WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES

SJIF Impact Factor 6.647

Volume 6, Issue 7, 1509-1525 Research Article ISSN 2278 – 4357

BIOLOGICAL EVALUATION OF *MERREMIA EMARGINATA* FOR ANTIBACTERIAL, ANTIFUNGAL AND ANTIOXIDANT ACTIVITIES

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Article Received on
06 May 2017,

Revised on 06 June 2017,
Accepted on 26 June 2017,

DOI: 10.20959/wjpps20177-9571

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ABSTRACT

The main aim of this research work is to evaluate the Antibacterial, Antioxidant and Anti-fungal activity from the pooled extract (ethyl acetate) of the dried leaves of *Merremia emarginata* and screening of the extract for antibacterial, antifungal and antioxidant activity. The extracts were subjected to preliminary phytochemical screening as per the standard methods and found to have the presence of secondary metabolites such as alkaloids, carbohydrates and reducing sugars, glycosides, proteins and amino acids, steroids and triterpinoids, phenolic compounds and tannins, flavanoids, fixed oils and fats, volatile oils, gums and mucilages. These biological evaluations are done by means of standard *in vitro* assays (agar well (6mm) diffusion or Tube dilution) utilizing a broad spectrum of pathogenic and non-pathogenic bacteria. The antibacterial activity of the extract against these organisms was studied by agar well diffusion method. The antimicrobial activity was carried out in dose dependent manner at 800 µg and 1000 µg per well. The highest antibacterial activity was exhibited by extract against *E. faecalis, S. aureus, P. aeruginosa, K. pneumoniae* at 1000 µg. The anti fungal activity was carried out against *Asperillus niger and Candida albicans* using Fluconazole as a standard. The invitro antioxidant activity was carried out by using DPPH method. Physicochemical studies were performed to determine alcohol soluble extraction, Water soluble extraction, Total ash value, Acid insoluble ash value (dilute HCl), Sulphated ash value, Water soluble ash value. The foreign matter in one gram of plant powder was found to be 1.1%. The moisture content of the powdered drug was found to be 0.12%.

KEYWORDS: Antibacterial, Antioxidant, Anti-fungal, screening, antimicrobial activity.

INTRODUCTION

Merremia emarginata Burm. F (Convolvulaceae) is a perennial, much branched herb (creeper).[1] It is commonly known as *Ipomoea reniformis*. The Convolvulaceae is commonly known as the morning glory family containing about 60 genera and probably 1650 species. It is an uncultivated food crop used as a green leaf vegetable by poor people in India and a creeping perennial herb rooting at the nodes. The leaves are simple, long-stalked and reniformis or ovate-cordate. The flowers are yellow and auxiliary with 1–3 flowers with very short peduncles and the fruits look similar to sub-globose capsules with 2–4 light brown, glabrous seeds.

Fig. 1 *Merremia emarginata*

Table 1: Taxonomic Classification of *Merremia emarginata*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliosida
Order	Convolvulales
Family	Convolvulaceae
Genes	Merremia
Species	Emarginata
Botanical	Merremia emarginata

Synonyms

Convolvulus reniformis Roxb, Evolvulus emarginatus Burm.fil, Evolvulus glechoma welw, Ipomoea reniformis (Roxb)choisy, Convolvulus excisus Zipp. ex Span, Convolvulus gangeticus L, Convolvulus gangeticus Roxb, Convolvulus reniformis Roxb, Ipomoea emarginata (Burm. fil). Kuntze, Ipomoea gangetica (Roxb.) sweet, Lepistemon reniformis (Roxb.) Hassk, Lettsomia emarginata (Burm.fil.) Hall. Fil., Merremia gangetica (L) cufodontis.[2]

Table 2: Vernacular names

Tamil	Elikathu keerai or paratai keerai
English	Kidney leaf morning glory
Bengali	Bhuikamri; Indurkani
Hindi	Musaakaani
Sanskrit	Akhukarni, Mooshikakarnee
Marati	Undirkaani
Telugu	Elika-Jemudu

