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Full Length Research Paper

Phytochemical screening and antibacterial activity of four *Cnidoscolus* species (Euphorbiaceae) against standard strains and clinical isolates

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The aim of this study is to investigate phytochemical composition and antibacterial activity of four species of *Cnidoscolus* [*Cnidoscolus infestus* Pax and K. Hoffman, *Cnidoscolus pubescens* Pohl, *Cnidoscolus quercifolius Pohl* and *Cnidoscolus urens* (L.) Arthur] used as ethnopharmacologicals in Caatinga. The qualitative phytochemical composition was analyzed by thin layer chromatography using eluent and specific revealing. Antimicrobial activity was evaluated using the agar diffusion method and determining the Minimum Inhibitory Concentration (MIC). The phytochemicals present in all samples were coumarins, phenolic compounds and terpenoids, however, alkaloids and naphthoquinones were not observed. The extract of the barks of *C. quercifolius* was active against *Staphylococcus* strains, with a MIC between 250 and 500 µg/ml and its dichloromethane fraction had MIC between 62.5 and 250 µg/ml against methicillin-resistant *Staphylococcus aureus* (MRSA). The antimicrobial activities of the bark of *C. quercifolius* indicated that the mechanism of multidrug resistance of *Staphylococcus* to current antibiotics does not confer resistance to the compounds present in samples. Thus, identification of the chemical constituents responsible for the antimicrobial activities of *C. quercifolius* may lead to the identification of new antimicrobial drugs against these pathogens.

Key words: Antimicrobial, Caatinga, multidrug resistance, phytochemistry, *Staphylococcus*, urtiga.

INTRODUCTION

Many ethnopharmacological studies in Brazil have shown that a large number of plant species are used by the local population to treat their diseases particularly the family Euphorbiaceae, which has great medicinal use, as well as the highly cited genus *Cnidoscolus*, popularly known as “urtiga” or “favela”, this genus has 50 to 75 representatives, which are predominantly concentrated in tropical America, almost exclusively in Mexico and northeastern Brazil (Webster, 1994). The distinct feature of this genus is the presence of stinging trichomes that, when stimulated by contact with skin, can cause severe, localized pain (Melo and Sales, 2008).

In the Caatinga, the genus is represented by four *Cnidoscolus* medicinal species [*Cnidoscolus infestus* Pax and K. Hoffm., *Cnidoscolus pubescens* Pohl, *Cnidoscolus quercifolius* Pohl and *Cnidoscolus urens* (L). Arthur], which are utilized for a variety of indications, including as an anti-inflammatory, an antitumor agent for the genito-urinary system, an antiseptic and to treat kidney infections, dermatological and ophthalmic lesions, bruises, fractures, wounds, warts, dysentery, hemorrhage, appendicitis and rheumatism (Agra et al., 2008; Albuquerque, 2006; Albuquerque et al., 2007; Almeida et al., 2005).

Worldwide, ethnopharmacological studies guide the

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search for new antimicrobial drugs from medicinal plants used by traditional communities (Sartoratto et al., 2004). Bacterial diseases cause a profound economic impact on public health, especially in tropical regions and in immunodeficient or immunosuppressed patients (Saraiva, 2007). Despite the existence of powerful antibiotics, the emergence of multidrug resistant strains of bacteria cause infections with high mortality, particularly in hospitals (Nascimento et al., 2000; Stapleton, 2004). Therefore, there is a need for ongoing research devoted to understanding the genetic mechanisms of resistance and to identify new drugs that have different mechanisms of action than the current antibiotics (Silver and Bostian, 1993; Alves et al., 2000).

Considering the fact that the genus *Cnidoscolus* is used for a large number of folk remedies, there is a paucity of studies assessing the therapeutic potential of these plants. Here, we have investigated the phytochemical profile and antibacterial activity of four species of *Cnidoscolus* referenced in ethnopharmacological surveys performed in the Caatinga.

