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Research Article

TO STUDY THE ANTIBACTERIAL ACTIVITY OF VARIOUS EXTRACTS OF *CLAUSENA DENTATA* (WILLD.) ROEM.

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ABSTRACT

Objectives: The objectives of the study were to study the antibacterial activity for the various extracts of *Clausena dentata* against human pathogens. *Clausena* (Rutaceae) is a genus of about 23 species of unarmed trees and shrubs. The stem bark of *C. dentata* is used in veterinary medicine for the treatment of wounds and sprains. Even though *C. dentata* has a lot of potential medical uses, the study of microbiological properties is very scarce.

Methods: The plant *C. dentata* was collected from Kadagaman, near Tiruvannamalai, Tamil Nadu, India, and authenticated by Centre for Advanced Study in Botany, University of Madras, Chennai. The dry powder of stem bark was extracted with hexane, chloroform, and methanol. The extracts were subjected to qualitative phytochemical screening and antibacterial activity against human pathogenic bacteria such as *Escherichia coli*, *Salmonella* Typhi, *Klebsiella pneumonia*, *Vibrio cholerae,* and *Staphylococcus aureus* and compared with ciprofloxacin.

Results: Qualitative chemical tests revealed the presence of various phytochemicals such as alkaloids, glycosides, carbohydrate, proteins and amino acids, phytosterols, and volatile oil. The antibacterial activity result reveals that all the extracts were are more active against *V. cholerae*. The activity against *Pseudomonas aeruginosa* was mild.

Conclusion: The activity against *V. cholerae* was comparable with that of 5 µg/mL ciprofloxacin at the concentration of *C. dentata* 40 µg/mL. The orders of antibacterial activity against human pathogenic bacteria are hexane, methanol, and chloroform extract of *C. dentata*.

Keywords: *Clausena dentata*, Antibacterial activity, *In vitro*, Disc diffusion, Extracts.

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INTRODUCTION

The family Rutaceae consists of about 140 genera and 1300 species. These plants are aromatic trees, shrubs, and few herbs and are distributed throughout the warm and temperate regions of the world, being most abundant in South Africa and Australia. The aroma of the plant is due to the universal occurrence of lysigenous oil cavities in the leaves and other young organs. A number of plants of Rutaceae are of medicinal value and furnish several drugs and pharmaceutical products. Pilocarpine (used in the treatment of glaucoma) from *Pilocarpus jaborandi* and diosphenol (used in the treatment of diuretic) from *Barsoma betulina* are the best-known drugs. Essential "oil of Bergamot" and "oil of Rue" used in perfumery and medicine, respectively, are extracted from *Citrus* species and *Ruta graveolens*. *Clausena* (Rutaceae) is a genus of about 23 species of unarmed trees and shrubs mainly grow in Indo-Malayan with a few in China, Africa, and Australia. Ten species are known to grow in India, of which five are of economic importance. The stem bark of *Clausena dentata* is used in veterinary medicine for the treatment of wounds and sprains [1]. The dried powdered rootstock is also used by the Kols, the tribes in Chotanagpur region, India, for decayed teeth. In Cambodia, the stem is considered bitter tonic and astringent [2].The infusion is given for colic pain with diarrhea. *C. dentata* is used for digestion and as diuretic. α-, β- and γ-clausenans and diclausenan have been reported from the leaves of *C. dentate* [3]. Imperatorin and two new coumarins, dentatin and nordentatin, have been isolated from the root bark [4]. Even though *C. dentata* has lot of potential medical uses, the study on chemistry of the plant is very scarce [5]. Considering the importance of the plant, the present study was undertaken with the following objectives: To test the efficacy of the extracts and isolated compounds as antibacterial agents against human pathogens. There are reports on efficacies of pure coumarins against Gram-positive and Gram-negative bacteria as well as fungi. Free 6OH in the coumarin nucleus has been found to be important for antifungal activity, while the free hydroxyl group at position 7 is important for antibacterial activity. Interestingly, coumarins also have inhibitory effect on DNA gyrase which may be linked to the anti-human immunodeficiency virus activity [6]. Antimalarial activity has been addressed to daphnetin, extracted from the plants of the genus *Daphne* [7] as well as dentatin and clausarin isolated from *Clausena harmandiana* [8]. The organisms that are clinically important and used in this study to find out the antibacterial activity of this plant are listed below along with the common diseases which they cause in human [9]. Antibiotic has been defined by Hammond and Peter [10] as "chemical substances that are produced by microorganisms antagonistic to the growth or life of other microorganisms at high dilution." Ciprofloxacin is used in this study to compare the inhibitory effect of the extracts/isolated compounds on the human pathogenic bacteria. It is the most potent first-generation fluoroquinolone antibiotic active against a broad range of bacteria. The "first-generation" fluoroquinolones were introduced in the 1980s. The minimum inhibitory concentration of ciprofloxacin against microorganisms is usually <0.1 µg/mL. It is rapidly absorbed orally, effective in a broad range of infections including some difficult to treat ones. Due to wide-spectrum bactericidal activity, oral efficacy, and good tolerability, it is being extensively employed for a wide therapy of infections.

