Int J Pharm Bio Sci 2014 Jan; 5(1): (P) 344 - 351

Research Article **Phytochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

HPTLC ANALYSIS AND *IN VITRO* ANTIHEMOLYTIC ACTIVITY OF FLAVONOIDS FROM *CROTALARIA GLOBOSA*

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ABSTRACT

Antioxidants generally possess the ability to protect the body from damage caused by free radical induced oxidative stress and disorders. Polyphenolics, especially flavonoids from plants serve as a good source of antioxidants. Investigation on the efficiency of these phenolics is of interest during recent years. The purpose of this work is to determine the total phenolic content, total flavonoid content and total tannin content of ethyl acetate and ethanol fractions from a novel plant source; *Crotalaria globosa*. *In vitro* investigation of antihemolytic activity for the bioactive ethyl acetate fraction (IC_{50} = 184.06 ± 1.94) revealed *C.globosa* leaves to be a good antioxidant. Further, high pressure thin layer chromatographic analysis is also carried out for the extracts of varying polarity of solvents which shows the presence of 14 different flavonoids. Ethyl acetate fraction yields more number of flavonoids compared to other solvent fractions. Bioactive compounds in these leaves can be suitably isolated and identified to develop new drugs which may be used to treat many ailments.

KEY WORDS: ***Crotalaria globosa*, antihemolytic, TPC, TFC, TTC, HPTLC.**

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INTRODUCTION

Secondary metabolites derived from plants are found to be the richest bio-resource of drugs used in traditional systems of medicine [1, 2]. Flavonoids, a secondary metabolite derived from higher plants, constitute one of the most ubiquitous groups of all plant phenolics. So far, over 8,000 varieties of flavonoids have been identified [3]. The best-described property of flavonoid is their capacity to act as antioxidants, and they have the capability to scavenge the free radicals produced in the body by neutralizing them [4]. They also have long been recognized to possess anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities [5, 6]. Hence it is of interest to identify the novel source of drugs and to quantify the amount of flavonoids present in it [7]. *Crotalaria globosa (C.globosa) Wight* & *Arn*, is a weed distributed worldwide and used as food for cattle, commonly called as cattle pods. A few species of *Crotalaria* are known to be ornamental [8]. It is also used as a medicine in treatment of various diseases [9]. In the present study, crude ethanolic extract of *C.globosa* leaves has been determined for its total phenolic content (TPC), total flavonoid content (TFC) and total tannin content (TTC). The existence of flavonoids has been proved using high performance thin layer chromatography (HPTLC) technique. *In vitro* study on antihemolytic activity of the separated erythrocytes from Wistar rat blood provides evidence to the antioxidant behavior of the flavonoid rich fraction.

MATERIALS AND METHODS

Plant collection and identification

The plant material *C.globosa* is collected from the surrounding area of Sathyamangalam, Erode district, Tamil Nadu, India, during the month of September 2012. A voucher specimen is deposited (MH/174353) at Botanical survey of India, Southern circle, Coimbatore, Tamil Nadu. The plant specimens collected are shade dried at room temperature (25^0C), pulverized and used for further analysis.

Chemicals used

Folin-Ciocalteu reagent, sodium carbonate, polyvinyl polypyrrolidone (PVPP), sodium nitrite, sodium hydroxide, toluene, acetone, formic acid, gallic acid and tannic acid are obtained from Aldrich Co, India. All other chemicals and solvents used in this study are of analytical grade.

Preparation of extract and solvent partitioning

About 400 g of the powdered leaves of *C.globosa* is extracted with 1.7 L of aqueous ethanol (EtOH) for 24 h and filtered. Resulting extract is concentrated in vacuo at 40 °C using a rotary evaporator (Cyber lab, USA, CR 2000), to yield the crude extract of *C.globosa* (95.0 g). About half of the crude extract (45 g) is suspended in distilled water (50 ml) and separately partitioned (100 ml x 3) with hexane to remove wax and chlorophyll followed by ethyl acetate (EtOAc), ethanol (EtOH) and water. The organic layer of each solvent is concentrated to dryness in vacuo at 40 °C using a rotary evaporator to afford the hexane, EtOAc, EtOH and water fractions. The above said fractions are utilized for HPTLC analysis. The EtOAc fraction is centrifuged at 10000 rpm (Table top refrigerated centrifuge (swinging bucket rotor and fixed angle) Remi C24) to yield yellow solid. The EtOH and EtOAc(s) fraction obtained are used for the determination of TPC, TFC and TTC. To assess the radical scavenging activity of flavonoid rich fraction, antihemolytic assay for EtOAc(s) has been investigated.

