

Original Research Article

In vitro antimicrobial, antioxidant, haemolytic, thrombolytic activities and phytochemical analysis of *Cipadessa bacifera* leaves extracts

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Abstract

To study the leaves of *Cipadessa bacifera* for their antimicrobial, antioxidant, haemolytic, thrombolytic activities and to perform phytochemical evaluation.

The three extracts (methanol, cyclohexane, chloroform) of *Cipadessa bacifera* were screened for antimicrobial activity against eight pathogenic microorganisms by well diffusion method. In vitro antioxidant activity of extract was studied using hydrogen peroxide radical scavenging assay. The haemolytic activity was determined using agar diffusion techniques on blood agar plate, thrombolytic activity by clot disruption and phytochemical potential by qualitative analysis.

Among the different extracts tested, the methanol extract of leaves showed significant antimicrobial activities. The most susceptible micro-organisms were found to be Gram negative bacteria (*Sphingomonas*, *Klebsiella pneumoniae*, *Citrobacter*), Gram positive bacteria (*Enterococcus faecalis*) and fungi (*Candida albicans*). H₂O₂ scavenging activity of *Cipadessa bacifera* was found to increase with increasing concentration of the extract. IC₅₀ values of H₂O₂ scavenging activity was 100.92±0.41 µg/mL which was found in cyclohexane extract. The haemolytic activity was found to be higher in chloroform extract than methanol, cyclohexane and the methanol extract shows 14.63 % clot lytic whereas standard streptokinase shows 30.86 % clot lytic activity in thrombolytic assay. The phytochemical evaluation indicates the presence of chemical constituents.

This study shows that the methanol and chloroform extract of leaves of *Cipadessa bacifera* has bioactivity but further compound isolation is necessary to confirm the activities of individual compound.

Keywords: Antimicrobial activity, antioxidant, phytochemical evaluation, thrombolysis activity, haemolysis.

Introduction

In the recent years, researches on medicinal plants have attracted a lot of attention globally. Evidences have been accumulated to demonstrate the promising potential of medicinal plants used in various traditional, complementary, and alternative systems of treatment of human diseases [1]. About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful especially in the areas of infectious disease and cancer [2].

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found *in vitro* to have antimicrobial properties [3, 4].

Cipadessa bacifera belongs to the family of Meliaceae; *C. bacifera* is a shrub, usually 1- 4 m tall which has young branches grayish brown, ribbed, covered with yellow pubescence and sparse grayish white lenticels. Leaves are 8-30 cm; petiole and rachis cylindrical, glabrous or covered with yellow trichomes; leaflets usually 9-13 opposite; leaflet blades ovate to ovoid oblong, 3.5-10*1.5-5 cm smaller basally than apically on rachis. Secondary veins are 8-10

on each side of mid vein. Flowers are 3-4 mm in diameter. Petals white or yellow, linear to oblong-elliptic, 2-3.5 mm outside covered with sparse appressed pubescence.

The purpose of this work is to evaluate antimicrobial, antioxidant, haemolytic, thrombolytic activities and phytochemical analysis of extract of *C. bacifera* leaf.

Materials and methods

Collection of plant materials

The fresh and healthy leaves of the plant *C. bacifera* were collected from Pachaimalai hills region of Salem district, Tamilnadu, India. The area falls within the latitudes 11 10' 48" N and longitudes 78 21' 0" E. These areas consist of villages which are generally classified as rural area. The plant specimens were identified and authenticated by plant taxonomist, Dr. G. V. S.



Murthy, Botanical Survey of India, Southern Regional Centre, Coimbatore.

Extract preparation

The extraction of the *C. bacifera* leaves was carried out using known standard procedures [5]. The plant leaves were dried in shade and powdered in a mechanical grinder. The powder (20.0 g) of the plant leaves was soaked with 100 mL of chloroform, methanol and cyclohexane by using a Soxhlet extractor for 72 hours at a temperature not exceeding the boiling point of the solvent. The extracts were filtered using Whatman filter paper (No.1) while hot, concentrated in vacuum under reduced pressure using rotary flask evaporator, and dried in a desiccator. More yields of extracts were collected by this method of extractions. The extracts were then kept in sterile bottles, under refrigerated conditions, until further use. The dry weight of the plant extracts was obtained by the solvent evaporation and used to determine concentration in mg/ml. The extract was preserved at 2 to 4 °C. This extract was used for further investigation.