MATERIALS AND METHODS

Study area and plant material

Determination of the samples of plant species to be collected was based on the results of previous ethnopharmological surveys. For each plant species, parts of the plant traditionally used for folk remedies and parts which are not traditionally used were collected, as cited in the surveys. The collections were made in the regions of Caatinga de Pernambuco and Paraiba. In the municipality of Altinho-Pernambuco (08°35'13"S × 36°05'34"W), samples of *C. urens* were collected on July 9, 2008. Samples of *C. infestus* and *C. quercifolius* were collected in the municipality of Soledade-Paraíba (07°04'13"S × 36º20'52"W) on September 19, 2008. Samples of *C. pubescens* were collected in Buíque-Pernambuco (08º37'23"S × 37º09'21"W) on September 27, 2008. The voucher specimens were collected and incorporated into the Herbarium UFP Geraldo Mariz, Department of Botany, Federal University of Pernambuco, numbers 55.098 to 55.101.

Chemicals and reagents

Anisaldehyde, chloroform, dichloromethane, ethanol, ethyl ether, methanol, n-hexane and toluene were purchased from Vetec (Brazil). Dimethylsulfoxide, formic acid, glacial acetic acid and petroleum ether were obtained from Merck (Germany). Ethyl acetate and sulfuric acid were purchased from Synth (Brazil). Diethylamine was purchased from Fluka (Switzerland). The reagent 2, 3, 5-triphenyltetrazolium chloride (TTC) was obtained from Fluka (Switzerland). The antibiotics gentamicin and tetracycline were obtained from Sigma (USA).

Preparation of extracts and fractions

The samples were stabilized in an oven for three days at 45 ± 5°C, powdered utilizing a Willey mill with vertical knives and standardized by size using sieves, yielding a particle size of 1 mm (16 Mesh). The pulverized samples were macerated with methanol (80%, v/v) for 48 h and the extracts were obtained following concentration of the filtrates in a rotary evaporator under reduced pressure, obtaining yields between 4.01 and 22.48%. The extracts with high antimicrobial activity were subjected to filter column chromatography on silica gel and were eluted successively with n-hexane, dichloromethane, ethyl acetate and methanol (Santos et al., 2009). The solvents were evaporated to obtain the fractions.

Qualitative analysis of phytochemicals

The extracts were evaluated on thin layer plates of silica gel 60 F_{254} aluminum supports, applied with a micropipette and eluted in different solvent systems as described by Wagner and Bladt (1996), seeking to highlight the main groups of secondary metabolism (Table 1).

Evaluation of antibacterial activity

Bacterial strains

The extracts were tested on the following standard, strains and clinical isolates from the Microbiological Analysis Laboratory (LAM/UFPE) collection: *Staphylococcus aureus* - standard strain ATCC 6538 (AM103), *S. aureus* MRSA - isolated from secretion (AM642), *Staphylococcus saprophyticus* - isolated secretion LACEN (AM245), *Staphylococcus* coagulase negative - isolated from catheter secretion (AM789), *Enterococcus faecalis* - standard strain ATCC 51299 (AM1056), *Enterococcus faecalis* - isolated from urine (AM997), *Pseudomonas aeruginosa* - standard strain ATCC 14502 (AM206), *P. aeruginosa* - isolated from blood (AM428), *Klebsiella pneumoniae* - isolated from surgical wound secretion (AM379), *K. pneumoniae* - isolation of secretion (AM410), *E. coli* - standard strain ATCC 35218 (AM1050) and *Escherichia coli* - isolated secretion (AM247). Strains of *S. aureus* MRSA isolated from tracheal aspirates (AM793) and *Staphylococcus epidermidis* isolated from sperm (AM235) also were tested in the minimum inhibitory concentration (MIC).