Determination of total phenolic content

The method of Siddhuraju *et al* [10] is conceded to determine the total phenolic content. Aliquots of EtOAc(s) and EtOH fractions of leaves of *C. globosa* are diluted to the volume of 1 ml with distilled water. 0.5 ml of Folin – Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium

carbonate solution (20%) are added sequentially to the above fractions. After vortexing, the reaction mixture is allowed to stand in dark for 40 min and the absorbance is recorded at 725 nm against the reagent blank. The analysis is performed in triplicate and the results are expressed in gallic acid equivalents.

Determination of total tannin content

The total tannin content has been determined using the method of Siddhuraju *et al* [11]. 100 mg of polyvinyl polypyrrolidone (PVPP) is weighed in a test tube (100×12 mm), to this 1 ml distilled water and 1µg of EtOAc(s) are added, vortexed at 4 ˚C for 4 h. Finally the mixture is centrifuged at 3000 rpm for 10 min and the supernatant is collected which contains only simple phenolics other than tannins. The phenolic content of the supernatant is measured in gallic acid equivalents and expressed as the content of non-tannin phenolics (tannic acid equivalents) on a dry matter basis. The same procedure is repeated for EtOH fraction. From the above results, the tannin content of the sample is calculated as follows:

Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%)

Determination of total flavonoid content

Slightly modified colorimetric method of Zhishen et al [12] is followed. The EtOAc(s) and EtOH fractions of *C.globosa* (0.5 ml) are separately mixed with 2 ml distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ is added and allowed to stand for another 6 min, then 2 ml of 4% NaOH solution is added to the mixture. Further the mixture is diluted with 5 ml of distilled water and allowed to stand for 15 min. The absorbance is measured against water as reagent blank at 510 nm. Flavonoid concentration obtained is expressed as rutin equivalent per 100 g of extract.

HPTLC analysis of flavonoids

Of all the chromatographic methods, HPTLC technique is sophisticated and economic. It provides an excellent accuracy of separation, qualitative and quantitative analysis of crude samples containing wide range of compounds [13]. Different fractions obtained from the crude ethanol extract of *C.globosa* leaves (hexane, EtOAc, EtOH and water) are separated, dried and used for HPTLC analysis. The sample (100 mg) is weighed, dissolved in 1 ml ethanol and centrifuged at 3000 rpm for 5 min. The test solutions of 2 µL each are loaded as 5 mm band in the 8 x 10 silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 applicator. The mobile phase used is toluene-acetone-formic acid (4.5:4.5:1). The samples loaded plate is kept in TLC twin trough developing chamber (after saturating with solvent vapor) with respective mobile phase (flavonoid) and the plate is developed upto 90 mm. It is then dried in a hot air oven to evaporate the solvents and sprayed with 1% of ethanolic aluminium chloride reagent. The plate is kept in photo-documentation chamber (CAMAG REPROSTAR 3) and the images are captured under UV light at 366 nm.

Antihemolytic activity

Antihemolytic activity of the solid obtained from EtOAc fraction of *C.globosa* extract is assessed by the method of Naim *et al.* with slight modifications [14]. The erythrocytes are separated by centrifugation from Wistar rat blood and washed with 0.2 M phosphate buffer (pH 7.4) to give 4 % suspension [15]. About 200 – 1000 µg of extract in saline buffer is added to 2 ml of the erythrocyte suspension and the volume is made up to 5 ml using the same buffer solution. The mixture is incubated for 5 min at room temperature followed by the addition of 0.5 ml of H_2O_2 solution in saline buffer. The concentration of H_2O_2 in reaction mixture is adjusted to bring 90 % hemolysis of blood cells after 120 min. After incubation, the reaction mixture is centrifuged at 1500 rpm for 10 min and the extent of hemolysis is determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation. The analysis is performed in triplicate and the results are expressed in terms of percentage activity and IC_{50}.

RESULTS AND DISCUSSION

Quantitative analysis of TPC, TFC and TTC

The preliminary phytochemical investigation of EtOAc(s) and EtOH fraction of leaves of C.globosa reveals that the TFC and TPC are high in EtOAc(s) (98.81 mg/g and 75.65 mg/g) than that of EtOH fraction (1.24 mg/g and 9.32 mg/g). But the TTC is found to be high in EtOH fraction (34.28 mg/g) than EtOAc(s) (30.50 mg/g) as indicated in Table 1. Generally plants containing high phenolic and flavonoid content exhibit a better antioxidant activity and also act as a good therapeutic agent.

