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Research Article

IN VITRO ANTIOXIDANT CAPACITY AND FREE RADICAL SCAVENGING ACTIVITIES OF *CARDIOSPERMUM HALICACABUM* LINN.

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ABSTRACT

Objective: Free radicals and oxidants are produced in the body during normal cellular metabolism and exposure to pollutions and ionizing radiations. They exert beneficial and harmful effects to the human body. Harmful effects of these radicals are destroyed by either antioxidants of our body *in situ* or antioxidants which are supplied through foods. Hence, the aim of this present study is to assess the antioxidant and free radical scavenging capacity of the easily available edible plant *Cardiospermum halicacabum* Linn. (Mudakathan keerai).

Methods: Antioxidant and free radical scavenging activities of the aerial parts extract of *C. halicacabum* was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, ferrous ion chelating assay, nitric oxide (NO) radical scavenging assay, superoxide radical scavenging assay, hydroxyl radical scavenging assay, and lipid peroxidation assay.

Results: Remarkable free radical scavenging potential was observed in the ethanolic extract of *C. halicacabum* with IC_{50} values on all tested radicals, namely DPPH (IC_{50}=34.06 µg/ml), ABTS (IC_{50}=21.45 µg/ml), ferrous ion chelating (IC_{50}=19.56 µg/ml), NO (IC_{50}=25.16 µg/ml), superoxide (IC50=35.16 µg/ml), hydroxyl (IC50=28.56 µg/ml), and lipid peroxidation (IC50=33.12 µg/ml). The results revealed that ethanolic extract of *C. halicacabum* Linn. has significant antioxidant activity.

Conclusion: The present study suggested that the aerial part extract of *C. halicacabum* serves as a good source of natural antioxidants.

Keywords: Antioxidants, Cardiospermum halicacabum Linn., Free radicals scavenging activity.

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INTRODUCTION

Free radicals are chemically reactive oxygen or nitrogen containing molecules that are generated as a natural by-product of the cell metabolism. Free radicals are constantly generated and eliminated under normal physiologic conditions and have important functions in cell signaling, homeostasis, and clearance of microbial infections. Free radicals have unpaired electron in the outer shell of the molecule. Hence, these free radicals are highly reactive. These radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in disruption of a living cell. Free radicals may be either oxygen-derived reactive oxygen species or nitrogen-derived reactive nitrogen species. The oxygen-derived molecules are superoxide (O^-_2), hydroxyl (HO), hydroperoxyl (HO_2), peroxyl (ROO), and alkoxyl (RO) as free radicals and H_2O_2, oxygen as non-radical. Nitrogen-derived oxidant species are mainly nitric oxide (NO), peroxynitrate (ONOO), nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3). In a normal cell, there is a balance between oxidant and antioxidant. During times of environmental stress such as exposure to ionizing and ultraviolet radiation (UV) or heat, environmental pollutants, or during infection, free radicals are generated more in the body and the oxidant-antioxidant balance is disturbed which leads to oxidative stress. Oxidative stress can result in damage to cellular proteins, lipids, and DNA, leading to variety of human diseases such as inflammation, autoimmune diseases, diabetes, cardiovascular disease, cancer, and aging [1,2].

Antioxidants are the substances that significantly delay or prevent oxidation of oxidizable substrates when present at lower concentrations than the substrate [3]. The excessive production of free radicals should be neutralized or minimized by effective antioxidative system comprising of non-enzymatic as well as enzymatic antioxidants [4]. The enzymatic antioxidants which include superoxide dismutase, catalase, and glutathione peroxidase are present in plasma and non-enzymatic antioxidants include albumin, transferrin, ceruloplasmin, tocopherol (Vitamin E), quinines, bilirubin, ascorbic acid (Vitamin C), uric acid, copper, manganese, zinc, and carotenoids (Vitamin A) [5].