Habitat and distribution

Merremia emarginata is a procumbent herb and possess yellow colour flowers. it mainly grows in rainy season and winter season and widely distributed all over the India, especially in damp places in upper gangetic plain, Gujarat, Bihar, West Bengal, Western- Ghats, ascending up to 900m in the hills, Goa, Karnataka in India, Ceylon and Tropical Africa.[3]

Survey of literature and collection of data

Literature was collected from various reputed national and international journals and official communications of various Pharmacopeias and from internet. Literature survey reveal that the *Ipomoea reniformis* has been claimed to be useful for cough, headache, neuralgia, rheumatism, inflammation, troubles of nose, fever due to enlargement of liver and also in kidney diseases. Powder of leaves is used as snuff during epileptic seizures, juice acts as purgative and root is having diuretic, laxative, and applied in the diseases of the eye and gums[4], Antioxidant activity[5], Nephroprotective activity[6,7], Anti-pyretic activity[8], Anti-bacterial[9], Antidiabetic effect[10], Analgesic activity[11], Anti cancer activity[12], Rheumatoid arthritis[13], Anti-inflammatory, anti arthritis and analgesic activity[14], In Vitro antioxidant and antimicrobial activities[15], GC-MS analysis[16], Cytotoxicity, antioxidant, anti-inflammatory activities[17], Antihypertensive[18-19], Anthelmintic activity[20], Hepatoprotective Activity[21] and also reported some Pharmacognostic studies.[22, 23] Though several biological works have been carried out on the plant there is no specific procedure for isolation of phenolic constituents is observed and moreover the extracts prepared from several works may be provided with many kinds of plant constituents which might have given the non-specific biological responses. The main objective of the research work is to prepare an extract which consists of biologically important plant phenolics and evaluate its antibacterial, antifungal and antioxidant potential of dried leaves of Merremia emarginata.

EXPERIMENTAL

Materials and methods

All the chemicals and reagents used were of analytical grade and procured from various manufactures. Gentamicin I.P. (98.75%), Ascorbic acid I.P. (99.5%), Fluconazole I.P. (98.5%) was procured form Indian pharmacopoeia commission, Ghaziabad. All the microorganisms used were of ATCC.

Preparation of plant extracts

About 20 g of powdered crude plant powder is weighed in to a 1000ml beaker. Macerated with 500ml of dehydrated ethanol for 24 hours. To the macerate, 400ml of 0.1M HCl was added and heated for 10minutes for hydrolysis the solution was cooled and filtered through a Whatman filter paper. About 50ml of the filtrate was transferred in to a 250ml separating funnel and extracted with 3×10 ml portions of ethyl acetate. Ethyl acetate fractions was collected and evaporated to dryness and it was subjected to antibacterial, antifungal, antioxidant activities.

Phytochemical screening methods[24, 25]

All the crude extracts were subjected to preliminary phytochemical screening as per the standard methods to test the presence of alkaloids, carbohydrates and reducing sugars, glycosides, proteins and amino acids, steroids and triterpinoid, phenolic compounds and tannins, flavanoids, fixed oils and fats, volatile oils, gums and mucilage. Typical procedures are described as follows.

A Test for carbohydrates

Molisch test: To2-3 ml test solution few drops of alpha- naphthol solution in alcohol are added and finally concentrated H_2SO_4 from sides of test tube.

Fehling's test (for reducing sugars): To 1 ml Fehling's A and B solutions boil for 1 min, added equal volume of test solution heat in boiling water bath for 5-10 min.

Test for gums: Hydrolyse test sample using dilute HCL perform Fehling's or Benedict's test red colour is developed.

Test for mucilage: The powdered drug is treated with ruthenium red solution.

B Test for proteins

Biuret test: To 3ml of test solution 4%NaoH is added and finally few drops of 1%$CuSO_4$.

C Test for amino acids

Ninhydrin test: To 1ml of test solution, added 5drops of Ninhydrin Reagent and heated in a boiling water bath for 10 min.

D Test for glycosides: To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer.