Preliminary phytochemical screening

The extracts were subjected to preliminary phytochemical testing to detect for the presence of different chemical groups of compounds. *C. bacifera* leaves extract were screened for the presence of alkaloids, flavonoids, carbohydrates, glycosides, phenolic compound, tannins, triterpenoids, cardiolides, anthraquinones as described in literatures [6, 7, 8].

Test microorganisms and growth media

The following microorganisms *Sphingomonas*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Citrobacter* and *Proteus mirabilis* and fungal strains *Candida albicans*, *Aspergillus niger* were chosen based on their clinical and pharmacological importance [9]. All microbial cultures were obtained from Plant Biotechnology Laboratory, Department of Biotechnology, University College of Engineering – BIT campus, Trichy. The bacterial and fungal stock cultures were incubated for 24 hours at 37 °C on nutrient agar and potato dextrose agar (PDA) medium respectively, following refrigeration storage at 4 °C. The bacterial strains were grown in Mueller-Hinton agar (MHA) plates at 37 °C (the bacteria were grown in the nutrient broth at 37 °C and maintained on nutrient agar slants at 4 °C, whereas the fungal strains were grown in PDA media, respectively, at 28 °C. The stock cultures were maintained at 4 °C.

Antimicrobial activity

In vitro antibacterial and antifungal activities were examined for chloroform, methanol and cyclohexane extracts. Antibacterial and antifungal activities of plant part extracts against six pathogenic bacteria (one Gram positive and six Gram negative) and two pathogenic fungi were investigated by the well diffusion methods.

Agar plates were inoculated with 100 µL of standardized inoculums (1.5×10^8 CFU/mL) of each selected bacterium (in triplicates) and spread with sterile swabs. Wells of 6 mm size were made with sterile borer into agar plates containing the bacterial inoculums and the lower portion was sealed with a little molten agar medium. The sets of three dilutions (10, 25 and 50 µg/mL) of plant leaves extracts (chloroform, methanol and cyclohexane solvent) were poured into a different well of inoculated plates. Control experiments were carried out under similar condition by using cefotaxime for antibacterial activity and nystatin for antifungal activity as standard drugs. Chloroform, methanol and cyclohexane were used as a negative control which was introduced into a well instead of plant extract. The zones of growth inhibition around the well were measured after 18 to 24 hours of incubation at 37 °C for bacteria and 48 to 96 hours for fungi at 28 °C. The sensitivities of the microorganism species to the plant extracts were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks, and values <8 mm were considered as not active against microorganisms.

Antioxidant activity

The ability of the extracts to scavenge hydrogen peroxide was determined according to Ruch *et al.*, [10]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Different concentrations of chloroform, methanol and cyclohexane leaf extracts (2-10 µg/mL) were added to a hydrogen peroxide solution (0.6 mL, 40 mM). The total volume was made up to 3 mL with phosphate buffer. The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % Scavenged $[H_2O_2] = [(A_0 - A_1)/A_0] \times 100$ where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard.

Haemolytic assay

The haemolytic activity of the extract was determined using agar diffusion technique on blood agar plate [11]. Blood agar was prepared and well measuring 5 mm were made on the agar using cork borer. The wells were filled with 20 µL of different concentration of plant extracts solution. The plates were then incubated at 37 °C for 5 hours.

