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GV Sasi Rekha
Research Scholar, Department of Biochemistry, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India

Dr. PT Devika
Assistant professor and Research Supervisor, PG and Research Department of Biochemistry, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India

Correspondence
Dr. PT Devika
Assistant professor and Research Supervisor, PG and Research Department of Biochemistry, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India

Antioxidant activities and GC-MS analysis of ethanol extract of creeper stems of *Cissus quadrangularis* L.

GV Sasi Rekha and Dr. PT Devika

Abstract
Cissus quadrangularis belongs to the family Vitaceae, which is a perennial climber, found mostly in the hotter parts of the world such as India, Sri Lanka, Tropical Africa, Thailand, Java, and Philippines. The plant is mentioned in the ancient system of medicine such as Ayurveda, and is useful for treatment of bloody diarrhoea, skin disorders, earache, haemorrhoids, irregular menstruation, and accelerates healing of bone fracture. The aim of the study was to evaluate antioxidant activities and GC-MS analysis of ethanol extract of *C. quadrangularis*. Antioxidant activities such as DPPH radical, phosphomolybdenum reduction and Fe^{3+} reducing power assays were carried out. The maximum DPPH radical scavenging activity was 51.17% at 300 µg/mL concentration. The maximum phosphomolybednum reduction and Fe^{3+} reduction were 84.21% and 58.57% at 300 µg/mL concentration. GC-MS analysis was carried out for the identification of known active compounds present in the ethanol extract of *C. quadrangularis*.

Keywords: Antioxidant, DPPH˙ radical, ABTS, GC-MS

1. Introduction
Cissus quadrangularis, commonly known as (Hadjod) which is a perennial plant of the family Vitaceae. It is also known as pirandai, Adamant creeper, veldt grape, Square stalked vine, devil's backbone, adamant creeper, asthisamharaka, Sannalam, Nalleru, Vajravelli, Mangara valli. The native of Cissus quadrangularis is India, Bangladesh and Sri Lanka. This plant also found in Africa and Southeast Asia. It also being imported to Brazil and the southern United States. *Cissus quadrangularis* which reaches a height of 1.5 m and has quadrangular-sectioned branches with internodes of about 8 to 10 cm long and 1.2 to 1.5 cm wide. Along each angle is a leathery edge. The Toothed trilobe creeper stems 2 to 5 cm wide appear at the nodes. Each has a tendril emerging from the opposite side of the node. Racemes of small white, yellowish, or greenish flowers, globular berries are red when ripe [1].
Traditionally it was mostly used in treatment of female disorders (libido and menstrual disorders) and treating bone disorders (increasing bone mass or accelerating fracture healing rates) which gives it the traditional name of the 'Bone Setter'. The stemss are most useful for healing of fracture of the bones [2]. The plant has been documented in Ayurveda for the treatment of osteoarthritis, rheumatoid arthritis and osteoporosis. The stems juice of plant is used to treat scurvy, menstrual disorders and epistaxis. This herb is fed to cattle to induce flow of milk. The stout fleshy quadrangular stems is traditionally used for treatment of gastritis constipation, eye diseases, piles and anemia [3].

1.1. Taxonomy
Kingdom: Plantae
Subkingdom: Tracheobionta
Super division: spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Vitales
Family: Vitaceae
Genus: *Cissus*
Species: *quadrangularis*

Fig 1: Habitat of *C. quadrangularis*

2. Materials and Methods

2.1. Collection of creeper stems and preparation of extract

The creeper stems of *C. quadrangularis* were collected from Ambattur, Tamilnadu, India. The creeper stems were washed, shade dried for 5 days and coarse powdered by mechanical blender. About 10 g of creeper stems powder was soaked in ethanol for 72 h. The greenish supernatant liquid was filtered by filter paper and condensed in rotor evaporator at 50 °C, which yields gummy extract.