METHODS

Plant collection

The plant *C. dentata* (Willd.) Roem. was collected from Kadagaman, near Tiruvannamalai, Tamil Nadu, India, and the identification was confirmed as *C. dentata* at Centre for Advanced Study in Botany, University of Madras, Chennai. A voucher specimen of the plant has been deposited at the herbarium. The collected plant material was free from disease and also free of contamination of other plants.

Preparation of extracts

The dry powder of stem bark (2.5 kg) was first soaked, at room temperature, in hexane (1:4 w/v) for 24 h. The extract was suction filtered using Whatman filter paper. This was repeated for 2 or more days and similar extracts were pooled together and concentrated at 40°C under reduced pressure using Buchi R-153 Rotavapor. The residual plant material was extracted successively with chloroform and methanol in the same manner as followed for hexane [11,12].

Thin-layer chromatography (TLC)

Pre-coated silica gel TLC sheet (E. Merck) was used for TLC. The crude extracts were spotted at 2 cm from the edge of the sheet. The chromatogram was developed with a mixture of suitable solvent system and dried at room temperature. The spots were visualized with ultraviolet light at 254 and 346 nm. The dried TLC plates were then sprayed with 10% H_2SO_4 and heated at 110°C for 5 min [10]. Alternatively, the developed TLC plates were placed in iodine chamber. The R_f values of the colored spots were recorded.

Qualitative phytochemical screening

The different qualitative chemical tests were performed for establishing the profile of given extract for its chemical composition. The extracts were subjected to test for alkaloids, glycosides, carbohydrate, proteins and amino acids, phytosterols, fixed oils and fats, gums and mucilages, and volatile oil.

Antimicrobial activity studies

The concentrate of all the three extracts was tested for antibacterial activity against human pathogens.

Activity against human pathogenic bacteria

The organisms tested against were one Gram-positive and five Gram-negative aerobic bacteria. They were *Escherichia coli* (ATCC No25922), *Klebsiella pneumoniae* (ATCC No70063), *Pseudomonas aeruginosa* (ATCC No27853), *Salmonella* Typhi (ATCC No6539), *Staphylococcus aureus* (ATCC No25923), and *Vibrio cholerae* (ATCC No-24312). All the organisms had been identified and obtained from the clinical specimens in the Department of Microbiology, SRM Medical College, Chennai. Fresh

Table 1: Qualitative phytochemical screening of various extracts of *Clausena dentata*