Thrombolytic assay

Whole blood (6 mL) was collected from the healthy volunteers without a history of oral contraceptive or anticoagulant therapy. For each treatment six tubes were taken and experiment was repeated thrice. Blood sample (1 mL) was distributed in pre weighed sterile micro centrifuge tubes and incubated at 37 °C for 90 mins for clot formation. After clot formation, the serum was completely aspirated

without disturbing the clot and the tubes were again weighed to determine the clot weight (clot weight = weight of the tube containing clot – weight of the empty tube). To the each Eppendorf tube containing pre weighed clot, 20 μ L, 40 μ L, 60 μ L, 80 μ L and 100 μ L of chloroform, methanol and cyclohexane extract were added. For negative control, 50 μ L of sterile distilled water was used. All the tubes were incubated at 37 °C for 18 hrs and observed for clot lysis. The fluid obtained after the incubation was removed carefully and the tubes were weighed again to observe the difference in weight after clot disruption. Difference in the weight taken before and after clot lysis was expressed as percentage of clot lysis.

Results

Preliminary phytochemical investigation

The preliminary phytochemical investigation of the methanolic extract of *C. bacifera* showed that it contains alkaloids, flavonoids, anthraquinones, glycosides, tannins, cardinolides, phenolic compound, carbohydrates. Triterpenoids were not present in methanol extract. Glycosides, tannins, triterpenoids, cardinolides

were not detected in both chloroform and cyclohexane extracts (Table 1).

Microbial activity

The antimicrobial activity of the leaf extracts of *C. bacifera* were tested in different concentrations (10, 25 and 50 μ g/mL) against six pathogenic bacterial strains, one Gram positive (*Enterococcus faecalis*) and five Gram negative (*Sphingomonas*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Citrobacter* and *Proteus mirabilis*), and two fungal strains (*Candida albicans*, *Aspergillus niger*). The extracts showed varying degrees of antimicrobial activity against tested microorganisms, values which are presented in Table 2. Methanol extracts exhibited higher degrees of antimicrobial activity than the other extracts. Among the three extract, cyclohexane extracts showed least inhibition of growth of microorganisms. The inhibitory effects of the extracts were compared with the standard antibiotics such as cefotaxime for bacteria and nystatin for fungal strain.

Table 1 Results of preliminary phytochemical tests for the presence of active constituents in *C. bacifera* leaves

Phytochemicals	Test performed	Extracts		
		Chloroform	Methanol	Cyclohexane
Alkaloids	Mayer's test	+	+	+
Flavonoids	Alkaline reagent test	+	+	+
Anthraquinones	Ammonia test	+	+	+
Glycosides	Borntragar's test	-	+	-
Tannins	FeCl ₃ test	-	+	-
Terepenoids	Salkowski test	-	-	-
Cardinolides	FeCl ₃ test	-	+	-
Phenolic compounds	FeCl ₃ test, lead acetate test	+	+	+
Carbohydrates	Molish's test, Fehlings's test and Benedict's test	+	+	+

Scavenging of hydrogen peroxide

The ability of *C. bacifera* leaf extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al., [10] and is shown in Table 3 and compared with that of ascorbic acid as standard and the highest IC50 was estimated as 100.92 \pm 0.41 μ g/mL. There was a statistically significant correlation between those values and the control (p < 0.01).

Haemolytic activity

Table 4 explains the haemolytic activity of the *C. bacifera* leaf extract in various concentrations. The zone of haemolysis was directly proportional to concentration of the extract. Chloroform extract of *C. bacifera* showed moderate haemolytic activity than methanol and cyclohexane. The activity of the extract to lyse the blood cell can be linked with the antimicrobial factors like flavonoids and phenolic compound which has been distributed in *C. bacifera*.