2.2. *In vitro* antioxidant assays

2.2.1. DPPH˙ radical scavenging assay

The antioxidant activity of ethanol extract of creeper stems of *C. quadrangularis* was measured on the basis of stable DPPH free radical reduction method [4]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations (50-300 µg/mL) of creeper stems extract. The mixture was then allowed to stand for 30 min incubation in dark. One mL methanol and 1 mL DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis Spectrophotometer at 517 nm. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\% \text{ of DPPH}^{\cdot} \text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

2.2.2. Superoxide radical ($O_2^{\bullet-}$) scavenging assay

Superoxide radical scavenging activity was carried out by the method of Ravishankara *et al*. [5]. Different concentrations of creeper stems extract (50-300 µg/mL) of *C. quadrangularis* was mixed with 50 mM of phosphate buffer (pH 7.8), 1.5 mM of riboflavin, 12 mM of EDTA and 50 mM of NBT solutions and added in that sequence. The reaction was started by illuminating the reaction mixture for 15 min. After illumination, the absorbance was measured at 590 nm in UV-Vis Spectrophotometer. Ascorbic acid was used as standard reference. The percentage of inhibition was calculated as:

$$\% \text{ of superoxide radical } (O_2^{\bullet-}) \text{ inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

2.2.3. ABTS$^{\bullet+}$ radical cation scavenging assay

The antioxidant capacity was estimated in terms of the ABTS$^{\bullet+}$ radical cation scavenging activity [6]. ABTS$^{\bullet+}$ was obtained by reacting 7 mM ABTS solution in 5 mM of phosphate-buffered saline (pH 7.4) with 2.45 mM potassium persulfate and the mixture was left to stand in dark at room temperature for 12-16 h before use. The ABTS solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) till to reach an absorbance of 0.70±0.02 at 734 nm. To the various concentrations (5-30 µg/mL) of ethanol extract of creeper stems of *C. quadrangularis*, 500 µL of diluted ABTS$^{\bullet+}$ solution was added. The absorbance was measured after 10 min incubation at 734 nm. Ascorbic acid was used as the standard reference. The ABTS$^{\bullet+}$ radical cation scavenging activity was expressed as:

$$\% \text{ of ABTS}^{\bullet+} \text{ radical cation inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

2.2.4. Phosphomolybdenum reduction assay

The antioxidant capacity of ethanol extract of creeper stems of *C. quadrangularis* was assessed by Mo^{6+} reduction method [7]. The creeper stems extract with concentrations ranging from 50-300 µg/mL was combined with 1 mL of reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in water bath at 95ºC for 90 min. The absorbance of the coloured complex was measured at 695 nm in UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\% \text{ of phosphomolybdenum reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

2.2.5. Ferric (Fe $^{3+}$) reducing power assay

The reducing power of ethanol extract of creeper stems of *C. quadrangularis* was determined by Fe^{3+} reduction method with slight modification [8]. One mL of creeper stems extract of different concentrations (50 - 300 µg/mL) was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1 % (w/v) potassium ferricyanide [$K_3Fe(CN)_6$] solution. The mixtures were then incubated at 50 °C in water bath for 30 min. One mL of 10 % (w/v) trichloroacetic acid was added to each mixture. Then 1 mL of 0.1% (w/v) freshly prepared $FeCl_3$ solution was added and the absorbance was measured at 700 nm in UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\% \text{ of } Fe^{3+} \text{ reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

2.3. Gas chromatography–Mass Spectrometry (GC–MS)

In GC-MS analysis, the ethanol extract of creeper stems of *C. quadrangularis* was injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Following conditions were used: Helium as carrier gas, flow rate of 1 mL/min; and the injector was operated at 200 °C and column oven temperature was programmed as 50-250 °C at a rate of 10 °C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV; ion source

temperature of 250 °C; interface temperature of 250 °C; and mass range of 50-600 mass units [9].