Antioxidants may be synthetic or natural. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole are widely used in food industry, cosmetics, and therapeutic industry. Synthetic antioxidants are highly volatile and instable in elevated temperature and carcinogenic in nature [6]. Due to these reasons, people have switched to nature. Many phytocompounds present in plants, vegetable, and fruits have free radical scavenging or active oxygen scavenging capacity. These are called natural antioxidants. Flavonoids and phenolic acids are most important groups of secondary metabolites and bioactive compounds in plants, and they are good sources of natural antioxidants in human diets. Due to their antioxidant properties, the plants show antiulcer, anti-inflammatory, antimicrobial, cytotoxic, and antitumor activities [7].

The family Sapindaceae has a wide spread of distribution with 136 genera and 2000 species. Ethnomedicinal information revealed that the extracts from members of this family are commonly used for the treatment of boils, ulcers, pain, dermatological troubles, wound healing, rheumatism, diarrhea, and dysentery [8]. *Cardiospermum halicacabum* Linn. belongs to family Sapindaceae. Common name is

Balloon vine or Love in a puff. Tamil name is Mudakathan [9]. They are widely distributed in tropical and subtropical areas of the world. This plant is produced in the plains of Asia and Africa. It is used in the treatment of rheumatism, nervous diseases, stiffness of the limbs, and snakebite [10,11]. Preliminary phytochemical screening of the ethanolic extract of *C. halicacabum* revealed the presence of alkaloids, flavonoids, saponins, proteins, carbohydrates, tannins, and glycosides [12,13]. Hence, the objective of the present study is to assess the antioxidant and free radical scavenging properties of the aerial part extract of *C. halicacabum* by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, ferrous ion chelating activity, NO radical scavenging activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity, and lipid peroxidation activity.

METHODS

Dried powdered form of aerial parts of *C. halicacabum* extract was obtained from Green Chem, Bengaluru.

Antioxidant and free radical scavenging activities of the aerial parts extract of *C. halicacabum* were determined by DPPH radical scavenging assay, ABTS radical scavenging assay, hydroxyl radical scavenging assay [8,14,15], ferrous ion chelating assay, NO radical scavenging assay, superoxide radical scavenging assay, and lipid peroxidation assay method [16].

DPPH scavenging activity

The antioxidant activity of the extract was estimated on the basis of the radical scavenging effect of the stable DPPHi. Various concentrations of the extract were added to a methanolic 0.4 mM DPPHi solution (0.1 ml) in a 96-well plate. The reaction mixture was shaken vigorously and allowed to stand for 30 min at 37°C. The degree of DPPHi purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract. The absorbance of the mixture was determined at 517 nm using UV-visible microplate reader, and ascorbic acid was served as a positive control. The scavenging activity against DPPHi was calculated using the following equation:

Scavenging activity (%) = [1 - (A1 - A2)/A0]×100%

Where
A0 was the absorbance of control (DPPHi solution without the extract),
A1 was the absorbance of DPPHi solution in the presence of the extract, and
A2 was the absorbance without DPPH solution.

ABTS radical scavenging activity

ABTS radical cation was produced by the reaction of a 7 mmol/L ABTS solution with 2.45 mmol/L potassium persulfate. The mixture was stored in the dark at room temperature for 12 h before use. The $ABTS^{+}$ solution was diluted with ethanol to an absorbance at 734 nm. After addition of 25 µL of sample or standard to 2 mL of diluted $ABTS^{+}$ solution, absorbance at 734 nm was read after 6 min. A standard curve was prepared by measuring the reduction in absorbance of $ABTS^{\bullet+}$ solution at different concentrations of extract. Appropriate blank measurements were carried out and the values were recorded with ascorbic acid as the positive control.

Ferrous ion chelating activity

The extract (2 ml) was added to a solution of 2 mM $FeCl_2$ (0.1 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), and the mixture was shaken vigorously and left standing at room temperature for 5 min. Absorbance was measured at 562 nm and ethylenediaminetetraacetic acid (EDTA) was used as a positive control. The percentages of inhibition of ferrozine-Fe^{2+} complex formation were given by the following formula:

Ferrous ion chelating activity (%) = [1 - (A1 - A2)/A0] × 100%

Where
A0 was the absorbance of the control (the mixture without the extract),
A1 was the absorbance of the mixture in the presence of the extract, and
A2 was the absorbance without ferrozine.

