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Research Article

PHYTOCHEMICAL SCREENING, ANTIBACTERIAL, AND ANTIOXIDANT ACTIVITIES OF *CASSIA MONTANA* HEYNE EX ROTH LEAF EXTRACTS

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ABSTRACT

Objective: The leaf extracts of *Cassia montana* (Caesalpiniaceae) were being used for leucorrhea and rheumatic pains used by the certain tribal people in Andhra Pradesh. The present studies focused on the antibacterial, antioxidant activity of leaf extracts of *C. montana* which provide the scientific basis for its folklore applications in various ailments.

Methods: Ethyl-alcohol and water extracts of *C. montana* (leaf) were screened for phytochemicals, antimicrobial activity and antioxidant activity using *in vitro* methods. The total phenolics were estimated using Folin–Ciocalteu reagent with reference to gallic acid, whereas the antioxidants of the extracts were quantified using ammonium molybdate reduction assay with reference to the ascorbic acid. The antibacterial activity was studied by disk-diffusion method and the radical scavenging assay by DPPH discoloration method with reference to ascorbic acid.

Results: The leaf extracts were tested for antibacterial, antioxidants, and phytochemical screening and found the significant phytoconstituents as steroids, lignins, flavonoids, phenols, glycosides, tannins, cardiac glycosides, and reducing sugars in ethanol and aqueous extracts. The tested extracts exhibited significant antibacterial activity. It is also observed that ethanolic extracts are more effective with minimum inhibition concentrations values at 28 and 32 µg/disk against *Bacillus subtilis* and *Staphylococcus aureus* followed by *Klebsiella pneumonia* and *Pseudomonas aeruginosa* with 80 and 90 µg/disk. Total polyphenols quantified in alcohol and water extracts with reference to the gallic acid is 108 and 267 mg/g dry weight of leaf, respectively. Total antioxidants quantified in alcohol and water extracts with reference to the ascorbic acid is 70 and 85 mg/g dry weight of leaf, respectively. The tested extracts were exhibited strong effect on the discoloration of DPPH indicated the significant scavenging activity.

Conclusion: The present study revealed that the tested extracts were exhibited significant anti-bacterial antioxidant activity along with the diversified phytochemicals. Hence, the leaf extracts of *C. montana* were having potential role in the treatment of ethno botanical health claims.

Keywords: *Cassia montana*, Caesalpiniaceous, Phytochemicals, Antioxidants and antibacterial activity.

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INTRODUCTION

Since the human civilization, people relay on plants for domestic activities and human ailments, which lead to the development of branch of ethnobotany. The herbs have been using for their medicinal, flavoring, and aromatic properties since beginning of the human civilization and proved as safety and affordable cost. The overwhelming use of synthetic drugs surpassed their importance and leads to the development of drug resistance among the microorganisms and scope for emerging new diseases. In this connection naturally occurring plant products provide alternate medicaments in treating human pathogenic diseases. Several leaves and leaf extracts have been found to have medicinal properties due to the presence of several phytochemical constituent such as such as terpenoids, tannins, flavonoids, saponins, steroids, alkaloids, and phenolic compounds. The phytochemicals have pharmaceutical properties such as antimicrobial, antioxidant, anti-inflammatory, anti-helminthic, diuretic, and laxative. Hence, the plant materials have been using to prevent and treat infectious diseases successfully over the period of time [1-6].

The genus *Cassia* L. is one of the largest taxon which is represented by 600 species worldwide and reported for various medicinal properties [7]. *Cassia auriculata* L., *C. occidentalis* L., and *Cassia fistula* L. have many medicinal properties such as anti-arthritic, anticancer antidiabetic, antimicrobial, anti-inflammatory, anti-oxidant, antipyretic, anthelminthic, and antiulcer nephro- protective etc., [8-10]. *Cassia montana* Heyne ex. Roth. belongs to the family Caesalpiniaceae (syn. *Senna montana* (Roth) V. Singh, as accepted name in The Plant List.). It is commonly called mountain cassia. It is a large shrub to tree. Flowers yellow, fruits are pods which are straight, flat with prominent nerve. It is an endemic plant in southern Peninsular India and appears in hill slopes and open places. The leaf extract of *C. montana* is used in leucorrhea [11], rheumatic pains [12], and body pains [13]. The phytoconstituents such as Kaempferol, Quercetin, Kaempferol 3 - *O*-rutinoside, and Rutin C were reported from this plant [14]. Moreover, the antioxidant and antimicrobial activities of the leaf extract of *C. montana* have hither to not reported so far as per the available literature. Hence, the selected plant is evaluated for the antioxidant and antimicrobial studies. The preliminary phytochemicals screening has conducted for the extracts to support the studies and basis for the activities.