Antibacterial test using the agar diffusion method (well)

To determine the antibacterial activity of extracts, the agar diffusion method was chosen, using the well technique (CLSI, 2009a) as this method shows better performance, inhibition of a greater number of species of bacteria (Alves et al., 2008). The inoculates (10^8 CFU/ml) were plated with sterile swabs into 6 mm wells containing 20 ml of Mueller-Hinton agar with 50 µl of the extract dissolved in dimethyl sulfoxide (DMSO) (40%, v/v) at 100 and 200 mg/ml (5 and 10 mg/well, respectively) (Sakagami et al., 1998). As a positive control, we used 50 µl of gentamicin at 2 mg/ml (100 µg/well) and as negative control we used 50 µl of DMSO (40%, v/v). The plates were pre-incubated for 3 h at room temperature, allowing for the complete diffusion of the extracts (Möller, 1966). Next, these diffused extracts were incubated aerobically at 37 ± 1°C for 24 h and the antibacterial activity was assessed by measuring the inhibition zones.

Determination of minimum inhibitory concentration

The MIC evaluation was adapted from the microdilution methodology proposed by the Clinical and Laboratory Standards Institute (CLSI, 2009b) with bacteria that showed an inhibition zone ≥ 13 mm. Using microplate sterile 96-well (with U-bottom), each well received 90 µl of sterile physiologic serum, 80 µl of Mueller-Hinton broth, 20 µl of inoculum corresponding to 0.5 on the McFarland scale (10^8 CFU/ml) and 10 µl solutions of different

Table 1. Elution systems, standards and revelators used to characterize the main secondary metabolites from the extracts of four species of *Cnidoscolus* by thin layer chromatography (Wagner and Bladt, 1996).

Phytochemical	Elution systems	Standards	Revelators
Alkaloids	Toluene: ethyl acetate: diethylamine (70:20:10, v/v)	Yohimbine quinine	Dragendorff reagent
Anthocyanins	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26, v/v)	Methylene blue	Anisaldehyde-sulfuric acid reagent
Anthracene derivatives	Ethyl acetate: methanol: water (100:13.5:10, v/v)	Aloin	10% ethanolic KOH reagent
Anthraquinones	Petroleum ether: ethyl acetate: formic acid (75:25:1, v/v)	Anthraquinone	Phosphomolybdic acid / 10% ethanolic H_2SO_4
Coumarins	Toluene: ethyl ether (1:1 saturated with acetic acid 10%, v/v)	Scopoletin	10% ethanolic KOH reagent
Flavonoids and tannins	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26, v/v)	Gallic acid Rutin Quercetin	NEU reagent
Lignans	Chloroform: methanol: water (70:30:4, v/v)	Flaxseed extract	Vanillin phosphoric reagent
Monoterpenes and diterpenes	Toluene: ethyl acetate (93:7, v/v)	Thymol carvacrol	Vanillin sulfuric reagent
Naphthoquinones	Toluene: formic acid (99:1, v/v)	Biflorina Lapachol	10% ethanolic KOH reagent
Saponins	Chloroform: acetic acid: methanol: water (64:32:12:8, v/v)	Saponin	Anisaldehyde-sulfuric acid reagent
Triterpenes and steroids	Toluene: chloroform: ethanol (40:40:10, v/v)	Lupeol Sitosterol	Lieberman-Burchard reagent
Xanthines	Ethyl acetate: methanol: water (100:13.5:10, v/v)	Caffeine Theobromine	Iodine - KI - HCl

concentrations of extract (31.25 to 2000 µg/ml) and fractions (7.81 to 1000 µg/ml). The controls used were as follows: (1) 100 µl of sterile physiologic serum, 80 µl of Muller-Hinton broth and 20 µl of inoculum (control of microbial growth); (2) 100 µl of sterile physiologic serum and 80 µl of Muller-Hinton broth (control of sterility); (3) aqueous solution gentamicin and tetracycline solutions at concentrations of 0.125 to 64 µg/ml (positive control); (4) 90 µl of sterile physiologic serum, 80 µl of liquid culture medium Mueller-Hinton broth, 20 µl of inoculum to 0.5 McFarland scale and 10 µl DMSO (40%, v/v) (negative control). The microplates were incubated at 37 ± 1°C for 24 h, and thereafter 50 µl of aqueous chloride 2, 3, 5-triphenyltetrazolium (2.5 mg/ml, w/v) was added as developer. This test was performed in duplicate.