Table 1
TPC, TFC and TTC of EtOAc and EtOH fractions of leaves of C.globosa

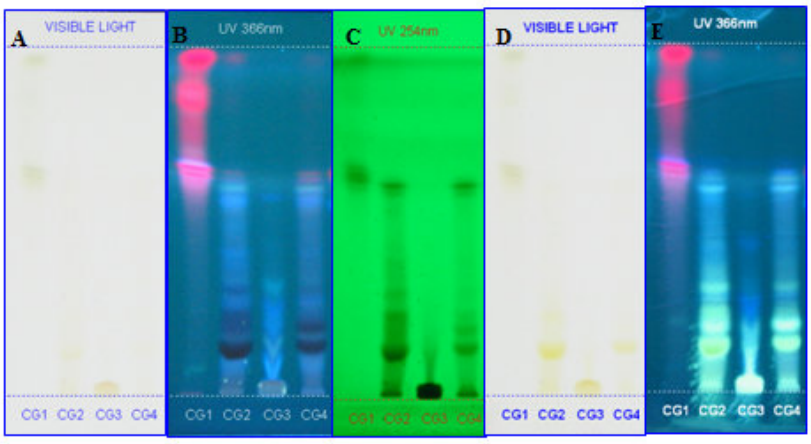
Quantification of Phytochemicals	EtOAc Fraction	EtOH Fraction
[a]Total phenolic content (TPC)	75.65±4.26	9.32±0.81
[b]Total flavonoid content (TFC)	98.81±1.97	1.24±2.18
[c]Total tannin content (TTC)	30.50±1.64	34.28±1.97

[a]TPC is expressed as mg tannic acid equivalents(RE) in 1g of dry weight material ± std dev.
[b]TFC is expressed as mg tannic acid equivalents (TAE) in 1 g of dry weight material ± std dev.
[c]TTC is expressed as mg rutin equivalents(RE) in 1g of dry weight material ± std dev.
Values are means of three independent analyses of the extract ± std dev.

HPTLC profile of C.globosa leaf extract

The optimization of mobile phase for HPTLC analysis is carried out using different solvent systems of varying polarity and the most suitable solvent system is found to be toluene-acetone-formic acid (4.5 : 4.5 : 1). The preparation of test sample, application of amount of sample, spot development and scanner speed are also standardized before proceeding the analysis. Using HPTLC technique, the chromatogram, baseline peaks and peak densitogram of applied samples (hexane, EtOAc, EtOH and water extract of *C.globosa* leaves) are recorded. The presence of yellow and yellowish blue fluorescent bands indicates the presence of flavonoids as revealed by the chromatogram derivation images [Fig 1] obtained under day light and UV (366 nm).The above fact is further supported by the analysis of R*f* values and peaks present in the densitogram [Table 2, Fig 2]. Finally it is found that EtOAc fraction of *C.globosa* shows the presence of seven flavonoid compounds whereas hexane fraction exhibits presence of one flavonoid peak, EtOH fraction shows presence of four flavonoid peaks and water fraction shows the presence of five flavonoid peaks respectively.