S. No.	Phytochemxical test	Hexane	Chloroform	Methanol
1.	Alkaloids			
	a. Mayer's reagent	–	–	+
	b. Wagner's reagent	–	–	+
	c. Hager's reagent	–	–	+
	d. Dragendorff's reagent	–	–	+
2.	Carbohydrates and glycosides			
	a. Molisch's test	+	+	+
	b. Fehling's test	+	+	+
	c. Barfoed's test	+	+	+
	d. Benedict's test	+	+	+
	e. Borntrager's test	+	+	+
	f. Legal's test	+	+	+
3.	Saponins			
	Foam test	–	–	–
4.	Proteins and amino acids			
	a. Millon's reagent	+	+	+
	b. Biuret reagent	+	+	+
	c. Ninhydrin reagent	+	+	+
5.	Phytosteroids			
	Liebermann–Burchard's test	–	–	–
6.	Fixed oils and fats			
	a. Spot test	–	–	–
	b. Saponification test	–	–	–
7.	Phenolic compounds and flavonoids			
	a. Ferric chloride test	+	+	+
	b. Gelatin test	+	–	–
	c. Lead acetate test	+	–	–
	d. Alkaline reagent	+	–	–
	e. Magnesium and hydrochloric acid reduction	+	–	–
8.	Gums and mucilages			
	Alcohol 95% test	+	–	–
9.	Volatile oils			
	Steam distillation	+	+	+

–: Negative, +: Positive

Table 2: Effect of crude hexane extract of *Clausena dentata* on human pathogenic bacteria

Organism	Control	Percentage inhibition				Antibiotic ciprofloxacin (5 µg/mL)
		Concentration of extract (µg/mL)				
		5	10	20	40	
Escherichia coli	8	11 (10)	12 (11)	18 (16)	21 (19)	42 (38)
Klebsiella pneumoniae	8	16 (14)	21 (19)	23 (21)	27 (24)	41 (37)
Salmonella Typhi	8	12 (11)	14 (13)	18 (16)	23 (21)	42 (38)
Vibrio cholerae	8	22 (20)	31 (28)	33 (30)	35 (32)	42 (38)
Pseudomonas aeruginosa	8	10 (9)	12 (11)	16 (15)	15 (14)	11 (10)
Staphylococcus aureus	8	12 (11)	14 (13)	17 (15)	23 (21)	43 (39)

( ) Inhibition zone in mm. Values are average of three determinations

cultures were prepared by inoculating in Mueller-Hinton (MH) broth and incubating at 37°C for 24 h. Each microorganism was suspended in sterile broth and diluted to contain 106 colony-forming units (CFUs)/mL. This was checked by matching the turbidity of the tube with McFarland standard 0.5 of standardized bacterial suspension [13].

MH broth composition for inoculation

A loopful (4 mm diameter loop) of organism from the culture plate was added to 4.5 mL of sterile MH broth taken in a test tube. It was incubated at 37°C for overnight. After the incubation period, 0.5 mL of this bacterial suspension was added to 4.5 mL of sterile broth. This was incubated for 2–3 h at 37°C. The turbidity was adjusted with sterile broth so as to correspond to McFarland standard 0.5.

Preparation of McFarland standard (0.5)

McFarland standard was prepared by adding 1.175 g of barium chloride in 100 mL of distilled water and 1 mL of 0.36 N sulfuric acid in 100 mL distilled water was prepared separately. From the McFarland standard, 99.5 mL of sulfuric acid and 0.5 mL of barium chloride were added and mixed well. It was distributed in test tubes with a screw cap of same size as those containing bacterial cultures and the turbidity was evaluated. The standard was stored in ambient temperature in the dark. The cap was closed tightly and sealed to prevent evaporation. It was agitated vigorously before use [13].

Evaluation of antibacterial activity *in vitro* by agar well diffusion method

MH agar medium was used for the preparation of plates; 3.8 g of medium was dissolved in 100 mL of distilled water and sterilized. The medium (25 mL) was poured to the depth of 4 mm in sterile Petri plates of 90 mm diameter. The agar was allowed to set at ambient temperature. In each plate, using a sterile cork borer, 8 mm diameter well was cut from the agar in the center of the plate. A sterile cotton swab was immersed into the standardized bacterial suspension and pressed against the wall of the tube to express excess fluid. The plates were inoculated by streaking with the swab. Streaking was done successively in three different directions to obtain even inoculum. The concentrated extracts were weighed and dissolved in dimethyl sulfoxide to prepare extract solution of conc. 1 mg/mL. To each well, 5–40 µL of this solution was delivered using a sterile micropipette [14]. The inoculated plates were incubated within 15 min of inoculation at 37°C for 24 h. The plates were examined for the zone of inhibition. Inhibition zones were recorded as the diameter of growth free zones including the diameter of the well in mm at the end of incubation period.