Test for cardiac glycosides (Legal's test): To test solution 1 ml of pyridine is added finally 1ml of sodium nitroprusside solution mixed.

Test for saponin glycosides (Foam test): Shake the drug extract or dry powder vigorously with water.

E Tests for organic acids

Test for malic acid: To 2-3 ml of Test solution 2-3 drops of 5% $FeCl_3$ solution is added.

Test for tartaric acid: To 3ml of test solution 2 drops of 2% resorcinol solution is added and mix 3ml of Con H_2SO_4 and the solution was boiled for few minutes.

F Test for vitamin-C To 2ml of 2% w/v solution 2ml of water is added and 0.1g $NaHCO_3$ is added finally mix 20 g of ferrous sulphate shake and allowed to stand.

G Test for flavonoids (Magnesium and Hydrochloric acid reduction)

Shinoda test: To test solution 5 ml of 95% of ethanol is added and also few drops of concentrated HCl finally 0.5 g of magnesium turnings were added.

H Test for alkaloids

Dragendorff's Test: To 2-3ml of test solution few drops of Dragendorff's, reagent were added.

Wagner's test: 2-3 ml filtrate with few drops Wagner's were added.

I Test for phytosterols (*Libermann-Burchard's test):* The extract (50 mg) is dissolved in of 2 ml acetic anhydride. To this, 1 or 2 drops of concentrated sulphuric acid are added slowly along the sides of the test tube. An array of colour change shows the presence of phytosterols.

J Test for phenolic compounds

i. 2ml of extract is treated with few drops of 5% of ferric chloride solution
ii. 2ml of extract is treated with few drops of lead acetate solution
iii. 2ml of extract is treated with few drops of gelatin solution
iv. 2ml of extract is treated with few drops of bromine water solution
v. 2ml of extract is treated with few drops of acetic acid solution
vi. 2ml of extract is treated with few drops of potassium dichromate
vii. 2ml of extract is treated with few drops of dilute iodine solution.
viii. 2ml of extract is treated with few drops of nitric acid solution.
ix. 2ml of extract is treated with few drops of ammonium hydroxide solution, potassium and ferric cyanide.
x. 2ml of extract is treated with one drop of ammonium hydroxide solution, excess 10% silver nitrate, and heat for 20minutes in boiling water bath.
xi. 2ml of extract is treated with few drops of dilute potassium permanganate solution

Biological screening of the extract

1. Evaluation of antibacterial activity

The susceptibility of a micro-organism to antibiotics and other chemo therapeutic agents can be determined by paper disc plate technique.[26]

a) Disc Plate Technique

The paper-disc plate method is the most commonly used technique for determining susceptibility of microorganisms to chemotherapeutic agents. Small paper discs impregnated with known amounts of chemotherapeutic agents are placed upon the surface of inoculated plate. After incubation, the plates are observed for any zones of inhibition surrounding the disc. A zone of inhibition (A clear area) around the disc indicates that the organism was inhibited by the drug which diffused into agar from the disc. The single disc method susceptibility testing currently recommended by the FDA is a slight modification of the procedure developed by Bauer, Kirby Sheris and Turck in 1966. This is a highly standardized technique. The amount of anti microbial agent contained in the disc is specified as well as the test medium, size of the inoculums, conditions of incubation and other details. When the

susceptibility test is performed in conformity with FDA procedure, one can correlate the sizes of the zones of inhibition with the MIC of the drug for the microorganism in question. It is possible to determine whether the microorganism is resistant or susceptible to anti microbial agent. Strategies for testing the anti-bacterial activities of herbal products against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Enterococcus faecalis, Klebsiella pneumoniae, Pseudomonas aeruginosa,* are not yet standardized. Various methods to determine the MIC (Minimum Inhibitory Concentration) of number of antibacterial agents have been reported. The present study was designed to determine the sensitivity pattern of various compounds of *Merremia emarginata* against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Enterococcus faecalis, Klebsiella pneumonia, Pseudomonas aeuriginosa.*

b) Agar well diffusion method

To determine the susceptibility patterns of bacteria against compounds of *Merremia emarginata* the overnight grown culture in nutrient broth served as inoculums. The sterile agar medium was poured into sterile Petri plates at 40-45^{0}c and allowed to solidify. The nutrient agar plates were inoculated with the overnight grown cultures. The wells were prepared on the inoculated plates equidistantly. The extract were dissolved in DMSO (Dimethyl Sulfoxide) and each well is filled with 600µg and 800µg of crude extract with the help of micro pipette. After proper diffusion of extract into the media the plates were incubated for 24hrs at 37^{o}c in aerophilic conditions.