2.3.1. Identification of components
The database of National Institute Standard and Technology (NIST) having more than 62,000 patterns was used for the interpretation on mass spectrum of GC-MS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

3. Results and Discussion
3.1. DPPH˙ radical scavenging assay
DPPH˙ (1,1-Diphenyl-2-picrylhydrazyl) is a stable nitrogen-centered free radical which has an unpaired valence electron at one atom of nitrogen bridge. Scavenging of DPPH free radical is one of the popular antioxidant assays. DPPH radical scavenging assay is a decolorization assay that will measure the capacity of antioxidants to directly scavenge DPPH˙ radicals by monitoring its absorbance using spectrophotometer at wavelength of 517 nm [10]. The DPPH assay provided rapid and an easy way to evaluate the antioxidant activity of most of the plant extracts. The ethanol creeper stems extract of *C. quadrangularis* to scavenge free radicals was assessed by using DPPH˙ radical as the substrate, which measures the hydrogen or electron donating ability of creeper stems extract. The creeper stems extract of *C. quadrangularis* reducing the stable purple colour DPPH (1,1-diphenyl-2- picrylhydrazyl) free radical to the yellow coloured 1,1-diphenyl-2-picrylhydrazine and the reduction capacity increases with increasing concentration of the extract. The maximum DPPH˙ radical scavenging activity was 51.17±0.19% at 300 µg/mL concentration (Table 2 and Fig 2). The IC_{50} was 83.88 µg/mL concentration and was compared with standard ascorbic acid (IC_{50} = 6.31 µg/mL concentration).

3.2 Superoxide ($O_2^{\cdot-}$) radical scavenging assay
Superoxide anion is also very harmful to cellular components and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents [11]. Flavonoids are effective antioxidants, mainly because they scavenge superoxide anions. Superoxide anions derived from dissolved oxygen by the riboflavin-light-NBT systems will reduce NBT in this systems. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to blue formazan, which is measured at 590 nm in UV-Vis spectrophotometer. Antioxidants are able to inhibit the blue NBT formation and the decrease of absorbance with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The maximum superoxide radical scavenging activity of *C. quadrangularis* was 54.24±0.28% at 300 µg/mL concentration (Table 2 and Fig 2) and the IC_{50} was 20.60 µg/mL concentration. It was compared with the standard of ascorbic acid (IC_{50} = 9.65 µg/mL concentration).

Table 1: DPPH˙ radical and superoxide ($O_2^{\cdot-}$) radical scavenging activities of ethanol extract of creeper stems of *C. quadrangularis*

S. No.	Concentration (µg/mL)	% of inhibition	
		DPPH˙ radical at 517 nm	Superoxide ($O2^{\cdot-}$) radical at 590 nm
1	50	12.01	17.95
2	100	24.28	27.41
3	150	31.59	32.04
4	200	40.20	39.58
5	250	44.12	42.66
6	300	51.17	54.24

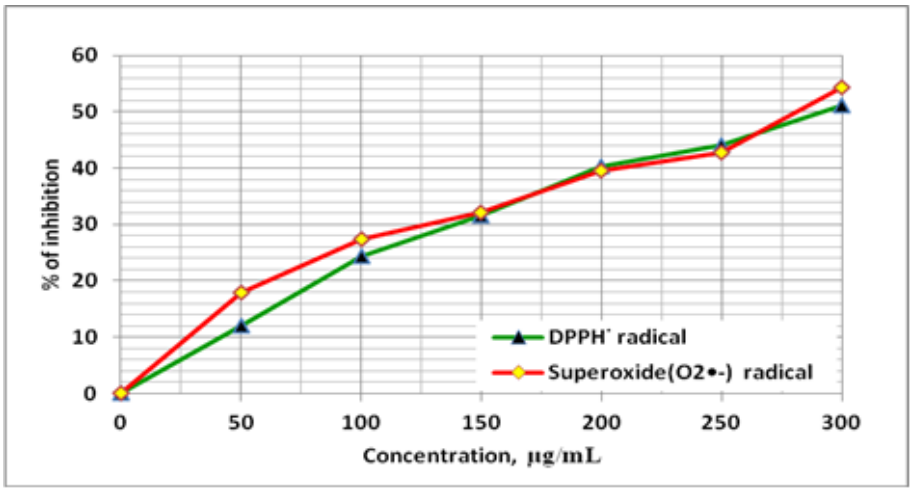


Fig 1: DPPH˙ radical and Superoxide ($O_2^{\cdot-}$) radical scavenging activity of ethanol extract of creeper stems of *C. quadrangularis*