METHODS

Plant material

The leaves of *C. montana* (Fig. 1) were collected from Ardhagiri hills of Chittoor District. Provisional identification of voucher specimen (No. MHL 314) was done by Prof. N. Yasodamma, Department of Botany, S. V. University, Tirupati, and herbarium specimen was deposited in the Department of Botany, S.V. University Tirupati, and identified with the help of [15] Flora of presidency of madras, Flora of Andhra Pradesh, and Flora of Chittoor district. Identification is confirmed after comparing with authentic specimens in Herbarium, Department of Botany, S. V. University Tirupati.

Preparation of plant extracts

The leaves were washed with water to remove dust and shade dried and pulverized to powder and stored in air tight glass jar for further work.

Fifty grams of leaf powder were weighed and immersed in 500 ml of distilled ethanol and water respectively for 24 h and the extracts were filtered using Whatman No. 1 filter paper. The filtrate was evaporated to dryness on water bath at 50°C. The extracts were stored in screw capped glass bottles in refrigerator for further use.

Preliminary secondary metabolite screening

Preliminary phytochemical screening of secondary metabolites was carried out with ethanol and aqueous extracts for alkaloids, flavonoids, phenols, glycosides, tannins, steroids, lignins, saponins, terpenoids and anthocyanidins by standard qualitative methods [16-19].

Test for alkaloids

To the plant extract chloroform was added and the residue obtained was digested with 1% HCl. The acidic solution was taken and made into two parts. 2 ml of Mayer's reagent were added to the first part and 2 ml of Wagner's reagent was added to the second part.

I. Mayer's reagent test: 1.3 g of mercuric chloride and 5 g of potassium iodide were dissolved separately in 60 ml and 10 ml of double distilled water then both the solutions were mixed and diluted to 100 ml. Appearance of precipitation and turbidity shows the presence of alkaloids
II. Wagner's reagent test: 2 g of Potassium Iodide and 1.27 g of Iodine were dissolved in distilled water and make up to 100 ml distilled water. Appearance of yellowish white precipitate shows the presence of alkaloids.

Test for flavonoids

- Shinoda's test: 2 ml of the extract were taken and added few drops of Conc. HCl and then small pieces of magnesium ribbons were added. Pinkish red color was developed indicates the presence of Flavonoids
- Ferric chloride test: To 2–3 ml of the extract, few drops of Ferric Chloride solution were added. Production of black color indicates the presence of flavonoids.

Test for phenolic compounds

- Ferric chloride test: To 2–3 ml of plant extract 1 or 2 drops of 1% ferric chloride solution was added, appearance of intense blue color denotes the presence of phenols.

Test for cardiac glycosides

I. Keller-Kiliani test: 5 ml of the extract is added with glacial acetic acid and 2 drops of ferric chloride and transferred to test tube containing 2 ml of Conc. H_2SO_4. Formation of reddish brown color ring at the junction of two layers indicates the presence of glycosides.
II. Bromine water test: Each extract is added with bromine water, formation of yellow color precipitate indicates positive test for glycosides.

Test for tannins

a. Gelatin test: The concentrated methanolic extract of *C. montana* leaf was dissolved in water and mixed with 1% gelatin solution. Formation of white precipitate indicates the presence of tannins
b. Ferric chloride test: To 5 ml of the extract a few drops of ferric chloride was added. Development of black precipitate indicates the presence of tannins.