Nitric oxide radical scavenging assay

At physiological pH, aqueous solution of Sodium nitroprusside (SNP), spontaneously generated iNO which interacted with oxygen to produce nitrite ion, which was estimated by using Griess reagent. The reaction mixture containing 2 ml of the extract at different concentrations and 50 mM SNP (0.5 ml) in 10mM PBS was incubated at 37°C for 60 min. An aliquot (0.5 ml) of the incubated solution was pipetted out and diluted with 0.5 ml of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride [NED]). The absorbance of the chromophore that formed during diazotization of nitrite with sulfanilamide and subsequent coupling with NED was immediately recorded at 540 nm. The absorbance from various concentrations of sodium nitrite salt treated the same way with Griess reagent was plotted for a standard curve. α-tocopherol was used as a standard. The capability to scavenge iNO radicals was calculated using the following equation:

Scavenging activity (%) = [1 - (A1 - A2)/A0]×100%

Where
A0 was the absorbance of the control (the reaction mixture without the extract),
A1 was the absorbance in the presence of the extract, and
A2 was the absorbance without Griess reagent.

Superoxide radical scavenging activity

The assay was based on the capacity of the plant extracts to inhibit nitro blue tetrazolium (NBT) up to 50% in the presence of riboflavin-light-NBT system. The reaction medium contains 50 mM phosphate buffer pH 7.6, 20 µg riboflavin, 12 mM EDTA, different concentrations of extract (5-200 µg/ml), and NBT 0.1 mg/3 ml, and BHT was taken in different test tube and the same reagents were added. The reaction was started by illuminating the sample cuvette at regular intervals of 30 s, and increases in absorbance were measured at 590 nm up to 2.5 min. The superoxide radical scavenging activity was calculated using the following formula:

% inhibition of superoxide radical= OD (extract absent) - OD (extract present)/OD (extract absent).

Hydroxyl radical scavenging activity

The effect of extract on hydroxyl radical was assessed using the deoxyribose method. The reaction mixture contained 450 µl of 0.2 M sodium phosphate buffer (pH 7.0), 150 µl of 10 mM 2-deoxyribose, 150 µl of 10 mM $FeSO_4$-EDTA, 150 µl of 10 mM H_2O_2, 500 µl of H_2O, and 100 µl of sample solution (5–200 µg/ml). The reaction was started by the addition of H_2O_2. After incubation at 37°C for 4 h, the reaction was stopped by adding 750 µl of 2.8% trichloroacetic acid and 750 µl of 1% thiobarbituric acid in 50 mM NaOH, and the solution was boiled for 10 min and then cooled in ice water. The absorbance of the solution was measured at 520 nm. Here, ascorbic acid was used as positive control.

Lipid peroxidation assay

The liver homogenate was washed several times with ice-cold buffer solution (0.15 M KCl, pH 7.4). A 10% liver homogenate was prepared, and lipid peroxidation was initiated by the addition of 25 µM $FeSO_4$, 100 µM ascorbate, and 10 mM KH_2PO_4. The homogenates were incubated at 37°C for 30 min with different concentrations of extract (5–200 µg/ml). Lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) by extracting with n-butanol and pyridine (15:1.v/v). TBARS formed was determined at 532 nm OD.

The amount of effective concentration of extract needed to inhibit free radicals by 50% IC_{50} was estimated from regression analysis between scavenging activities (%) and various concentration of the extract [14].

RESULTS

The antioxidant and free radical scavenging activities of aerial parts extract of *C. halicacabum* Linn. was examined by DPPH, ABTS, NO, superoxide, hydroxyl radical scavenging activities, ferrous ion chelating activity, and lipid peroxidation activity.

DISCUSSION

The results of antioxidant activities of *C. halicacabum* extract revealed that the extract has significant antioxidant activities.