3.3. $ABTS^{\bullet+}$ radical cation scavenging assay
$ABTS^{\bullet+}$ is a blue-green chromophore produced by the reaction between ABTS and potassium persulfate. The blue-green chromophore radical cation gets reduced while reacting with an antioxidant in the creeper stems extract of *C. quadrangularis* and the remaining radical cation concentration was quantified with the loss of colour[12]. The antioxidant reduces $ABTS^{\bullet+}$ to ABTS and decolorize the blue-green chromophore. The maximum ABTS•+ radical cation scavenging activity was 88.93±0.23% at 30 µg/mL concentration (Table 3 and Fig 3) with the IC_{50} of 11.75 µg/mL concentration and was compared with standard ascorbic acid (IC_{50} = 4.21 µg/mL concentration).

Table 2: ABTS$^{\cdot+}$ radical cation scavenging assay of ethanol extract of creeper stems of *C. quadrangularis*

S. No	Concentration (µg/mL)	% of inhibition
		ABTS$^{\bullet+}$ radical cation
1	5	22.63
2	10	36.16
3	15	45.11
4	20	48.50
5	25	52.09
6	30	54.44

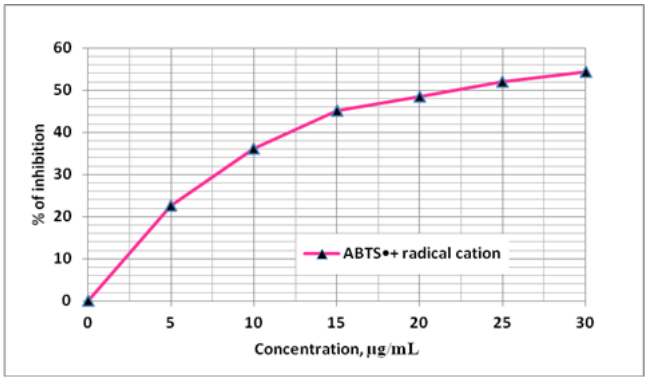


Fig 2: ABTS$^{\bullet+}$ radical cation scavenging assay of ethanol creeper stems extract of *C. quadrangularis*

3.4. Phosphomolybdenum reduction assay

Metal-Catalyzed Oxidation (MCO) systemss catalyze the reduction reaction, which alters the nature of proteins at the metal-binding site and cause DNA and protein damage [13]. The total antioxidant activity of ethanol creeper stems extract of *C. quadrangularis* was measured by phophomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm. The maximum phosphomolybdenum reduction was 79.68±0.41% at 120 µg/mL concentration with the RC_{50} of 56.35 µg/mL concentration (Table 4 and Fig 4). It was compared with the standard ascorbic acid (RC_{50} =6.34 µg/mL).

Table 3: Phophomolybdenum reduction and Fe^{3+} reducing power assay of ethanol extract of creeper stems of *C. quadrangularis*

S. No.	Concentration (µg/mL)	% of reduction	
		Phosphomolybdenum reduction at 695 nm	Fe^{3+} reducing power at 700 nm
1	50	47.57	35.98
2	100	64.36	51.98
3	150	70.65	52.99
4	200	73.20	54.50
5	250	81.91	57.69
6	300	84.21	58.57