Test for steroids

- Salkowski test: Few ml of the extract were added with $CHCl_3$ followed by the addition of Conc.H_2SO_4. Formation of red chloroform layer and greenish yellow acid layer shows the positive test for steroidal compounds.

Test for lignins

- Lignin test: The plant extract was added with 2 ml of conc. HCl and 2 ml of 2% furfur aldehyde. Appearance of red color infers the presence of lignin.

Test for saponins

The aqueous extract was shaken vigorously and kept for 5 min without disturbing the solution. The persistence of honey comb froth indicates the presence of saponins.

Test for anthocyanidins

The extract was added with methanolic HCl. The development of red or purple color indicates the presence of anthocyanidins.

Test for reducing sugars

- Fehling's test: Filtrate is added with Dilute Hydrochloric Acid, neutralized with Alkali and heated with Fehling's A & B solutions; formation of red precipitate indicates the presence of reducing sugars.

Antibacterial activity

Antibacterial assay

The antimicrobial activity of the crude extracts was carried out by adopting the standard disk diffusion technique [20]. The minimum inhibition concentration (MIC) was determined based on dose depending antibiotic sensitivity assays using NCCLS guidelines [21].

Preparation of the bacterial media (nutrient agar media)

Nutrient agar medium prepared by dissolving 3 g of beef extract, 3 g of peptone, ad 15 g of agar in 1 l of distilled water. The ingredients were accurately weighed using digital electronic balance and dissolved in 1 l of distilled water before the addition of agar; the media were adjusted to p^H 7.0 with 0.1N NaOH/HCl. Later this medium was poured into conical flasks and plugged with non-absorbent cotton. Medium was then sterilized by autoclaving at 15 lbs for 20 min, cooled to 4°C, and used for the study. The crude extracts of all the samples are diluted to get a concentration of 25 µg/ml, 50 µg/ ml, 75 µg/ml, and 100 µg/ml.

Bacterial strains such as *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 737, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* MTCC 1688, and *Shigella dysenteriae* were procured from the Department of Microbiology, S.V. University and SVIMS, Tirupati and utilized in present investigation. These cell lines were further maintained on nutrient agar slants and stored at 4°C until further use.

Preparation of inoculums

The pure cultures of the tested bacteria were stored and the stock cultures were preserved at 4°C. Active cultures for experiments were prepared by transferring a loop full of cells from the stock cultures to test tubes of nutrient agar medium and were incubated for 24 h at 37°C.

Disk diffusion method

Disk diffusion method was used for antibacterial activity. 100 mg of each extract was dissolved in 10 ml of the respective solvents (distilled water and absolute ethanol) made into uniform solution (10 mg/ml). The stock solution was then diluted to concentrations of 2.5, 5, 10, and 20 mg/ml of extract. 20 µl of each dilution was impregnated into sterile, blank disks of 6 mm in diameter. 5 µl of extract was spotted on both sides of the discs and allowed to dry and the process is repeated. Distilled water and ethanol – were served as negative controls, whereas *Amoxicillin* was used as positive control. Antibacterial activity was conformed and evaluated by measuring the diameter of inhibition zone (IZ) around the disks. The assay was repeated twice. Antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by the leaf extracts [20] and MIC were determined using NCCLS guidelines [21].

Quantification of polyphenols

Total phenol content in the extracts was quantified using Folin–Ciocalteu reagent [22] 10 µl (100 µg) of each extract was taken and added to 100 µl of Folin–Ciocalteu reagent and 300 µl of 20% aqueous sodium

carbonate solution, then the reaction mixture was made up to 1 ml. The reaction mixture was incubated in dark for 1 h and the absorbance was recorded at 725 nm. The total polyphenols in *C. montana* leaf extracts were calculated from the calibration curve prepared, in the same method with gallic acid, a known polyphenol as a standard and expressed as mg standard was conducted equivalent/g of dry plant.

Antioxidant studies

Total antioxidant capacity

100 µl of plant extract was added with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and heated up to 95°C for 90 min in water both. The cooled extracts were tested for the blue color and measured the intensity at 695 nm against a blank. The same procedure is followed and prepared the standard graph using ascorbic acid and compared with the plant extracts and the total antioxidant capacity is expressed as equivalents of ascorbic acid [23].

