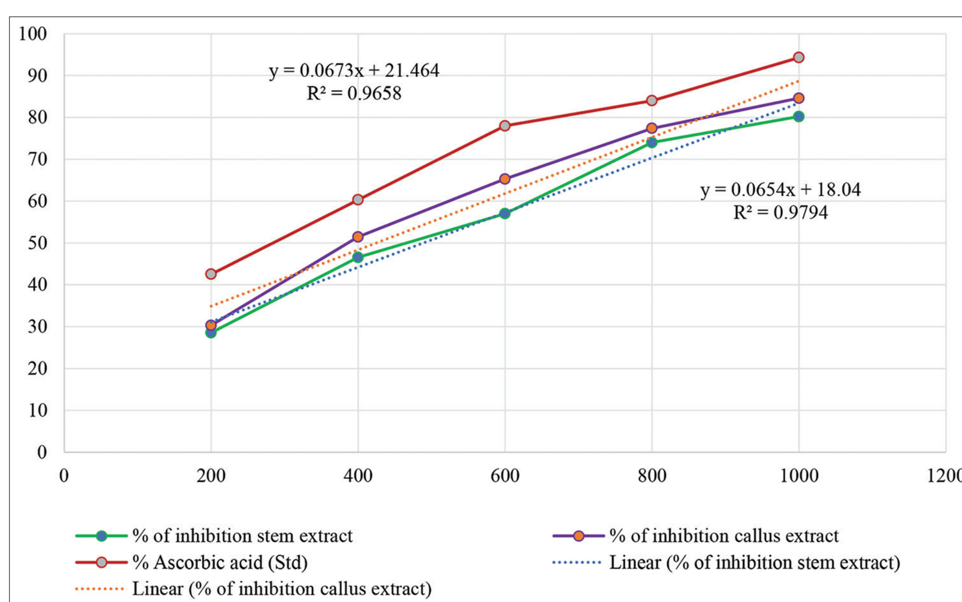

Fig. 9: Percentage of Ferric reducing antioxidant potential inhibition in *in vivo* stem and *in vitro* callus extract with respect to ascorbic acid standard

in disc diffusion method. The ethanol extract exhibits significant antibacterial activity against all the tested bacteria. The action of different solvent extracts was comparable to the standard Cefepime. Among the different solvent extracts, petroleum ether and aqueous extracts showed lower antibacterial activity compared to other solvent extracts. The callus chloroform extracts displayed maximum activities against three of the strains; *P. mirabilis* (10 mm), *Moraxella* sp (10 mm), and *S. aureus* (12 mm), followed by stem extract *P. mirabilis* (8 mm), *Moraxella* sp (10 mm), and *S. aureus* (10 mm). The acetone callus extracts of maximum activities against two of the strains; *S. aureus* (10 mm) and *E. faecalis* (8 mm). The result of antibacterial activities is presented in Tables 5 and 6. Among the various solvent extracts tested, the least antibacterial activity exhibited by aqueous extracts against all the tested strains. Among the different solvent extract tested, callus ethanolic extracts exhibited the highest activity of 17 mm (400 μl) inhibition zone against *S. aureus* this was followed by 13 mm *E. coli*, 14 mm *E. faecalis*, 8 mm *P. mirabilis,* and 10 mm inhibition zone against *Moraxella* sp. The *in vivo* stem extract showed the highest activity of 14 mm (400 μl) inhibition zone against *S. aureus* this was followed by 12 mm *E. coli*, 12 mm *E. faecalis*, 9 mm *P. mirabilis,* and 10 mm inhibition zone against *Moraxella* sp (Tables 5 and 6). Among the different tested strains, *S. aureus* showed the most resistance to various solvents of extracts and *E. coli* showed moderate activity. The ethanolic extract was most effective when compared to the standard antibiotic Cefepime. The lowest activity of the extract is 3 mm against *E. coli* and *P. mirabilis.* The extracts at the lower concentrations displayed a maximum zone of inhibition compare to higher concentrations. In particular, *S. aureus* showed the fact that *C. halicacabum* ethanolic extract could be effective in chronic lung disorders and skin disorders, which are often induced by *S. aureus.* Based on the results, Gram-positive strains exhibited more effectiveness than Gram-negative strains, for the reason that