RESULTS

Qualitative analysis of phytochemicals

The phytochemical analysis carried out with the methanol extracts showed the presence of anthocyanins, anthracene derivatives, anthraquinones, coumarone, flavonoids, steroids, lignans, saponins, tannins, terpenoids and xanthines. Alkaloids and naphthoquinones were not detected in extracts, while the terpenoids and coumarins were present in all samples (Table 2). The groups that most stood out were the phenolic compounds, coumarin and anthracene derivatives. The species studied had similar profiles for some classes of metabolites. The phytochemical profile of the roots of *C. infestus* was identical to the roots of *C. urens*, which corroborates the phenotypic similarity between the two species.

Antibacterial activity

The antibacterial activity was assessed by the measurement of inhibition zones according to the parameters suggested by Alves et al. (2000): inhibition zones < 9 mm, inactive; 9 to 12 mm, less active; 13 to18 mm, active; > 18 mm, very active.

Extracts were inactive against *P. aeruginosa*, *K. pneumoniae* and *E. coli*. The samples of *C. infestus*, *C. urens* and the leaves of *C. pubescens* did not show antibacterial activity. The two extracts of *C. quercifolius* were active against strains of *Staphylococcus* and were

Table 2. Phytochemical characterization of four species of Cnidoscolus. (1) Aerial parts of *C. infestus* (2) root of *C. infestus* (3) leaves of *C. pubescens* (4) root of *C. pubescens* (5) barks of *C. quercifolius* (6) leaves of *C. quercifolius* (7) aerial parts of *C. urens* and (8) root of *C. urens*.

Phytochemical	1	2	3	4	5	6	7	8
Alkaloids	-	-	-	-	-	-	-	-
Anthocyanins	-	-	+++	-	-	+++	+++	-
Anthracene derivatives	-	++	+++	-	-	+++	+	++
Anthraquinones	-	-	+++	-	-	+++	+	-
Coumarins	+	+++	+	+++	+++	++	+++	+++
Flavonoids and tannins	+	-	+++	+	-	+++	+++	-
Lignans	-	+++	-	-	-	++	-	+++
Mono and diterpenes	+++	+++	++	++	++	+	+++	+++
Naphthoquinones	-	-	-	-	-	-	-	-
Saponins	-	+	-	-	++	-	-	++
Triterpenes and steroids	++	+	++	+	+	++	++	+
Xanthines	++	-	+	-	-	+	+	-

- = not detect; + = low; ++ = moderate; and +++ = strong.

less active against *E. faecalis*. The extract of roots from *C. pubescens* was also active against *Staphylococcus*. Growth inhibition of the standard strains and clinical isolates by the Cnidoscolus samples are presented in Table 3.

Plant extracts are considered to have good antimicrobial activity with a MIC < 100 µg/ml, moderate activity with a MIC of 100 to 500 µg/ml, weak activity with a MIC of 500 to 1000 µg/ml and inactive with a MIC to 1000 µg/ml (Tanaka et al., 2005). The extract from the leaves of *C. quercifolius* was inactive against *Staphylococcus*, while the extract from the roots of *C. pubescens* showed weak activity against two strains of *S. aureus* (AM103 and AM642) and was inactive against the *S. coagulase negative* strains. The extract from the bark of *C. quercifolius* was active against all strains, with a MIC of 250 and 500 µg/ml. The dichloromethane fraction from the bark of *C. quercifolius* was the most active, with the MIC values ranging between 62.5 and 250 µg/ml and was the most effective of the samples examined against the *S. aureus* MRSA strain (AM793) which is currently known to be sensitive to only vancomycin (Saraiva, 2007).

The results of minimum inhibitory concentrations (MIC) are shown in Table 4.