1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

The free-radical scavenging activity of *C. montana* leaf ethanol and aqueous extracts was measured by the decrease in absorbance of methanol solution of DPPH (Yaushisakono, 1978). A stock solution of DPPH 33 mg/l was prepared in methanol and 5 ml of this stock solution was added to 100 µl of the *C. montana* leaf ethanol and aqueous solutions at concentration of 1000 mg/ml. After 30 min, absorbance was measured at 517 nm and compared with the ascorbic acid as a standard (50 µg/ml). The discoloration of blue color is considered as scavenging activity and was expressed as the percentage inhibition. The percentage scavenging of DPPH, nitric oxide, and hydrogen peroxide radicals with *C. montana* leaf ethanol and aqueous extracts and standard compounds were calculated using the following formula:

The inhibitory effect on the DPPH radical was calculated as:

% DPPH scavenging capacity = $(1 - A_S/A_C)\times 100$

A_C = Absorbance of control, A_S = Absorbance of sample solution.

Table 1: Preliminary phytochemical screening of *Cassia montana* leaf extracts

S. No.	Qualitative phytochemical screening test	CMAL extract	CMWL extract
1	Alkaloids		
	Mayer's test	–	–
	Wagner's Test	–	–
2	Flavonoids		
	Shinoda's test	++	+++
	Ferric chloride test	++	+++
3	Phenolic compounds		
	Ferric chloride test	++	++
4	Cardiac glycosides		
	Keller-Kiliani test	+	++
	Bromine water test	+	+
5	Tannins		
	Gelatin test	+	++
	Ferric chloride test	++	++
6	Steroids: Salkowski test	+	–
7	Lignins	+	–
8	Saponins	–	+
9	Anthocyanins	+	–
10	Reducing sugars	+	++

CMAL: *Cassia montana* leaf ethanol extracts, CMWL: *Cassia montana* leaf aqueous extracts

RESULTS

Qualitative analysis of phytochemicals of *C. montana* leaf extracts

The preliminary screening of secondary metabolites with respect to the *C. montana* leaf extracts resulted the presence of flavonoids, phenols, steroids, lignins, tannins, anthocyanins, cardiac glycosides, and reducing sugars in alcoholic and flavonoids, phenols, tannins, cardiac glycosides, anthocyaninns, saponins, and reducing sugars in water extracts, respectively (Table 1).

Antibacterial activity

Antibacterial activity of *C. montana* leaf extract (Table 2) exhibited significant inhibition activity against tested bacterial strains. The maximum zone of inhibition was observed on *B. subtilis* and *S. aureus* with both the extracts. Moderate activity on by *S. dysenteriae,* less activity shown on *Klebsiella pneumoniae and P. aeruginosa.* The similar activity has been compared with standard antibiotic *Amoxicillin* as a positive control. The efficacy of extracts was represented in the form of MIC in Table 3. Alcohol and aqueous extracts exhibited on *S. dysenteriae* with the MIC 20 and 40 µg/disk respectively, indicates its significant inhibition activity, whereas alcohol and aqueous extracts exerted MIC of 28 and 32 µg/disk indicates its significant inhibition activity of both the extracts on *B. subtilis* and *S. aureus.*

Total phenolic contents

The alcohol and aqueous leaf extracts of *C. montana* total phenolic content, since they are important components of biological activities. The aqueous extract shown high total phenols which were estimated using gallic acid as a standard compound. The alcoholic and aqueous extracts showed 66 and 165 mg/g dry weight of leaf, respectively (Table 4, Figs. 2 and 3).

Antioxidant studies

The alcoholic and aqueous extracts of *C. montana* tested for the presence of total antioxidant activity. The aqueous extract shown high total phenols which were estimated using ascorbic acid/Vitamin C as a standard. The alcoholic and aqueous extracts possessed quantity of 12.72 and 18 mg/g dry weight of leaf respectively in ammonium molybdate reduction assay (Fig. 3).