DPPH radical scavenging assay is an easy, rapid, and sensitive method for the antioxidant screening of plant extracts [17]. It was observed that free radical scavenging activity of the extract was increased with an increasing concentration of the extract. Percentage of inhibition on DPPH radical activity of extract was 19.74±1.3 for the concentration of 5 µg/ml and 84.19±2.06 for 200 µg/ml and IC_{50} value was 34.06 µg/ml (Table 1 and Fig. 1a and b).

ABTS radical is stable and soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/samples [18]. Percentage of inhibition on ABTS radical activity of extract was 3.86±0.26 for the concentration of 5 µg/ml and 89.6±0.24 for 200 µg/ml and IC_{50} value was 21.45 µg/ml (Table 2 and Fig. 2a and b).

Ferrous ions are the most powerful pro-oxidant among the various species of metal ions. Hence, it acts as an important lipid oxidation pro-oxidant, which causes severe oxidative damage. Minimizing ferrous ion may exhibit protection against oxidative damage by inhibiting production of ROS and molecular damage [19]. Ferrous ion chelating ability of the extract was increased with concentration. Percentage of ferrous ion chelation of the extract was 29.13±2.1 for the concentration of 5 µg/ml and 85.17±1.58 for 200 µg/ml and IC_{50} value was 19.56 µg/ml (Table 3 and Fig. 3a and b).

Low concentrations of NO are essential for the physiological function because NO is an intracellular messenger for modulating blood flow, thrombosis, and neural activity [20]. The toxicity of NO increases greatly when it reacts with the superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$) [21]. It was observed that the extract scavenged the nitric oxide radical in a dose-dependent manner. Percentage of inhibition on NO radical scavenging activity of the extract was 10.41±0.89 for the concentration of 5 µg/ml and 78.15±0.78 for 200 µg/ml and IC_{50} value was 25.16 µg/ml (Table 4 and Fig. 4a and b).

In the living cells, superoxide radicals are generated by reduction of molecular oxygen into water in electron transport chain and they are formed by activated phagocytes such as monocytes, macrophages, eosinophils, and neutrophils. It is an important factor in the killing

Table 1: DPPH radical scavenging activity of *C. halicacabum* Linn.

Sample	Concentration (µg/ml)	% of inhibition	IC_{50} (µg/ml)
Extract	5	19.74±1.3	34.06
	25	47.19±1.7	
	50	61.75±0.68	
	100	83.17±2.01	
	200	84.19±2.06	
Ascorbic acid - Standard	100	86.14±1.45	17.13

C. halicacabum: Cardiospermum halicacabum, DPPH: 2,2-diphenyl-1-picrylhydrazyl

Table 2: ABTS radical scavenging activity of *C. halicacabum* Linn.

Sample	Concentration (µg/ml)	% of Inhibition	IC_{50} (µg/ml)
Extract	5	3.86±0.26	21.45
	25	16.7±1.05	
	50	30.12±2.4	
	100	60.3±0.69	
	200	89.6±0.24	
Ascorbic acid - Standard	100	76.5±3.15	18.3

C. halicacabum: Cardiospermum halicacabum, ABTS: 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

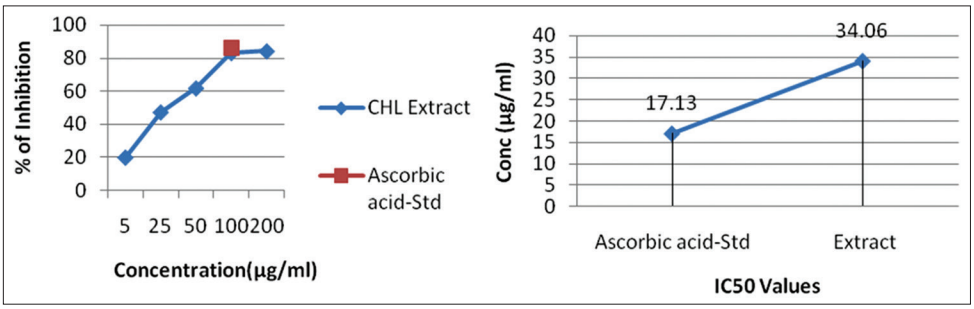

Fig. 1: (a) % inhibition of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of *Cardiospermum halicacabum* Linn. (b) IC_{50} values of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of *C. halicacabum* Linn.