RESULTS

TLC

The TLC profile of hexane, chloroform, and methanol extracts of *C. dentata* reveals that presence of coumarin and alkaloids.

Table 3: Analysis of variance for the effect of hexane extract of *Clausena dentata* on human pathogenic bacteria

S. No.	Extract	Organism	Concentration (µg/mL)	Mean inhibition zone (mm)±SD	F	95% CI mean	
						Lower	Upper
1.	Hexane	*Escherichia coli*	5	10±1.00	1018.500	7.52	12.48
			10	11±1.00	985.500	8.52	13.48
			20	16±1.00	865.500	13.52	18.48
			40	19±1.00	829.500	16.52	21.48
		Klebsiella pneumoniae	5	14±1.00	904.500	11.52	16.48
			10	19±1.00	829.500	16.52	21.48
			20	21±1.00	820.500	18.52	23.48
			40	23±1.00	829.500	21.52	24.48
		Salmonella Typhi	5	11±1.00	985.500	8.52	13.48
			10	13±1.00	928.500	10.52	15.48
			20	16±1.00	865.00	13.52	18.48
			40	21±1.00	800.500	18.52	23.48
		Vibrio cholerae	5	20±1.00	640.00	17.52	22.48
			10	28±1.00	883.500	25.52	30.48
			20	30±3.00	928.500	27.52	32.48
			40	32±2.00	985.500	29.52	34.48
		Pseudomonas aeruginosa	5	9±3.00	1054.500	6.52	11.48
			10	11±1.00	985.500	8.52	13.48
			20	13±1.00	928.500	10.52	15.48
			40	14±1.00	904.500	11.52	16.48
		Staphylococcus aureus	5	11±2.00	985.500	8.52	13.48
			10	13±2.00	928.500	10.52	15.48
			20	15±1.00	883.500	12.52	17.48
			40	21±1.00	820.500	18.52	23.48

All values are expressed as mean±SD. p<0.001 (ANOVA). Compared with control and antibiotic (ciprofloxacin). CI: Confidence interval, SD: Standard deviation, ANOVA: Analysis of variance

Table 4: Effect of crude chloroform extract of *Clausena dentata* on human pathogenic bacteria

Organism	Control	Percentage inhibition				Antibiotic ciprofloxacin (5 µg/mL)
		Concentration of extract (µg/mL)				
		5	10	20	40	
Escherichia coli	8	10 (9)	17 (15)	19 (17)	23 (21)	36 (40)
Klebsiella pneumoniae	8	9 (8)	12 (10)	17 (15)	22 (21)	41 (37)
Salmonella Typhi	8	12 (11)	14 (13)	20 (18)	23 (21)	36 (38)
Vibrio cholerae	8	28 (25)	31 (34)	35 (32)	32 (34)	36 (40)
Pseudomonas aeruginosa	8	10 (9)	11 (10)	12 (11)	14 (13)	10 (11)
Staphylococcus aureus	8	11 (10)	13 (12)	17 (15)	22 (20)	36 (40)

( ) Inhibition zone in mm. Values are average of three determinations

Table 5: Analysis of variance for the effect of chloroform extract of *Clausena dentata* on human pathogenic bacteria