DISCUSSION

Qualitative analysis of phytochemicals

The negative response to alkaloids in extracts *Cnidoscolus* species was unexpected, since there are reports in the literature of this class (Awoyinka et al., 2007; Rouse and Bienfang, 2006; Yakubu et al., 2008). As expected, anthocyanins were found in extracts of leaves and aerial parts. Among the phenolic compounds, flavonoids and tannins occurred more frequently. Yuan et al. (2007) performing a phytochemical with *Cnidoscolus texanus* (Müll. Arg.) Small, isolated 26 compounds, being fifteen flavonoids, four triterpenoids, three coumarins, three coumaric acid derivatives and a phytosterol. Coincidentally, there was the characterization of a coumarin same in all samples (Rf = 0.35), which can be used as a chemical marker for these species.

In recent years, research on plants of Caatinga that are traditionally used to treat various diseases has identified promising pharmacological activities. These activities have been generally attributed to the presence of phenolic substances because several species that have medicinal uses also contain high levels of these compounds (Almeida et al., 2005; Monteiro et al., 2006). For example, a study by Araújo et al. (2008), in a rural community, observed that there is a strong association between the content of tannins, and the healing and anti-inflammatory activities effects popularly attributed to the plants.

Antibacterial activity

The growth of bacterial resistance to antibiotics is a threat to the world population with an increasing recurrence of infectious diseases due to the emergence of multidrug resistant bacteria that hinder chemotherapy (Aqil et al., 2005). For example, nosocomial infections produced by *S. aureus*, especially Methicillin-resistant *S. aureus* (MRSA), is well recognized by its frequency, morbidity and mortality due to the difficulty of treatment (Nascimento et al., 2000). Virtually all strains of MRSA

Table 3. Growth inhibition zones (in mm) of standard strains and clinical isolates multidrug resistance from gram-positive and gram-negative bacteria by *Cnidoscolus* extracts.

Species	Part used	Conc./well	*S. aureus*		*S. coagulase* negative		*E. faecalis*		*P. aeruginosa*		*K. pneumoniae*		*E. coli*	
			AM103	AM642	AM245	AM789	AM997	AM1056	AM206	AM428	AM379	AM410	AM1050	AM247
C. infestus	Aerial parts	5 mg	-	-	-	-	-	-	-	-	-	-	-	-
		10 mg	-	-	-	-	-	-	-	-	-	-	-	-
	Roots	5 mg	-	-	-	-	-	-	-	-	-	-	-	-
		10 mg	-	-	-	-	-	-	-	-	-	-	-	-
	Leaves	5 mg	-	-	-	-	-	-	-	-	-	-	-	-
		10 mg	-	-	-	-	-	-	-	-	-	-	-	-
C. pubescens	Roots	5 mg	13	10	12	12	-	-	-	-	-	-	-	-
		10 mg	14	13	14	12	-	-	-	-	-	-	-	-
	Barks	5 mg	15	14	13	11	-	-	-	-	-	-	-	-
		10 mg	16	14	14	13	12	12	-	-	-	-	-	-
C. quercifolius	Leaves	5 mg	14	15	10	11	-	-	-	-	-	-	-	-
		10 mg	16	16	12	15	11	12	-	-	-	-	-	-
C. urens	Aerial parts	5 mg	-	-	-	-	-	-	-	-	-	-	-	-
		10 mg	-	10	-	10	-	-	-	-	-	-	-	-
	Roots	5 mg	-	-	-	-	-	-	-	-	-	-	-	-
		10 mg	-	-	-	-	-	-	-	-	-	-	-	-
DMSO	-	40%	-	-	-	-	-	-	-	-	-	-	-	-
Gentamicin	-	100 µg	23.5	23.75	23.5	28.75	-	-	21.5	-	20.5	21.25	22.25	22

The strain numbers below each species are strain identification codes beginning with the letters AM and are described in detail in section bacterial strains.

are resistant to β-lactams, macrolides, tetracyclines and aminoglycosides. Currently, glycopeptides are the only treatment alternative for infections caused by *S. aureus* multidrug resistant (Shibata et al., 2005).