DPPH scavenging activity

The alcoholic and aqueous leaf extracts of *C. montana* subjected to the scavenging capacity of DPPH a free-radical commonly used in the testing of antioxidant activity. The tested extracts were exhibited strong scavenging activity based on the concentration and the IC_{50} (Inhibition

Table 2: Antibacterial activity of *Cassia montana* leaf extracts

S. No.	Micro organisms	Ethanol extract (µg/disk)				Aqueous extract (µg/disk)				Standard 25 (µg/disk)
		25	50	75	100	25	50	75	100	*Amoxicillin*
1	*Bacillus subtilis* - MTCC 121	-	10±0.16	14±0.16	16±0.28	-	10±0.22	12±0.16	14±0.36	18±0.14
2	*Staphylococcus aureus* –MTCC 737	-	10±0.15	14±0.08	16±0.29	-	10±0.22	12±0.14	14±0.24	16±0.08
3	*Klebsiella pneumoniae*	-	8±0.22	10±0.16	12±0.16	-	-	-	10±0.29	16±0.08
4	*Pseudomonas aeruginosa* MTCC 1688	-	+	10±0.16	12±0.45	-	-	-	10±0.08	16±0.16
5	*Shigella dysenteriae*	8±0.33	10±0.08	12±0.22	14±0.29	-	8±0.08	10±0.22	12±0.00	14±0.16

CMAL: *Cassia montana* alcoholic extract, CMWL: *Cassia montana* aqueous extract

concentration of extracts) values were calculated as 64 and 82 µg/ml of alcohol and aqueous extracts, respectively (Table 5, Figs. 4 and 5).

Statistical analysis

The statistical analysis has been performed with the help of MS-Excel to calculate the standard deviation and errors for mean values of the results. The values were plotted and trend line is obtained with the r^2 value of about 0.9985–0.9798 inferring the accuracy. In all experiments the inhibition of radical scavenging and percentage of inhibition of growth of bacteria is significant when compare to controls. The values reported are expressed as Mean±S.D. Standard calibration curves were prepared for the quantitative estimation of phenol and antioxidants in the extracts, and graphical representations were made with the help of MS-Excel.

DISCUSSION

The World Health Organization (2002) [24] mentioned that the medicinal plants are important source of antibiotics having potential source of secondary metabolites and can be used in the treatment of infectious diseases as urinary tract infections, gastrointestinal disorders, respiratory disease and cutaneous infections [25,26]. In the present study, the preliminary phytochemical screening of *C. montana* revealed the presence of the significant phyto-constituents like alkaloids, steroids, lignins, flavonoids, phenols, glycosides, tannins, cardiac glycosides and reducing sugars. These active phyto-constituents are known for their medicinal properties and physiological actions alkaloids, terpenoids, flavonoids and tannins have been reported to inhibit bacterial growth and protective to plants against fungal infections. Flavonoids act as a potent water-soluble antioxidant and free-radical scavenger, which prevent oxidative cell damage and also have strong anticancer activity [27].

Oxidative stress is an important risk factor in the pathogenesis of numerous chronic diseases. Free radicals and other reactive oxygen species are recognized as agents involved in the pathogenesis of sicknesses such as asthma, inflammatory arthropathies, diabetes, Parkinson's and Alzheimer's diseases, cancers as well as atherosclerosis. Reactive oxygen species are also said to be responsible for the human aging [28,29].

Antioxidants are special chemical agents that delay or inhibit oxidative damage to a target biomolecule [30]. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds such as phenolic acids, polyphenols, and flavonoids scavenge free radicals such as peroxides, hydroperoxides or lipid peroxyls and which inhibit the oxidative processes that lead to prevention of degenerative diseases especially in age-related diseases. Medicinal plants considered as good antioxidant agent which can be beneficial in reducing the stress in human since from time immemorial [31]. The studies indicate that the antioxidant activity of the extracts of *C. montana* have major role in playing the radical scavenging capacity to reduce the oxidative stress during the treatment might be useful in the prevention of radical generated degenerative diseases.