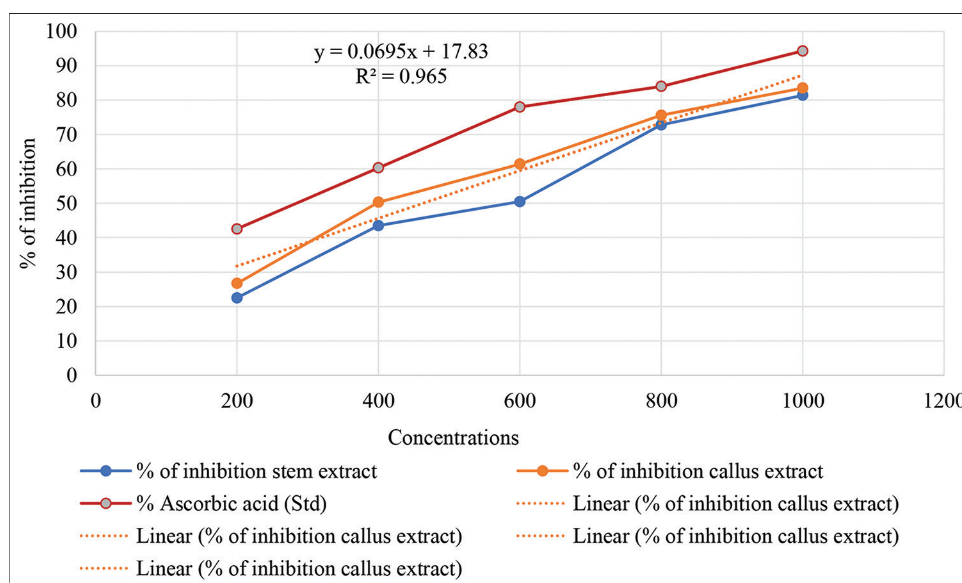

Fig. 10: Percentage of 2,2-diphenyl-2-picrylhydrazyl inhibition *in vivo* stem and *in vitro* callus extract with respect to ascorbic acid standard

Table 5: Zone of inhibition (mm) of Gram-ve and bacteria Gram+ve agents at various concentrations of different *in vivo* stem extracts of *C. halicacabum* and standard cefepime

Extracts	Concentration μg/ml	Zone of inhibition (in mm)					
		Gram negative			Gram positive		Cefepime 30 mcg/Disc
		P. mirabilis	*Moraxella* sp.	*E. coli*	*E. faecalis*	*S. aureus*	
Petroleum ether	200	3.0	6.0	5.0	4.0	–	22.5
	400	5.0	8.0	6.0	6.0	7.0	
	600	6.0	9.0	–	4.0	5.0	
	800	–	–	–	3.0	3.0	
chloroform	200	6.0	–		5.0	8.0	18.4
	400	8.0	10.0	6.0	7.0	9.0	
	600	9.0	6.0	7.0	9.0	10.0	
	800	–	8.0	9.0	10.0	5.0	
Acetone	200	5.0	4.0		–	–	17.0
	400	7.0	5.0	5.0	–	–	
	600	5.0	–	7.0	–	8.0	
	800	3.0	–	5.0	8.0	10.0	
Ethanol	200	6.0		7.0	6.0	8.0	15.0
	400	9.0	10.0	12.0	12.0	14.0	
	600	5.0	10.0	11.0	10.0	12.0	
	800	6.0	8.0	8.0	9.0	10.0	
Aqueous	200	–	–	–	–	–	18.0
	400	–	–	–	–	–	
	600	5.0	7.0	4.0	6.0	7.0	
	800	6.0	5.0	5.0	4.0	5.0	

(-): No response.

Table 6: Zone of inhibition (mm) of Gram-ve and bacteria Gram+ve agents at various concentrations of different *in vitro* callus extracts of *C. halicacabum* and standard cefepime

Extracts	Concentration μg/ml	Zone of inhibition (in mm)					
		Gram negative			Gram positive		Cefepime 30 mcg/Disc
		P. mirabilis	*Moraxella sp.*	*E. coli*	*E. faecalis*	*S. aureus*	
Petroleum ether	200	3.0	–	–	–	–	20.5
	400	5.0	–	–	6.0	8.0	
	600	6.0	9.0	5.0	4.0	6.0	
	800	–	7.0	4.0	3.0	5.0	
chloroform	200	6.0	–		5.0	8.0	18.5
	400	8.0	4.0	6.0	7.0	12.0	
	600	10.0	6.0	7.0	9.0	6.0	
	800	–	8.0	9.0	10.0	5.0	
Acetone	200	4.0	4.0	–	–	–	21.0
	400	5.0	5.0	5.0	–	–	
	600	6.0	–	7.0	–	8.0	
	800	3.0	–	5.0	8.0	10.0	
Ethanol	200	5.0		7.0	6.0	8.0	15.0
	400	8.0	10.0	13.0	14.0	17.0	
	600	7.0	9.0	11.0	12.0	13.0	
	800	6.0	6.0	8.0	10.0	12.0	
Aqueous	200	–	–	–	–	–	20.5
	400	–	5.0	–	–	–	
	600	5.0	4.0	4.0	6.0	6.0	
	800	6.0	3.0	5.0	4.0	5.0	

(-): No response.

the disrupt membrane of Gram-negative bacteria surrounding the cell wall, which prevents the diffusion of hydrophobic compounds across its lipopolysaccharide protection [33,34].

CONCLUSION

Well-developed green compact callus and maximum (78%) callus fresh weight was derived from stem explant on MS medium supplemented with 0.7 mg/l 2, 4-D and 0.5 mg/l BA. *C. halicacabum* exhibited the potential antibacterial activity against *S. aureus* in both the wild stem and *in vitro* callus extracts. The FT-IR spectrum of wild stem and callus ethanolic extracts confirm the presence of amino acids, which are widely reported for their antioxidant potential and major functional groups are present in the callus extract. The results confirmed the occurrence of conceivable phytoconstituents, promise effectiveness in the *C. halicacabum* callus extract also.

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AUTHORS' CONTRIBUTIONS

Both authors are equally contributed in performing the experiments and preparation of the manuscript.

CONFLICT OF INTERESTS

Both authors declare that they have no competing interests.

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