Table 2 Antimicrobial activity of leaves of *C. bacifera*

Microorganism	Zone of inhibition (mm)											
	Methanol ($\mu\text{g/mL}$)				Cyclohexane ($\mu\text{g/mL}$)				Chloroform ($\mu\text{g/mL}$)			
	10	25	50	+ve	10	25	50	+ve	10	25	50	+ve
Bacteria												
Gram Negative												
<i>Sphingomonas</i>	15 \pm 0.2	16 \pm 0.1	19 \pm 0.4	23 \pm 0.4	4 \pm 0.3	9 \pm 0.3	11 \pm 0.3	34 \pm 0.6	NA	NA	NA	30 \pm 0.6
<i>Pseudomonas aeruginosa</i>	14 \pm 0.1	14 \pm 0.3	17 \pm 0.1	27 \pm 0.3	12 \pm 0.2	11 \pm 0.3	12 \pm 0.4	30 \pm 0.2	10 \pm 0.3	13 \pm 0.1	13 \pm 0.3	27 \pm 0.2
<i>Klebsiella pneumonia</i>	14 \pm 0.3	14 \pm 0.2	15 \pm 0.3	28 \pm 0.3	10 \pm 0.4	11 \pm 0.2	13 \pm 0.3	30 \pm 0.3	13 \pm 0.3	13 \pm 0.2	14 \pm 0.4	25 \pm 0.6
<i>Citrobacter</i>	14 \pm 0.1	16 \pm 0.3	12 \pm 0.6	24 \pm 0.2	9 \pm 0.1	11 \pm 0.0	12 \pm 0.1	35 \pm 0.2	12 \pm 0.1	12 \pm 0.1	14 \pm 0.1	30 \pm 0.2
<i>Proteus mirabilis</i>	10 \pm 0.3	13 \pm 0.3	15 \pm 0.2	25 \pm 0.6	11 \pm 0.4	14 \pm 0.1	12 \pm 0.6	30 \pm 0.1	10 \pm 0.6	14 \pm 0.4	16 \pm 0.2	27 \pm 0.0
Gram Positive												
<i>Enterococcus faecalis</i>	13 \pm 0.1	14 \pm 0.4	20 \pm 0.3	26 \pm 0.2	12 \pm 0.6	11 \pm 0.2	11 \pm 0.1	25 \pm 0.4	10 \pm 0.6	12 \pm 0.0	15 \pm 0.2	29 \pm 0.1
Fungai												
<i>Candida albicans</i>	NA	2 \pm 0.1	4 \pm 0.3	6 \pm 0.0	6 \pm 0.2	8 \pm 0.1	10 \pm 0.2	16 \pm 0.3	5 \pm 0.2	6 \pm 0.1	7 \pm 0.4	12 \pm 0.2
<i>Aspergillus niger</i>	NA	NA	NA	6 \pm 0.1	NA	NA	NA	8 \pm 0.0	NA	NA	NA	6 \pm 0.0

+ve control for bacteria: cefotaxime; +ve control for fungai: nystatin ($\mu\text{g/ml}$); Results represented as means \pm standard deviation (n = 3); NA: No activity.

Table 3 Antioxidant activity of *C. bacifera* leaf extract

Concentration ($\mu\text{g/mL}$)	Percentage of H_2O_2 scavenging activity		
	Methanol	Cyclohexane	Chloroform
20	14.4 \pm 0.2	8.8 \pm 0.4	10 \pm 0.1
40	23.3 \pm 0.2	17.1 \pm 0.2	31 \pm 0.2
60	31 \pm 0.2	30 \pm 0.0	29 \pm 0.3
80	46 \pm 0.1	42 \pm 0.0	48 \pm 0.6
100	56 \pm 0.4	57 \pm 0.7	66 \pm 0.2
IC50 $\mu\text{g/mL}$	97.832 \pm 0.35	100.925 \pm 0.41	79.799 \pm 0.05

Results represented as means \pm standard deviation (n = 3).

Table 4 Zones of haemolysis (mm) of *C. bacifera* leaf extract at different concentration

Extract	Zones of haemolysis (mm)		
	Concentration of crude extract (mg/mL)		
	25	50	100
Methanol	6 \pm 0.2	6 \pm 0.1	6 \pm 0.4
Cyclohexane	6 \pm 0.1	6 \pm 0.1	6 \pm 0.3
Chloroform	7 \pm 0.2	6 \pm 0.3	7 \pm 0.1

Results represented as means \pm standard deviation (n = 3).