S. No.	Extract	Organism	Concentration (µg/mL)	Mean inhibition zone (mm)±SD	F	95% CI mean	
						Lower	Upper
1.	Chloroform	*Escherichia coli*	5	9±1.00	664.900	6.52	11.48
			10	15±1.00	558.700	12.52	17.48
			20	17±1.00	537.700	14.52	19.48
			40	21±2.00	688.900	18.52	23.48
		Klebsiella pneumoniae	5	8±1.00	642.200	5.52	10.48
			10	10±2.00	655.200	7.52	12.48
			20	15±1.00	558.700	12.52	17.48
			40	21±1.00	575.700	18.52	23.48
		Salmonella Typhi	5	11±1.00	622.300	8.52	13.48
			10	13±2.00	586.900	10.52	15.48
			20	18±1.00	529.900	15.52	20.48
			40	21±1.00	517.300	18.52	23.48
		Vibrio cholerae	5	25±3.00	525.700	22.52	27.48
			10	31±1.00	592.300	28.52	33.48
			20	32±3.00	609.700	29.52	34.48
			40	34±1.00	649.900	31.52	36.48
		Pseudomonas aeruginosa	5	9±1.00	850.875	6.52	11.48
			10	10±1.00	642.700	7.52	12.48
			20	11±1.00	769.375	9.90	12.77
			40	13±1.00	928.500	10.52	15.48
		Staphylococcus aureus	5	10±1.00	642.700	7.52	12.48
			10	12±1.00	603.700	9.52	14.48
			20	15±1.00	558.700	12.52	17.48
			40	20±1.00	519.700	17.52	22.48

All values are expressed as mean±SD. p<0.001 (ANOVA). Compared with control and antibiotic. CI: Confidence interval, SD: Standard deviation, ANOVA: Analysis of variance

Table 6: Effect of crude methanol extract of *Clausena dentata* on human pathogenic bacteria

Organism	Control	Percentage inhibition				Antibiotic ciprofloxacin (5 µg/mL)
		Concentration of extract (µg/mL)				
		5	10	20	40	
Escherichia coli	8	11 (10)	13 (12)	17 (15)	20 (18)	38 (42)
Klebsiella pneumoniae	8	12 (12)	14 (13)	17 (15)	23 (21)	30 (42)
Salmonella Typhi	8	12 (13)	18 (16)	23 (21)	22 (24)	37 (41)
Vibrio cholerae	8	20 (22)	28 (25)	29 (33)	33 (37)	38 (42)
Pseudomonas aeruginosa	8	9 (10)	11 (12)	14 (13)	17 (18)	10 (11)
Staphylococcus aureus	8	14 (13)	17 (15)	22 (20)	30 (27)	37 (41)

( ) Inhibition zone in mm. Values are average of three determinations

Qualitative phytochemical screening

Qualitative chemical tests revealed the presence of various phytochemicals in hexane, chloroform, and methanol extracts of *C. dentata* (Table 1). The methanol extract showed positive test for alkaloids. All the extracts contained carbohydrates, glycosides, amino acids, proteins, and volatile oils. Ferric chloride test showed the presence of phenolic compounds, in all the extracts. Saponins, phytosteroids, fixed oils, and fats were absent.

Antibacterial activity of the extracts of *C. dentata* on human pathogenic bacteria

The antibacterial activities of the extracts of hexane, chloroform, and methanol are presented in Tables 2-7. The hexane, chloroform, and methanol extracts showed significant antibacterial activity against *E. coli* (21, 23, and 20% inhibition at 40 µg of hexane, chloroform, and methanol extract of *C. dentata*), *Salmonella* Typhi (23, 23, and 24%), *K. pneumoniae* (27, 22, and 23%), *V. cholerae* (35, 37, and 37%), and *S. aureus* (23, 20, and 30%). They are more active against *V. cholerae*. The activity against *P. aeruginosa* was mild.

DISCUSSION

In the present-day context, the synthetic drugs have proved to result with numerous side effects. This situation has clearly created awareness in the global arena to concentrate on the area of research from plant products. Important plant secondary metabolites known as glycosides, flavonoids, coumarins, lignins, terpenoids, and alkaloids have been isolated over a period of time from natural sources. Coumarins have a variety of bioactivities including anticoagulant, estrogenic, dermal photosensitizing, antimicrobial, vasodilator, molluscacidal, anthelmintic, sedatives and hypnotic, analgesic, anti-inflammatory, and hypothermic activity [15].