2. Evaluation of antifungal activity

To determine the susceptibility patterns of fungi against compounds of the *Merremia emarginata* over night grown cultures were inoculated on the surface of solidified potato dextrose agar medium. The wells were prepared on the inoculated plates equidistantly. Each well is filled with 600µg and 800 µg of crude extract with the help of micro pipette. After proper diffusion of extract into the media the plates were incubated for 72hrs at 37^{o}C. Zones of inhibition were measured with ruler.

3 Evaluation of antioxidant activity

Stock solutions of the sample were prepared by dissolving 10 mg of dried ethyl acetate extract in 10 ml of methanol to give concentration of 1mg/ml. About 0.1mM solution of DPPH is used along with ethyl actate. 3ml of ethyl acetate extract at different concentrations of (5µg, 10µg, 15µg, 20µg, and 30µg/ml.) shake vigorously and allowed to stand at room

temperature for 30 minutes. Measure the absorbance at 516nm. Ascorbic acid is used as the reference standard. %scavenging was calculated using formula

$$\% \text{ Scavenged} = A - A_1/A \times 100$$

Where A= Absorbance of control

A= Absorbance of test or standards.

RESULTS AND DISCUSSION

a) Maceration: Extraction of phytoconstituents from dried leaves of *Merremia emarginata* was achieved by cold maceration procedures (figure 2). Its extractive values were determined as per procedure given under pharmacopoeia.[27]

Fig. 2 Extraction of crude drug by Maceration

b) Physicochemical constants

Physicochemical studies were performed to determine alcohol soluble extraction, Water soluble extraction, Total ash value, Acid insoluble ash value (dilute HCl), Sulphated ash value, Water soluble ash value. Determination of Physicochemical constants is performed as per the standard protocol followed in the Ayurvedic pharmacopoeia. The values are tabulated in (Table 3 and 4).The foreign matter in one gram powder was found to be 1.1%. The moisture content of the powdered drug was found to be 0.9%.

Table 3: Different extractive values of *Merremia emarginata*

Extractive value	Values in % w/w
Alcohol soluble extraction	1.5
Water soluble extraction	1.7

Table 4: Different ash values of *Merremia emarginata*

ASH VALUES	Values in %
Total ash value	9.0
Acid insoluble ash value (dil.Hcl)	3.6

Sulphated ash value (H_2SO_4)	20.8
Water soluble ash value (H_2O)	8.5

c) Preliminary phytochemical investigation: it was done in order to know main constituents present in the extract and the results overall catalog is given in table 5.

preliminary phytochemical screening was carried out and the presence of alkaloids, steroids, glycosides and flavanoids. The details are given in table 5.

Table 5: Reports of preliminary phytochemical screening

Name of constituents	Pet ether	n-hexane	Chloroform	Ethyl acetate
Carbohydrates	-	-	-	-
Reducing sugars	-	-	+	-
Gums	-	-	+	-
Mucilage	+	-	-	+
Proteins	-	-	-	-
Amino acids	-	-	-	-
Steroids	-	-	+	-
Glycosides	--	--	+	+
Alkaloids	--	--	-	+
Tannins and Phenolic	--	--	+	+
Oxalic acid	-	-	+	+
Tartaric acid	-	-	-	+
Malic acid	--	--	-	+
Vitamin –C	--	--	--	--
Flavonoids	--	--	+	+

Where, '+' indicates positive, '-' indicates negative.