Table 5 Effect of *C. bacifera* leaf extracts on *in vitro* clot lysis

Extract	% of clot lysis				
	Concentration of crude extract ($\mu\text{g/mL}$)				
	20	40	60	80	100
Methanol	5	7.5	11.9	14.29	14.63
Cyclohexane	2.63	5.26	7.89	10	12.19
Chloroform	2.44	4.88	7.32	7.69	12.82

Thrombolytic activity

The *in vitro* thrombolytic activity study revealed that methanol, cyclohexane and chloroform extract showed 14.63%, 12.19%, and 12.82% clot lysis respectively for 100 $\mu\text{g/mL}$ and compared with the negative control (methanol, cyclohexane, chloroform solvent). Statistical representation of the effective clot lysis percentage is tabulated in Table 5.

Discussion

Natural products are in great demand owing to their extensive biological properties and bioactive components which have proved to be useful against large number of diseases. It is proved that present extracts of *C. bacifera* leaves showed wide array of activities like antimicrobial, antioxidative, antihemolytic and anti thrombolytic.

In the present work, the extracts obtained from *C. bacifera* show strong activity against most of the tested bacterial and fungal strains. The results were compared with standard antibiotic drugs. In this screening work, extracts of *C. bacifera* were found to be active against Gram-positive, Gram-negative, and fungal strains were resistant to all the extracts of *C. bacifera* except *Aspergillus niger*. There was no activity was found in chloroform extract against *Sphingomonas*.

Hydrogen peroxide can be formed *in vivo* by many oxidase enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. The ability of *C. bacifera* leaves extract to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* The *C. bacifera* chloroform extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. IC50 for scavenging of H_2O_2 were 79.79 ± 0.05 $\mu\text{g/mL}$ for chloroform extract, 97.83 ± 0.35 $\mu\text{g/mL}$ for methanol extract and 100.92 ± 0.41 $\mu\text{g/mL}$ for cyclohexane respectively. The IC50 values for ascorbic acids were 21.4 ± 0.12 $\mu\text{g/mL}$. The effectiveness of the leaves might be due to the hydroxyl groups existing in the phenolic compounds chemical structure [12] that can provide the necessary component as a radical scavenger. A potent scavenger of free radicals may serve as a possible preventive intervention for the diseases [13].

This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index. The result of phytochemicals in the present investigation showed that the plant contains more or less same components like alkaloids, flavonoids, anthraquinones, glycosides, tannins,

cardinolides, phenolic compounds and carbohydrates. Results show that plant rich in anthraquinones and phenolic compounds have been shown to possess antimicrobial activities against a number of microorganisms.

The chloroform extract of *C. bacifera* shows antihemolytic activity. The activity of the extract to lyse the blood cell can be linked with bioactive components. It is well documented that flavonoids and the polyphenolic compounds which showed potential beneficial effects on human health and possess antiviral, anti-inflammatory, antitumor, antihemolytic and antioxidative activity [14]. The zone of hemolysis was directly proportional to concentration of the extract.

Now-a-days, about 61% of the pharmaceuticals are prepared from plants worldwide [2]. A number of studies have been conducted by various researchers to find out the herbs and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke [15, 16, 17, 18]. There are several thrombolytic drugs obtained from various sources. Some are modified further with the use of recombinant technology in order to make these thrombolytic drugs more site specific and effective [17]. Side effects related to these drugs have been reported that lead to further complications [18]. Sometimes the patients die due to bleeding and embolism [19, 20, 21, 22]. In our study the *in vitro* thrombolytic activity results revealed that methanol, cyclohexane and chloroform extracts showed 14.63%, 12.19%, and 12.82% clot lysis respectively for 100 mg/mL and compared with the negative control (methanol, cyclohexane and chloroform solvent).

Conclusion

The present study has demonstrated the antimicrobial, antioxidant, hemolytic, thrombolytic activities and phytochemical analysis of *C. bacifera*. We have observed that methanol extract has shown highest antioxidant, antimicrobial properties and chloroform extracts shown highest hemolytic, thrombolytic activities. To the best of our knowledge this is the first paper about antioxidant, hemolytic and thrombolytic activity of *C. bacifera*. Hence this study was conducted by leaves extract, further advanced studies should be carried out for compound isolation and it is necessary to observe which compounds are actually responsible for specific effects.

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