Since *Clausena* species contain coumarin derivatives and carbazole alkaloids, an attempt has been made to isolate the pure coumarin derivatives and carbazole alkaloid from the crude material of *C. dentata*. Hexane, chloroform, and methanol extracts were taken from stem bark of *C. dentata*. The hexane, chloroform, and methanol extracts were tested for antibacterial activity against human pathogenic bacteria in the present study.

The organisms tested against were one Gram-positive and five Gram-negative aerobic bacteria. They were *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella* Typhi, and *Vibrio cholerae*. The efficacy of plant extracts and isolated compounds as effective antibacterial agents was tested and compared with an antibiotic ciprofloxacin. Ciprofloxacin is a fluoroquinolone that is active against both Gram-negative and positive bacteria [16]. Have been reported that the free 6-OH in the courmarin

Table 7: Analysis of variance for the effect of methanol extract of *Clausena dentata* on human pathogenic bacteria

S. No.	Extract	Organism	Concentration (µg/mL)	Mean inhibition zone (mm)±SD	F	95% CI mean	
						Lower	Upper
1.	Methanol	*Escherichia coli*	5	10±1.00	1446.00	7.52	12.48
			10	12±1.00	1368.00	12.52	17.48
			20	15±1.00	1273.500	12.52	17.48
			40	18±3.00	1368.000	25.52	30.48
		Klebsiella pneumoniae	5	12±1.00	1206.000	9.52	14.48
			10	13±1.00	1333.500	10.52	15.48
			20	15±3.00	1273.500	12.52	17.48
			40	21±1.00	1165.500	18.52	23.48
		Salmonella Typhi	5	12±1.00	1368.000	9.52	14.48
			10	16±1.00	1248.000	13.52	18.48
			20	21±2.00	1166.500	18.52	23.48
			40	22±1.00	1158.000	19.52	24.48
		Vibrio cholerae	5	20±1.00	1176.000	17.52	22.48
			10	25±2.00	1153.500	22.52	27.48
			20	9±1.00	1189.500	26.52	31.48
			40	33±0.58	1897.000	31.23	34.10
		Pseudomonas aeruginosa	5	10±1.00	1446.000	7.52	12.48
			10	11±1.00	985.500	8.52	13.48
			20	13±1.00	1333.500	10.52	15.48
			40	14±1.00	904.500	11.52	16.48
		Staphylococcus aureus	5	13±1.00	1350.000	10.52	15.48
			10	15±1.00	1273.000	12.62	17.48
			20	20±2.00	1176.000	17.52	22.48
			40	27±1.00	1165.000	24.52	24.48

All values are expressed as mean±SD. p<0.001 (ANOVA). Compared with control and antibiotic. CI: Confidence interval, SD: Standard deviation, ANOVA: Analysis of variance

nucleus has been found to be an important one for antibacterial activity. Since all the 3 extracts are containing phenolic compounds responsible for antibacterial activity against human pathogen. Since *C. dentata* shows good antibacterial activity, further studies required in the isolated compounds and formulation level.

CONCLUSION

The hexane, chloroform, and methanol extracts showed significant antibacterial activity against *E. coli, Salmonella* Typhi, *K. pneumoniae*, *V. cholerae,* and *S. aureus.* The activity against *V. cholerae* was comparable with that of 5 µg/mL ciprofloxacin at the concentration of *C. dentata* 40 µg/mL. The orders of antibacterial activity against human pathogenic bacteria are hexane, methanol, and chloroform extract of *C. dentata*. The activity against *P. aeruginosa* was mild.

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AUTHORS' CONTRIBUTIONS

Raju Kamaraj performed the preparation and analysis of extract. Annmalai Maduram contributed to design of the experiment and manuscript writing.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

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