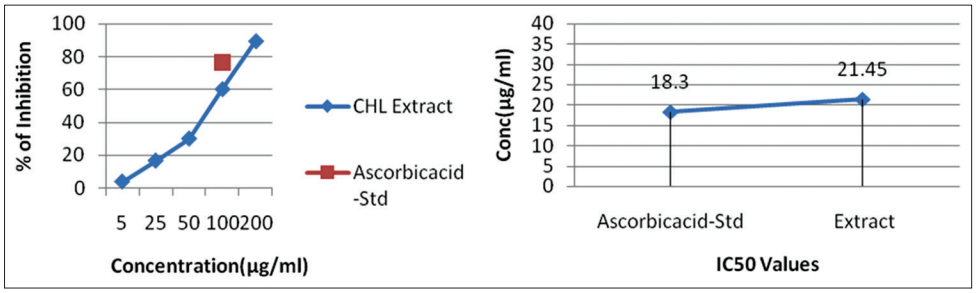

Fig. 2: (a) % of inhibition of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of *Cardiospermum halicacabum* Linn. (b) IC_{50} Values of ABTS radical scavenging activity of *C. halicacabum* Linn.

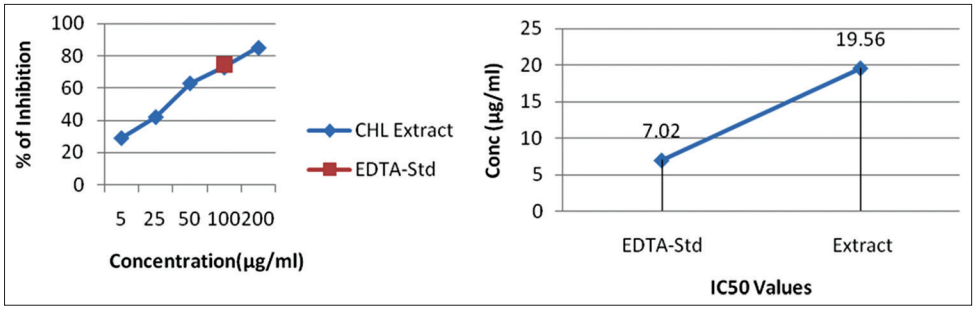

Fig. 3: (a) % of inhibition of ferrous ion chelating activity of *Cardiospermum halicacabum* Linn. (b) IC_{50} values of ferrous ion chelating activity of *C. halicacabum* Linn.

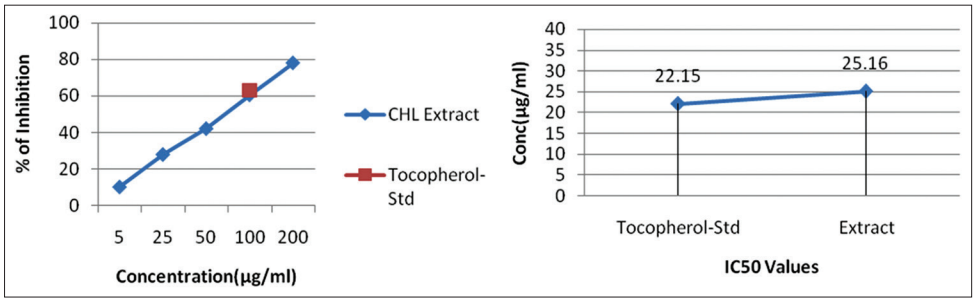

Fig. 4: (a) % of inhibition of nitric oxide (NO) radical scavenging activity of *Cardiospermum halicacabum* Linn. (b) IC_{50} values of NO radical scavenging activity of *C. halicacabum* Linn.

Table 3: Ferrous ion chelating activity of *C. halicacabum* Linn.

Sample	Concentration (µg/ml)	% of inhibition	IC_{50} (µg/ml)
Extract	5	29.13±2.1	19.56
	25	42.16±1.08	
	50	63.14±1.20	
	100	72.91±0.58	
	200	85.17±1.58	
EDTA - Standard	100	74.6±1.23	7.02

C. halicacabum: Cardiospermum halicacabum, EDTA: Ethylenediaminetetraacetic acid

Table 4: NO radical scavenging activity of *C. halicacabum* linn.