Table 6: Specific tests for phenolic compounds

Test	Observation	Inferences
2ml of extract + few drops of 5% of ferric chloride solution	Deep blue black colour appears	Presence of phenolic compound
2ml of extract + few drops of lead acetate solution	White precipitate is formed	Presence of phenolic compound
2ml of extract + few drops of gelatin solution	White precipitate is formed	Presence of phenolic compound
2ml of extract + few drops of bromine water solution	Decolourisation of bromine water	Presence of phenolic compound
2ml of extract + few drops of acetic acid solution	Red colour is formed	Presence of phenolic compound
2ml of extract + few drops of potassium dichromate	Red precipitate is formed	Presence of phenolic compound
2ml of extract + few drops of dilute iodine solution	Transcient red colour is formed	Presence of phenolic compound
2ml of alcoholic extract + few	Reddish to yellow colour is	Presence of phenolic

drops of nitric acid solution	formed	compound
2ml of extract + few drops of ammonium hydroxide solution +potassium ferric cyanide	Red colour is formed	Presence of phenolic compound
2ml of extract + one drop of ammonium hydroxide solution +excess 10% silvernitate + heat for 20minutes in boiling water bath	White precipitate is formed then dark silver mirror deposits on wall of test tube	Presence of phenolic compound
2ml of extract + dilute potassium permanganate solution	Decolourisation of potassium permanganate solution	Presence of phenolic compound

d) Anti bactrial acitivty of ethyl acetate extract

The extracts were screened for antimicrobial, antifungal, antioxidant activities and the results or shown in the figure 3-7, and the details are accessible in the tables 7-9. The biological screening of Ethyl acetate extract was carried out for determination of antibacterial activity. These evaluations are done by means of standard *in vitro* assays (agar well (6mm) diffusion or Tube dilution) utilizing a broad spectrum of pathogenic and non-pathogenic bacteria. In the present study gram positive (*S. aureus, B. subtilies, E. faecalis*), gram negative (*E. coli, K. pneumoniae, P. aeruginosa*) bacterial strains were used. The antibacterial activity of ethyl acetate extract against these organisms was studied by agar well diffusion method. The antimicrobial activity was carried out in dose dependent manner at 800 µg and 1000 µg per well. The antibacterial activity was exhibited by ethyl acetate extract against *E. faecalis, S. aureus, P. aeruginosa, K. pneumoniae* at 1000 µg. zone of inhibition due to ethyl acetate extract against the various test organisms were given in figure 3-6.

Table 7: Zone of inhibition (mm) caused by Ethyl acetate extract against bacteria

Name of the organism	**Gentamicin**	**DMSO**	**Ethyl acetate extract**			
	40 ug/ml	**10 ug/ml**	**400 ug/ml**	**600 ug/ml**	**800 ug/ml**	**1000 ug/ml**
S. aureus	31	--	0	0	11	12
B. subtilies	40	--	--	--	--	--
E .faecalis	34	--	--	--	--	--
E. coli	29	--	--	--	12	13
P. aeruginosa	30	--	--	--	--	--
K. pneumoniae	30	--	--	--	--	--