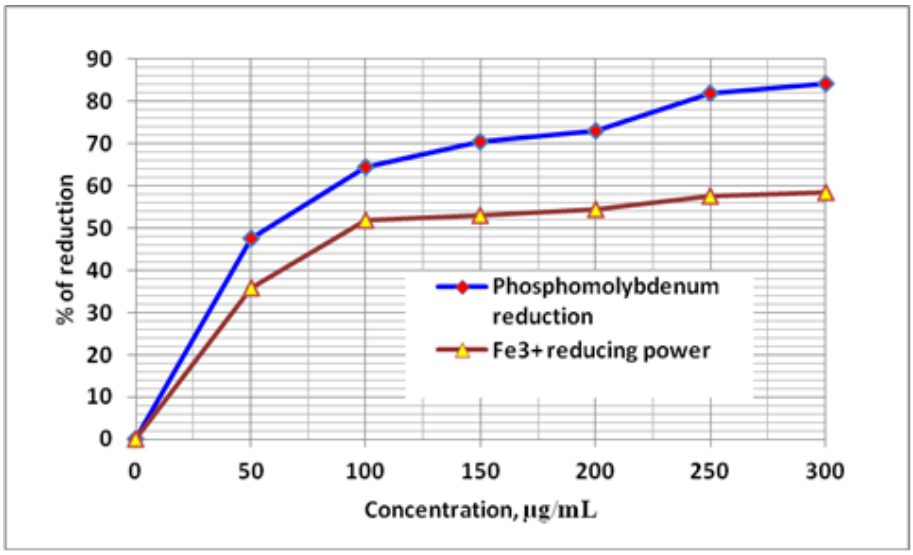


Fig 3: Phophomolybdenum reduction and Fe^{3+} reducing power assay of ethanol extract of creeper stems of *C. quadrangularis*

3.5. Ferric (Fe^{3+}) reducing power assay

The reducing power assay was carried out by the reduction of Fe^{3+} to Fe^{2+} by the ethanol extract of creeper stems of *C. quadrangularis* and the subsequent formation of ferro-ferric complex. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action [14]. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom or by neutralizing the free radicals by donating an electron and become lone pair of electrons instead of odd electron. The reduction ability increases with increase in concentration of the extract. The maximum Fe^{3+} reduction was 47.88±0.47% at 120 µg/mL concentration with the RC_{50} of 125.31 µg/mL concentration (Table 4 and Fig 4) and was compared with the standard ascorbic acid (RC_{50} =7.72 µg/mL).

3.6. GC-MS analysis

GC-MS analysis was carried out for ethanol extract of creeper stems of *C. quadrangularis* and the eluted compounds were showed in Table 6. An anti-inflammatory compound 3-(4-Hydroxyisopent-2-(Z)-enyl)-4-hydroxyacetophenone [15] antioxidant compounds such as pentadecanoic acid,14-methyl-methyl ester [16], n-hexadeconoic acid [17] and octadecanoic acid, methyl ester [18] , Polydatin [19] as well as visual pigments regulator 9-Cis- retinal [20] were eluted and recorded (Table 5).