Sample	Concentration (µg/ml)	% of Inhibition	IC_{50} (µg/ml)
Extract	5	10.41±0.89	25.16
	25	28.13±1.1	
	50	42.26±0.94	
	100	60.17±1.18	
	200	78.15±0.78	
Tocopherol - Standard	100	63.08±0.51	22.15

C. halicacabum: Cardiospermum halicacabum

of bacteria by phagocytosis [22]. It is observed that the percentage of inhibition of extract on superoxide radical activity was 10.31±0.73 for the concentration of 5 µg/ml and 87.6±1.53 for 200 µg/ml and IC_{50} value was 35.16 µg/ml (Table 5 and Fig. 5a and b).

Hydroxyl radical is the neutral form of hydroxide ion and is a highly reactive free radical. It is formed in a fenton reaction, in which hydrogen peroxide reacts with metal ions (Fe^{2+} or Cu^{+}) often bound in complex with different protein such as ferritin and ceruloplasmin or other molecules and it is formed by the reaction between superoxide radical and hydrogen peroxide in a reaction called Haber–Weiss reaction [23]. It was observed that the percentage of inhibition on hydroxyl radical scavenging activity of the extract was 5.67±0.42 for the concentration

Table 5: Superoxide radical scavenging activity of *C. halicacabum* Linn.

Sample	Concentration (µg/ml)	% of Inhibition	IC_{50} (µg/ml)
Extract	5	10.31±0.73	35.16
	25	19.86±0.46	
	50	40.18±0.94	
	100	71.16±1.06	
	200	87.6±1.53	
BHT - Standard	100	84.73±1.16	7.16

C. halicacabum: Cardiospermum halicacabum

Table 6: Hydroxyl radical scavenging activity of *C. halicacabum* Linn.

Sample	Concentration (µg/ml)	% of Inhibition	IC_{50} (µg/ml)
Extract	5	5.67±0.42	28.56
	25	11.56±0.25	
	50	28.56±0.89	
	100	57.88±1.1	
	200	79.69±2.3	
Ascorbic acid - Standard	100	84.51±5.3	12.24

C. halicacabum: Cardiospermum halicacabum

of 5 µg/ml and 79.69±2.3 for 200 µg/ml and IC_{50} value was 28.56 µg/ml (Table 6 and Fig. 6a and b).

Lipid peroxidation is a physiological process that takes place in all aerobic cells. Unsaturated fatty acids which are structural part of cell membranes are subjected to lipid peroxidation by a non-enzymatic and free radical mediated reaction chain. The destruction of cell membrane lipids and the end product of such lipid peroxidation reactions are dangerous for the viability of cells [24]. It was observed that the percentage of inhibition on lipid peroxidation activity of the extract

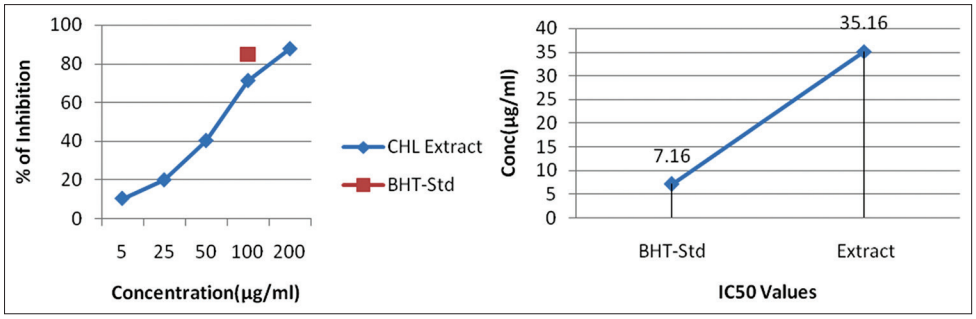

Fig. 5: (a) % of inhibition of superoxide radical scavenging activity of *Cardiospermum halicacabum* Linn. (b) IC_{50} values of superoxide radical scavenging activity of *C. halicacabum* Linn.