Fig. 3 Zone of Inhibition of ethyl acetate extract against *S. aureus*

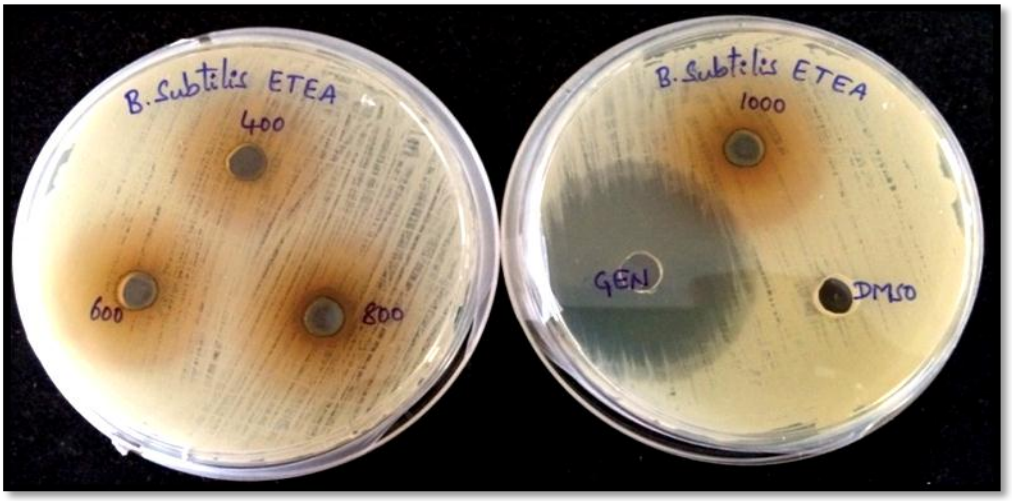

Fig. 4 Zone of Inhibition of ethyl acetate extract against *B. subtilies*

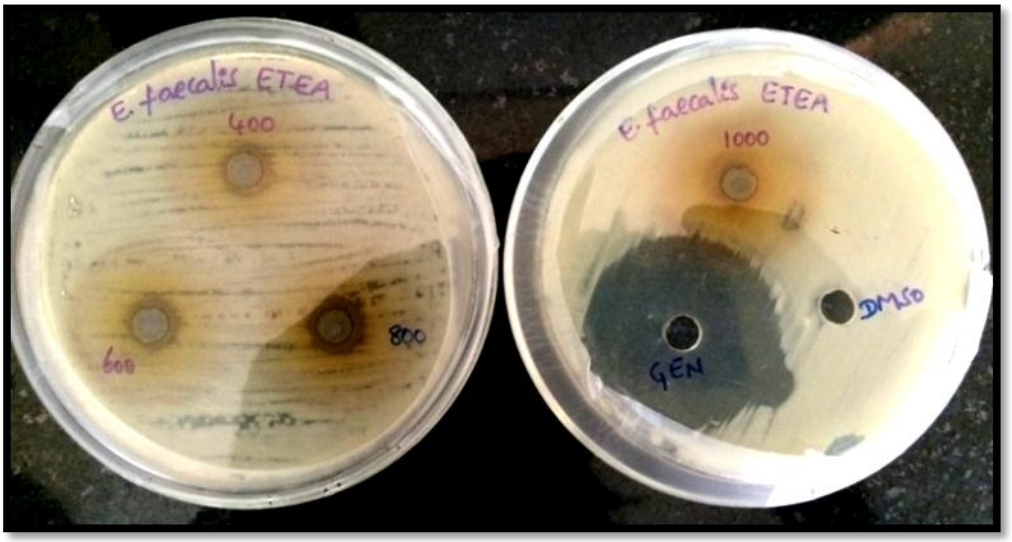

Fig. 5 Zone of Inhibition of ethyl acetate extract against *E .faecalis*

Fig. 6 Zone of Inhibition of ethyl acetate extract against *E. coli*

Well size: 6mm, Ethyl acetate extract DMSO: Dimethylsulphoxide, GEN: Gentamycin *S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis, E. faecalis: Enterococcus faecalis, E. coli: Escherichia coli.*

e) Antifungal activity

For antifungal activity we consider the organisms are *Candida albicans (ATCC 2091) Asperillus niger* (ATCC 16404). The results of antifungal activity are given in table 8.

Asperillus niger and Candida albicans

The ethyl acetate extracts exhibit antifungal against *Candida albicans* and *Asperillus niger* fungal organisms at 75 µg/ml concentration. The ethyl acetate extract were found to be more active with inhibition zone diameter of 25mm and 20 mm when compared with standard Fluconazole 24 mm and 26 mm.

Table 8: Antifungal activity of different extracts of leaves of M.emarginata against *Candida albicans* (ATCC 2091) *Asperillus niger* (ATCC 16404).