Antioxidants play an important role in prevention of various diseases such as cancer, chronic inflammation, atherosclerosis, cardiovascular disorder and ageing processes [32]. Plants serve as the bio-factory for the production of secondary metabolites which can express variety of biological activities such as antimicrobial activity [33,34]. The secondary metabolites such as alkaloids, phenolic compounds, tannins, and flavonoids were proved *in vitro* for the antimicrobial properties. They are highly beneficial in the treatment of infectious diseases such as urinary tract, gastrointestinal disorders, respiratory and cutaneous infections [25,26]. Hence, the present study on the antibacterial activity expressed by the extracts which may possess a variety of secondary metabolites as shown in Table 1. Both ethanol and aqueous extracts were effective against food pathogens such as the *S. aureus* and enteric organisms like *S. dysenteriae* and *Bacillus subtilis.* They are also effective against *P. aeruginosa* which might involve in urinary tract infections and *Klebsiella pneumonia* which is responsible for respiratory disorders such as pneumonia. Antibacterial activity of ethanolic and aqueous extracts of *Cassia tora* leaves were investigated with the MIC of 0.15 mg 0.31 mg respectively [35]. The ethanolic and methanolic extracts obtained from *C. fistula* flowers extracts showed significant activity

Table 3: Minimum inhibition concentration of *Cassia montana* leaf extracts

S. No.	Microorganisms	MIC (µg/disk)	
		CMAL	CMWL
1	*Bacillus subtilis* - MTCC 121	28	32
2	*Staphylococcus aureus* - MTCC 737	28	32
3	*Klebsiella pneumoniae*	80	90
4	*Pseudomonas aeruginosa*MTCC 1688	80	90
5	*Shigella dysenteriae*	20	40

CMAL: *Cassia montana* alcoholic extract, CMWL: *Cassia montana* aqueous extract

Table 4: Gallic acid standard

Concentration of gallic acid	OD Values 1	OD Values 2	OD Values 3	Average
0	0	0	0	0
10	0.15	0.15	0.14	0.15
15	0.30	0.31	0.29	0.30
20	0.46	0.45	0.46	0.46
25	0.61	0.62	0.61	0.61
30	0.70	0.71	0.71	0.71
35	0.85	0.85	0.84	0.85
40	0.97	0.97	0.98	0.97
45	1.1	1.0	1.1	1.1
50	1.21	1.21	1.20	1.21

Table 5: 1, 1-diphenyl-2-picrylhydrazyl standard (ascorbic acid)

Concentration of ascorbic acid	% of Inhibition
0	0
10	8
20	13
30	25
40	30
50	34
60	45
70	60
80	70
90	90
100	90