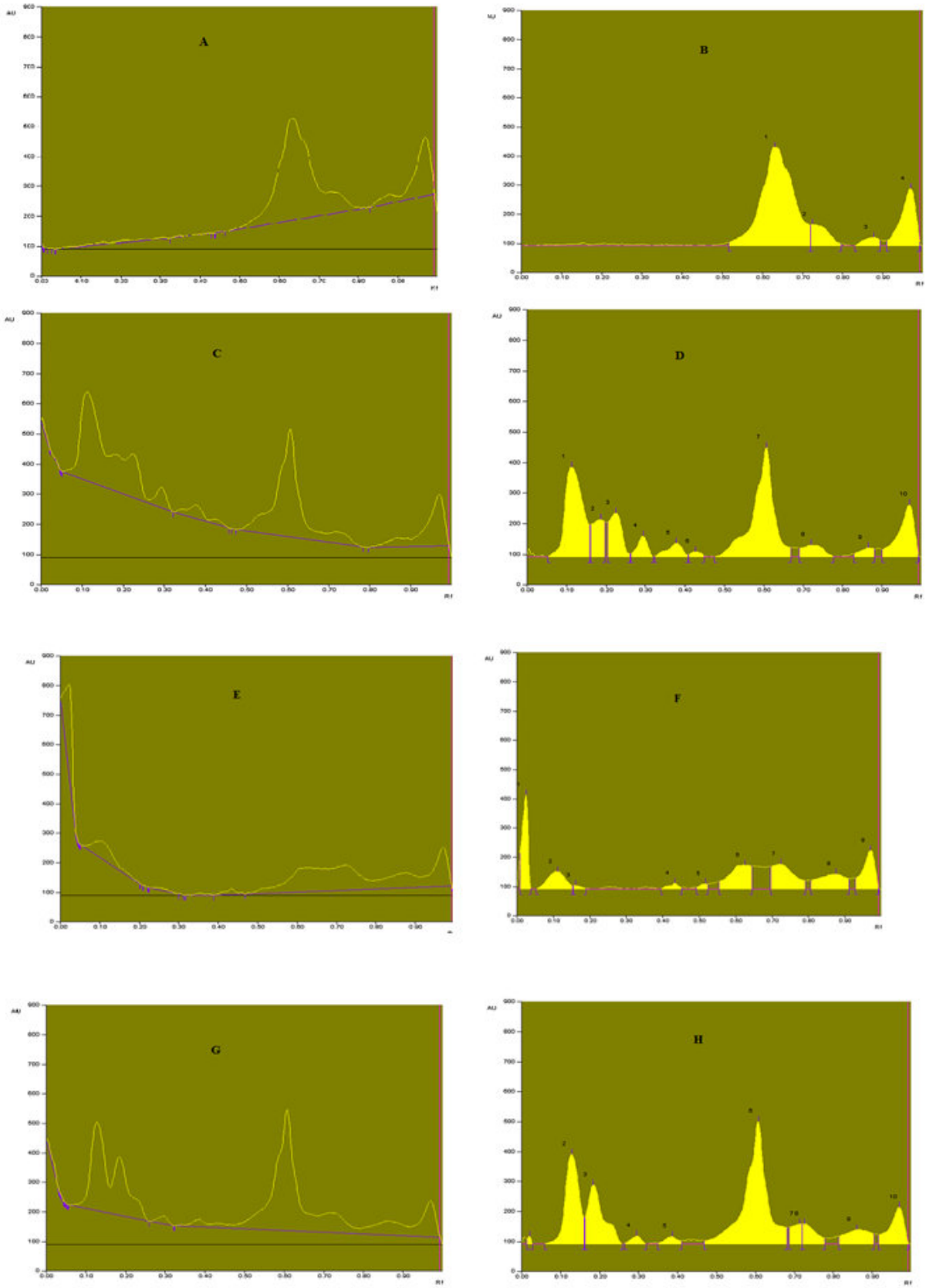
Figure 1
HPTLC studies on the flavonoids of C.globosa

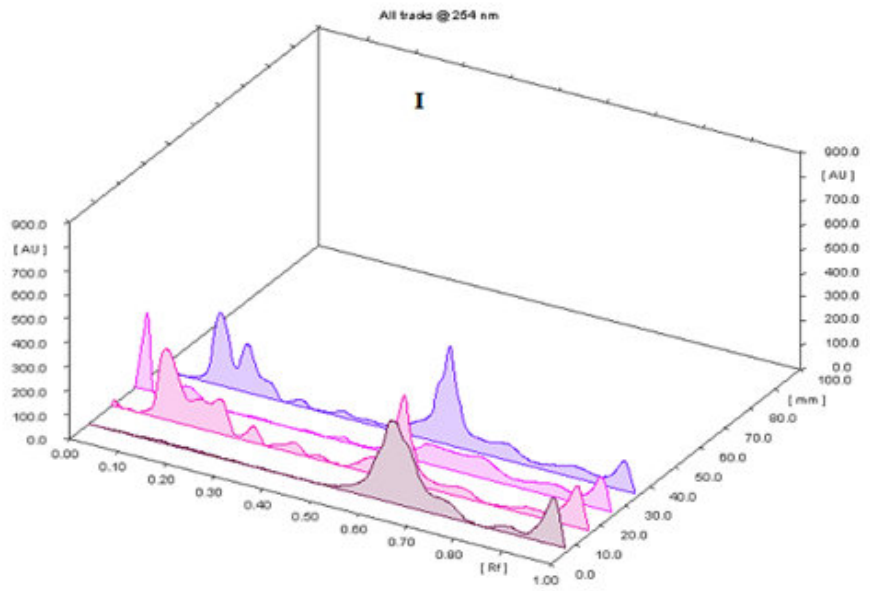


HPTLC profile of different fractions of C.globosa in A) daylight, B) UV at 366 nm (before derivatization), C) UV at 254 nm (before derivatization), D) daylight (after derivatization), E) UV at 366 nm (after derivatization).