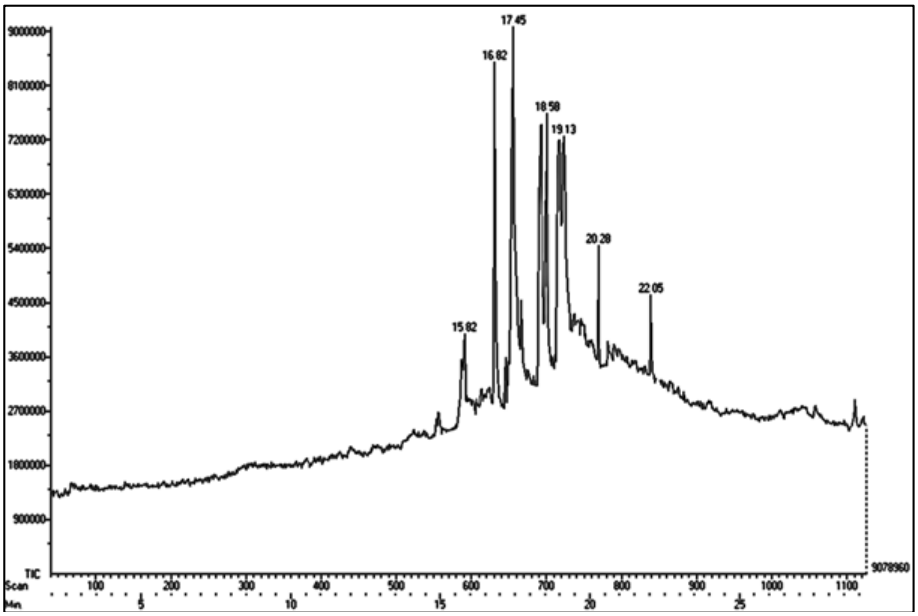
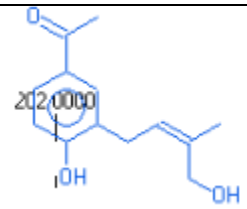
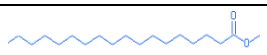
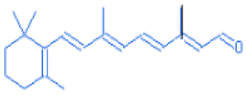
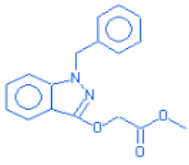
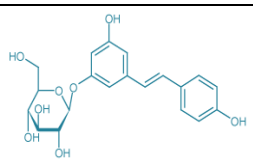


Fig 4: Gas chromatogram of ethanol creeper stems extract of *C. quadrangularis*

Table 4: GC-MS analysis of ethanol creeper stems extract of *C. quadrangularis*.

S. No	Compound Name	RT	Compound Structure	Molecular Weight (g/Mol)	Molecular Formula	Pharmacological activity
1	3-(4-Hydroxyisopent-2-(Z)-enyl)-4-hydroxyacetophenone	15.82		220.78	$C_{13}H_{16}O_3$	Anti-inflammatory
2	Pentadecanoic acid,14-methyl-methyl ester	16.82		269.88	$C_{17}H_{34}O_2$	Antioxidant
3	n-Hexadecanoic acid	17.45		255.87	$C_{16}H_{32}O_2$	Antioxidant, Hypochloesterolemic, Nematicide, Pesticide, Lubricant, Antiandrogenic, Haemolytic, 5-Alpha reductase inhibitor.
4	Octadecanoic acid, methyl ester	18.58		298	$C_{19}H_{38}O_2$	Antioxidant
5	Retinal,9-Cis-	19.13		283.88	$C_{20}H_{28}O$	Universal chromophore of the visual pigments in the eye, and the hormonal retinoids, mainly all-trans- and 9-cis-retinoic acid (RA),2 regulate the expression of target genes via activation of two classes of nuclear retinoid receptors, the retinoic acid receptors (RARs), and the retinoid X receptors (RXRs).
6	Bendazac methyl ester	20.28		296.87	$C_{17}H_{16}N_2O_3$	Used to treat Inflammation, Dermatitis, Eczema, Pruritis, hives, insect bites, burns, erythema
7	3,4,5-trihydroxystilbene-3-beta-monoglucoside (polydatin)	22.05		390.38	$C_{20}H_{22}O_8$	Antioxidant,Anticancer , Antibacterial agent, Anti-arteriosclerosis,Hepato protective, Neuroprotective, , Antibacterial activity.

4. Conclusion

C. quadrangularis is a traditional medicine in Ayurveda, which is used to treat menopause, libido, and menstrual disorders and to treat bones as increasing bone mass or accelerating fracture healing rates, which gives it the traditional name of the 'Bone Setter'. Also it has antiulcer properties, antihemhorroid properties and pain relieving properties. GCMS analysis showed the presence of antioxidant compounds and should be reflected in antioxidant activity significantly. Now-a-days *C. quadrangularis* has drawn much attention for its medicinal uses because of its efficacy and safety for human use. It does not produce any toxic effects when used orally and due to its various inherent pharmacognostic properties. Further research work is needed to recommend for human welfare.

5. Reference

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