Fig. 1: ***Cassia montana habit***

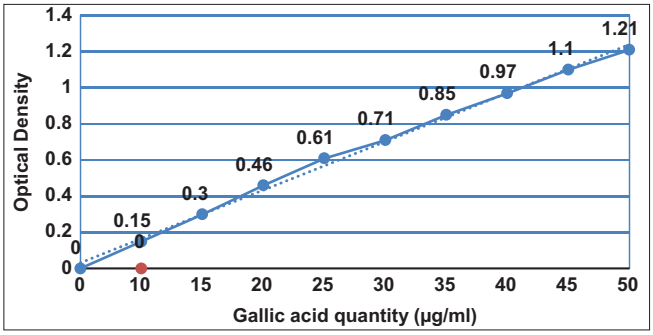

Fig. 2: Gallic acid standard curve

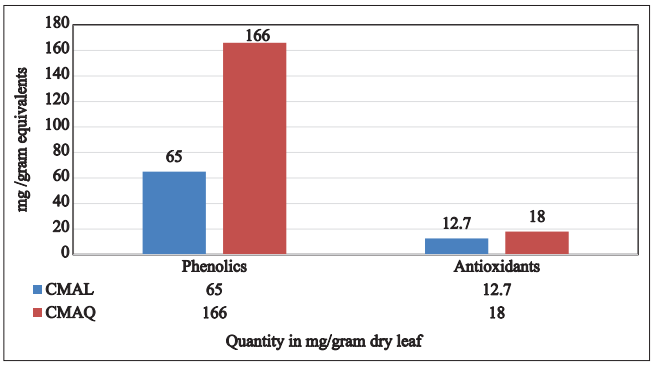

Fig. 3: Total phenolics and antioxidents of *Cassia montana* leaves

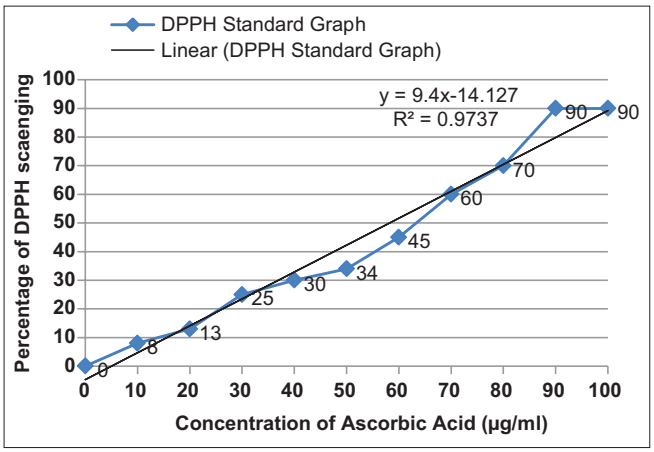

Fig. 4: 1, 1-diphenyl-2-picrylhydrazyl standard graph (ascorbic acid)

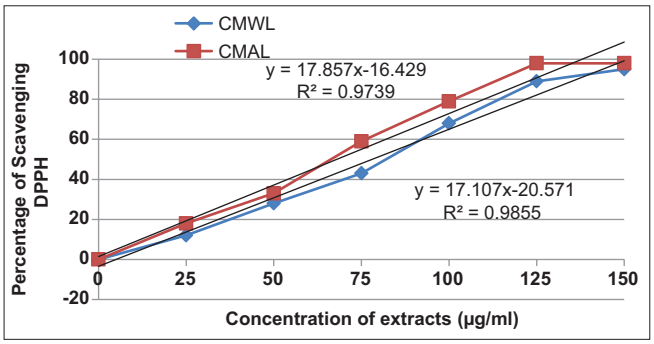

Fig. 5: 1, 1-diphenyl-2-picrylhydrazyl scavenging of *Cassia Montana*

against five of the tested strains with an effective dose 1.5 mg which has been assigned to the presence of secondary metabolites such as phenolic compounds [36]. Species of *Cassia* are rich source of anthraquinones which are well known as natural dyes and are gaining importance in recent years due to environmental pollution caused by synthetic dyes. This paper attempts to give an overview of literature on the isolated and characterized anthraquinones from various *Cassia* species. Anthraquinone compounds are used as laxatives mainly from their glycosidic derivatives and also used in the treatment of fungal skin diseases [37]. Similar type of antioxidant oxidant studies reported by the earlier workers where the phenolic compounds are responsible for antioxidant property and degenerative disease [38,39]. Saivenkatesh *et al*. reported the presence of alkaloids, tannins, and flavonoids from the different solvents extracts of leaf [40].

However, the in the present study with *C. montana* leaf extracts exhibited the antibacterial activity ranging 50–100 µg with the ethanolic and aqueous extracts which is found to be more effective. It reveals that the plant extracts act as an antibiotic for the management of gastrointestinal and respiratory troubles. The antioxidant study also revealed the potentiality as its usage for the different ailments. The antibacterial activities of the extracts are due to the presence of bioactive compounds such as phenols, flavonoids, tannins and terpenoids. Phenols, flavonoids and tannins are responsible for the antioxidant properties of *C. montana.*

CONCLUSION

The present investigation provided the justification for therapeutic potential of *C. montana* leaves and also used as medicinal plant. The leaf extracts of *C. montana* were beneficial in the treatment of many infectious as well as metabolic disorders which require further studies. Although, the significant results are shown on inhibition of the tested bacteria using *in vitro* studies it requires further studies on its efficacy.

AUTHORS CONTRIBUTIONS

All the authors contributed equally.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest in the publication.

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