S. No	Extracts	concentration µg/ml	Zone of inhibition(mm)	
			C. albicans	*A. niger*
1	EA	75	25	20
		50	15	13
		25	10	12
		10	R	R
		5	R	R
2	Fluconazole	75	24	26

EA: Ethyl acetate extract, R –Resistance against *C. albicans (*ATCC 2091), *A. niger (*ATCC 16404).

The ethyl acetate extract exhibit antifungal activity, these may be due the presence of secondary metabolites present in the extracts which are reported in phytochemical results. The extract exhibit comparable antifungal activities at a concentration of 75 µg/ml, the activity of this compound are less than standard drugs.

***f) In-vitro* antioxidant activity-DPPH free radical scavenging activity**

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 516 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was at 516 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. Fig.7 illustrates increase scavenging of DPPH radicals in dose dependent manner due to the scavenging ability of the ethyl acetate extract. IC_{50} values of the ethyl acetate extract were found as 72.45 µg.

Table 9: Evaluation of antioxidant activity

Concentration of extract	Absorbance at 516 nm			% scavenging activity Average of three trails
	Trail 1	Trail 2	Trail 3	
12.5	0.612	0.548	0.609	47.3511
50	0.524	0.426	0.518	56.3095
100	0.325	0.315	0.361	69.9107
200	0.247	0.254	0.236	78.0654
400	0.128	0.115	0.147	88.3928
Control	1.12			
Ascorbic acid	1.15			

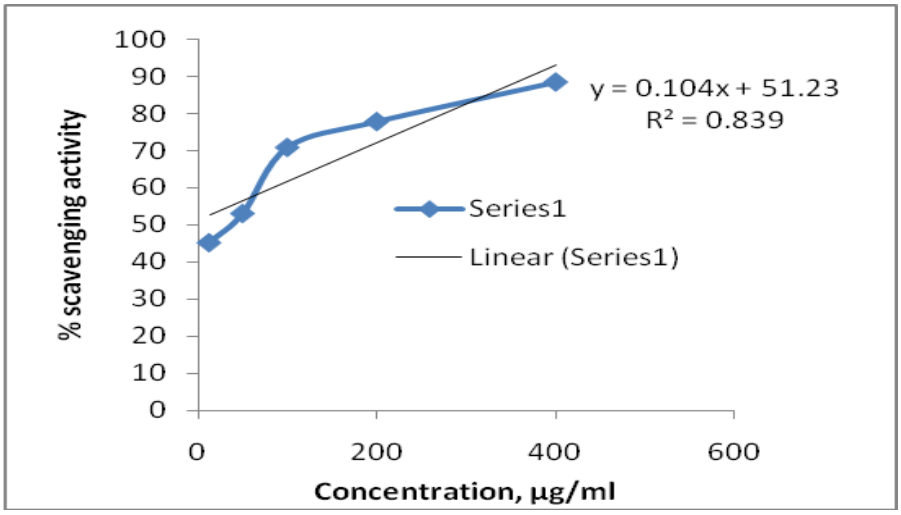

Fig. 7 IC 50 curve of ethyl acetate extract

CONCLUSION

A *Merremia genus* is distributed worldwide and comprises about 60 species and it is a native of Indian traditional medicine which includes *M.emarginata*. In this research work we have developed the optimized procedures for extraction of phytoconstituents in M*erremia emarginata* using cold maceration. Preliminary phytochemical screening results lead to find important plant metabolites viz. alkaloids, flavanoids, glycosides, steroids, phenolics etc. This study was also extended to know the potentiality of the plant extract to study its antibacterial agent against gram positive and gram negative bacteria and antifungal against Asperillus *niger and Candida albicans* and invitro antioxidant-DPPH free radical scavenging activity in association with other important crude drug standardization aspects. The extract exhibit comparable antifungal activity at a concentration of 75µg/ml, the activity of this compound is less than standard drugs. IC_{50} values of the ethyl acetate extract were found as 72.45 µg. based on the studies concerned, it is concluded that the procedures are accurate, reliable and reproducible so that the work is helpful for standardization of crude drug (*Merremia emarginata*) present in various Ayurvedic formulations and marketed polyherbal preparations containing the above said crude drug.

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