Figure 2

HPTLC densitometric display and bioautography on different fractions of C.globosa leaves





A, B– Hexane fraction of C.globosa Baseline and Peak densitogram display (Scanned at 254nm)
C, D– Ethyl acetate fraction of C.globosa Baseline and Peak densitogram display (Scanned at 254nm)
E, F– Ethanol fraction of C.globosa Baseline and Peak densitogram display (Scanned at 254nm)
G, H– Water fraction of C.globosa Baseline and Peak densitogram display (Scanned at 254nm)
I– 3D display of all Tracks

Table 2
HPTLC profile of various fractions of C.globosa leaves

Track	Peak	Rf	Height	Area	Assigned substance
Hexane	1	0.63	339.1	24829.9	Unknown
	2	0.72	74.3	2956.4	Flavonoid 1
	3	0.88	30.5	1024.1	Unknown
	4	0.97	197.8	6601.2	Unknown
EtOAc(s)	1	0.11	294.2	12267.6	Flavonoid 1
	2	0.19	122.3	3578.0	Flavonoid 2
	3	0.22	143.5	4344.3	Flavonoid 3
	4	0.29	68.1	1607.1	Flavonoid 4
	5	0.38	45.4	1596.8	Flavonoid 5
	6	0.43	17.4	418.7	Flavonoid 6
	7	0.61	358.0	16484.5	Flavonoid 7
	8	0.72	38.5	1921.9	Unknown
	9	0.86	30.3	957.4	Unknown
	10	0.97	170.7	6102.4	Unknown
EtOH	1	0.03	321.4	4691.9	Flavonoid 1
	2	0.11	60.0	2696.8	Flavonoid 2
	3	0.16	12.9	233.5	Flavonoid 3
	4	0.43	20.3	484.0	Flavonoid 4
	5	0.52	18.3	370.5	Unknown
	6	0.63	81.5	4574.4	Unknown
	7	0.72	85.5	4825.1	Unknown
	8	0.88	52.1	3569.0	Unknown
	9	0.97	132.5	3974.7	Unknown
Water	1	0.02	26.4	230.2	Flavonoid 1
	2	0.13	300.5	9817.2	Flavonoid 2
	3	0.18	197.8	7434.3	Flavonoid 3
	4	0.29	27.4	643.6	Flavonoid 4
	5	0.39	25.4	745.0	Unknown
	6	0.61	409.2	20060.0	Flavonoid 5

	7	0.71	67.6	1675.9	Unknown
	8	0.73	67.7	2146.8	Unknown
	9	0.86	48.7	2892.7	Unknown
	10	0.97	122.6	4038.1	Unknown

Antihemolytic assay

Erythrocytes are primary targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids and the O_2 transport associated with redox active haemoglobin molecules, which are potent promoters of reactive O_2 species. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activity. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical [16]. Hydroxyl radicals abstract hydrogen atoms from the membrane lipids, which results in lipid peroxidation [17]. The solid obtained from EtOAc fraction is found to be rich in flavonoids as assessed by HPTLC method. Antihemolytic activity for the above said solid is analysed as depicted in Table 3. It is found that the percentage activity increases with increase in concentration of the EtOAc(s) and it exhibit good scavenging activity.

Table 3

Antihemolytic activity of EtOAc fraction of leaves of C.globosa

Fraction	Concentration(µg)	Percentage activity (%)	IC_{50} (µg/ml)
EtOAc(s)	200	35.04 ± 0.56	184.06 ± 1.94
	400	37.50 ± 0.25	
	600	38.46 ± 0.26	
	800	39.46 ± 0.26	
	1000	43.45 ± 0.40	

CONCLUSION

In the present study, quantification of extract of *C.globosa* revealed high concentration of total phenolic content and total flavonoid content in EtOAc(s) than EtOH fraction. But in contrast to this, the total tannin content is found to be high in EtOH fraction. Analysis of different solvent fractions of *C.globosa* leaves by HPTLC method exhibits the presence of fourteen flavonoid compounds based on the R*f* values. The radical scavenging activity of flavonoids in EtOAc(s) by antihemolytic assay using erythrocytes of Wistar rat blood is found to be potent scavenger and hinder the extent of lipid peroxidation. The result of this study reveals that *C.globosa* can be used as a potent source of drugs in the pharmaceutical industry. Further purification and characterization of individual flavonoids from this plant is under investigation.

ACKNOWLEDGEMENT

The authors wish to thank Botanical Survey of India, Sothern circle for identification of plant specimen and the Manian Institute of Technology, Coimbatore for providing laboratory facility to conduct the antihemolytic assay.

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