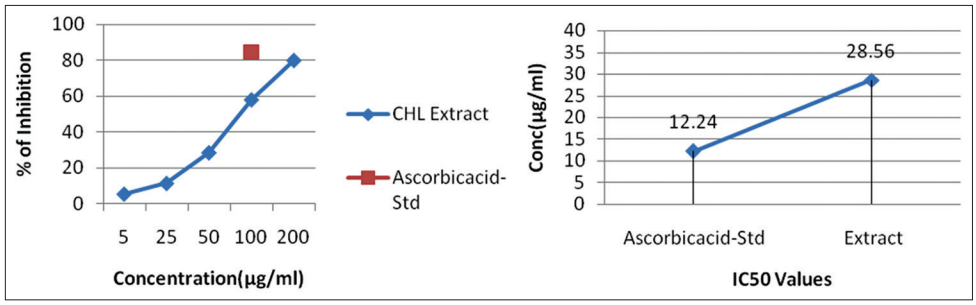

Fig. 6: (a) % of inhibition of hydroxyl radical scavenging activity of *Cardiospermum halicacabum* Linn. (b) IC_{50} values of hydroxyl radical scavenging activity of *C. halicacabum* Linn.

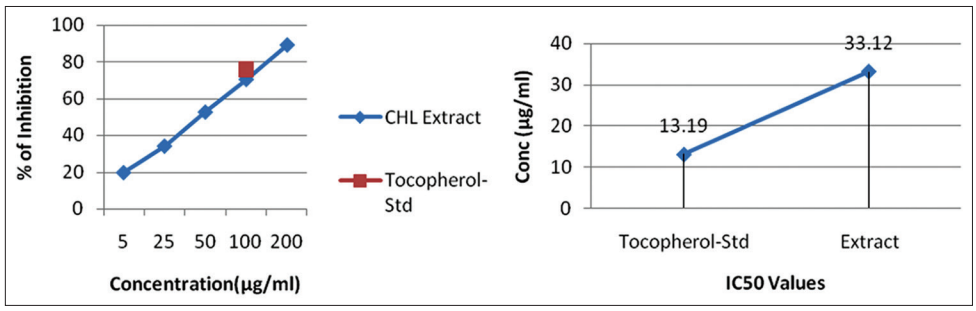

Fig. 7: (a) % of inhibition of lipid peroxidation of *Cardiospermum halicacabum* Linn. (b) IC_{50} values of lipid peroxidation of *C. halicacabum* Linn.

Table 7: Lipid peroxidation of *C. halicacabum* Linn.

Sample	Concentration (µg/ml)	% of Inhibition	IC_{50} (µg/ml)
Extract (Tocopherol - Standard)	5	19.78±1.05	33.12
	25	34.13±3.12	
	50	52.7±1.12	
	100	70.45±2.89	
	200	89.12±1.45	
Tocopherol - Standard	100	75.78±1.07	13.19

C. halicacabum: Cardiospermum halicacabum

was 19.78±1.05 for the concentration of 5 µg/ml and 89.12±1.45 for 200 µg/ml and IC_{50} value was 33.12 µg/ml (Table 7 and Fig. 7a and b).

CONCLUSION

Due to our busy and stressful lifestyle, the production of free radicals in the body is increased. Free radicals play a dual role in the human body either they may be harmful or helpful. Excessive production and accumulation of these radicals cause oxidative stress which leads to disease to human body. The harmful effects of these radicals are destroyed by the substances called antioxidants which may be natural or synthetic. Due to these dangerous side effects and expensive of synthetic antioxidants, finding the natural antioxidants from easily available sources is indispensable. Thus, the current study on forgotten, economical, and readily reachable plant *C. halicacabum* (Mudakathan keerai) revealed the momentous antioxidant activity. Addition of this plant in our day-to-day life may prevent the occurrence of many diseases to human health.

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CONFLICTS OF INTEREST

The authors of this article declare no conflict of interest in this study.

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