In this sense, the genus *Cnidoscolus* showsvery promising antimicrobial activity, especially against gram-positive bacteria because it showed an inhibitory effect on both sensitive and very resistant strains of bacteria. For example, the strain *S. aureus* MRSA (AM793) is multiresistant, being sensitive only to the glycopeptide vancomycin, which is effective but has significant toxic side effects and requires monitoring during its administration (Soares et al., 2000). The inhibitory activity of the extract from the bark of *C. quercifolius* on *S. epidermidis* (AM235), an important microorganism related to the occurrence

Table 4. MIC in µg/ml of *C. pubescens* and *C. quercifolius* on growth of *Staphylococcus* strains.

Species	Part used or fraction	*S. aureus*			*S. coagulase negative*		
		AM103	**AM642**	**AM793**	**AM245**	**AM789**	**AM235**
C. pubescens	Roots	500	500	1000	2000	2000	2000
C. quercifolius	Barks	250	250	250	500	250	500
	HEX	> 1000	> 1000	> 1000	500	> 1000	500
	DCM	125	250	62.5	250	125	125
	AcOEt	500	500	125	500	250	250
	MeOH	> 1000	> 1000	500	> 1000	1000	> 1000
	Leaves	> 2000	> 2000	> 2000	> 2000	> 2000	> 2000
Gentamicin (µg/ml)		0.125	0.25	> 64	1	0.125	1
Tetracycline (µg/ml)		0.25	0.0625	32	0.25	0.25	0.25

The strain numbers below each species are strain identification codes beginning with the letters AM and are described in detail in section determination of minimum inhibitory concentration (MIC); HEX = hexane fraction; DCM = dichloromethane fraction; AcOEt = ethyl acetate fraction; MeOH = methanol fraction.

of endocarditis, sepsis and other serious infections involved with implanted medical devices, such as catheters and prostheses (Marangoni, 1997), reveals the potential against this bacterial species. Therefore, in accordance with the established standard, the crude methanol extract from the bark of *C. quercifolius* showed the highest activity against Gram-positive species because the rings are considered excellent tools to determine the antimicrobial susceptibility of organisms (Andrews, 2001).

When checking the literature, we found that many studies have reported antimicrobial properties for species of the family Euphorbiaceae. Kuete et al. (2010), studying the antimicrobial activity of *Thecacoris annobonae* Pax and K. Hoffman showed that a methanol extract of the bark prevented the growth of all organisms tested, indicating that it may be a potential source of new drugs against tuberculosis and some fungal and bacterial diseases. In another study, a hydroalcoholic extract, fractions and some of the compounds isolated from *Croton urucurana* Baillon showed excellent antibacterial activity against strains of *S. aureus* and *Salmonella typhimurium* (Peres et al., 1997). Aiyelaagbe (2000) evaluated the antibacterial activity of *Jatropha multifida* L. roots extracted successively with hexane, ethyl acetate, chloroform and methanol, finding that the extracts inhibited the growth of *Bacillus subtilis*, *S. aureus* and *E. coli*. The methanol extracts of leaves, flowers, stems and roots of *Euphorbia hirta* L. were active against Gram-positive bacteria, Gram-negative bacteria and *Candida albicans*, with MICs between 3.13 and 100 mg/ml (Rajeh et al., 2010).

Despite having shown promising results, the extract was inactive against Gram-negative bacteria. This result may have occurred for two reasons: the absence or low relative concentration of potentially active compounds and structural differences of the Gram-negative bacteria has in relation to Gram-positive that can hinder the action of the active components of the extracts (Ayres et al., 2008).

Conclusion

The mechanism of resistance of *Staphylococcus* to current antibiotics does not confer resistance to the compounds present in the barks of *C. quercifolius*, which possesses coumarins predominantly in their phytochemical composition. Thus, identification of the chemical constituents responsible for the antimicrobial activities of the plant species may lead to the identification of new antimicrobial drugs against these pathogens. These results are very promising since there is a large amount of substances present in extracts and, after isolation, the compounds responsible for the activity may have enhanced their medicinal potential, enabling a reduction in concentration and increased activity.

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