Full Length Research Paper

Modulatory activity and chemical profile of a hydroalcoholic extract of *Crateva tapia* L.

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The scientific community is constantly searching for innovative substances that have antimicrobial potential and the ability to reduce or eliminate bacterial antibiotic resistance to specific drugs. This ability is known as the modulatory activity of antibiotics. *Crateva tapia* L., popularly known as "trapiá," is a plant belonging to the Capparaceae family and is used in northeastern Brazil as a tonic, anti-diarrheal, antipyretic, hypotensive and muscle relaxant. We investigated the phytochemical profile and the antibiotic and modulating activity of a hydroalcoholic crude extract (CECT) against multi-drug resistant strains of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, all of which were isolated from surgical wounds. A phytochemical assay detected the presence of phenols and alkaloids. The minimum inhibitory concentration (MIC) of CECT against all of the strains used was ≥ 1024 µg/mL. The extract synergistically modulated the antibiotic resistance to Amikacin of *S. aureus*, reducing the MIC from 312.5 to 39.06 µg/mL (an 87.5% reduction). The same occurred in the case of gentamicin against *S. aureus* and *P. aeruginosa*. In both cases, the MIC was reduced from 312.5 to 78.12 µg/mL, a reduction of 75%. Although the antimicrobial activity of *C. tapia* extract is clinically irrelevant, it showed significant modulatory activity, justifying further fractionation and tests with other strains and antibiotics.

Key words: Antimicrobial activity, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, amikacin, gentamicin, neomycin.

INTRODUCTION

The property of reducing or eliminating bacterial antibiotic resistance to specific drugs is known as the modulatory activity of antibiotics (Gunics et al., 2002). Some studies have reported substances or plant extracts with modulatory antibiotic activity against various microorganisms (Palaniappan and Holley, 2010; Hemaiswarya and Doble, 2009; Yap et al., 2013). It is even possible that a substance that has no antibiotic activity when used alone can present a potent modulatory activity when associated with antibiotics and antifungals (Morais-Braga et al.,

2013).

Proof of Darwinian natural selection can be observed in the increasing emergence of bacteria that have become resistant to multi-drug antibiotics and that are categorized as having "extensive drug-resistance" (Park et al., 2011). Upon acquiring resistance, pathogenic, saprophytic and commensal bacteria can potentially result in pathogens that can cause physiological damage (Razera et al., 2009). This has raised great concern in the scientific community, resulting in the constant search for innovative substances.

If the mutations responsible for resistance were to occur individually, this problem would not be so severe. However, many transposons and plasmids confer resistance on a variety of classes of antibiotics (Livermore, 2007). Such selection, in most instances, occurs in an artificial and accelerated manner through the indiscriminate use of antibiotic substances, causing serious problems with regard to antibiotic therapy.

Various species of bacteria now fall within the category of "extensive drug-resistance", as observed in the cases of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *E. coli*, despite being part of the normal flora of the gastrointestinal tract, has multi-drug resistant strains that have caused infections in other locations, such as the urinary tract, lungs, heart valve, and blood stream (Wright and Perinpanayagam, 1987; Caso et al., 2008). This microorganism has been responsible for serious nosocomial and community outbreaks in different parts of the world and is considered the sixth most dangerous pathogen to human health (Pacheco et al., 2013). In regard to hospital contaminations, *P. aeruginosa* is one of the main bacteria responsible for multi-drug resistant infections. Isolated versions of these strains of *P. aeruginosa* can be obtained from various sites of infection, such as hair follicles, nails, lungs, the urinary tract and surgical wounds (Kerr and Snelling, 2009). Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains also play a major role in hospital outbreaks. In more than half of Latin America, MRSA has been identified in more than 50% of all clinical isolates of *S. aureus* (Guzmán-Blanco et al., 2009). Data from 2001 showed a surprising increase, from 0.7 to 14.8%, in the prevalence of MRSA in clinical isolates from patients in the British Isles (Livermore et al., 2003).

Ethnobotany and ethnopharmacology have emerged as very important tools in the study of the customs and usages of medicinal plants in traditional communities. These tools have identified plants with promising antimicrobial activity in specific and even global ecosystems (Almeida et al., 2012; Mbosso et al., 2010; Canales et al., 2005).

In general, common popular knowledge is corroborated by biological assays (Leitão et al., 2013), often leading researchers in the fields of phytochemistry and pharmacology to adopt a directly ethnopharmacological approach. This is not only the quickest but also the least costly method for selection of species with biological activity (Albuquerque and Hanazaki, 2006).

The present study, therefore, aimed to study the antimicrobial activity of *Crateva tapia* L., a species used in popular medicine in Northeastern Brazil, selected on the basis of ethnopharmacological information. *C. tapia* L. belongs to the Capparaceae family and is found in a variety of environments, such as the Atlantic rain forest and Pantanal and Caatinga of the Brazilian Northeast. The plant is consumed as food by monkeys, fish and birds and humans have not only utilized it for timber but also for its medicinal properties (Lorenzi, 1998). The stem bark of this tree has traditionally been employed for a variety of purposes: as a tonic, an anti-diarrheal, an antipyretic (Araújo, 2008), a hypotensive and a muscle relaxant (Albuquerque et al., 2007), and in the treatment of urinary disorders and inflammation (Patil et al., 2010). One of such ethnobotanical study of plants with purported anti-inflammatory activity was carried out in the interior of the state of Pernambuco, Brazil.

In this study, *C. tapia* was also considered appropriate for treating kidney pain and sore throats (Ferreira Júnior et al., 2011). Inflammatory processes may result from tissue damage caused by bacteria (Guyton and Hall, 2006) and a species popularly known as an anti-inflammatory agent may thus in fact be producing this effect because it possesses antimicrobial properties.

With a view to furthering knowledge of this species, the present study determined the phytochemical profile of a hydroalcoholic extract and evaluated the antimicrobial and modulatory activity of the hydroalcoholic extract of *C. tapia* stem bark against multi-resistant strains of *E. coli*, *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

Drugs

Gentamicin, Amikacin, and Neomycin were purchased from SIGMA Co. (St. Louis, MO). The solutions of antibiotics were prepared in accordance with the recommendations of the Clinical and Laboratory Standards Institute (NCCLS, 2008).

Plant material

Cr. tapia L. bark was collected from a remnant of deciduous thorny vegetation (Caatinga) in the State of Pernambuco, Northeastern Brazil, in April and May of 2011 during the beginning of the rainy season. The species was identified by Dra. Lucilene Lima dos

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Table 1. Antibiogram of the strains used in the microbiological assays.

Bacteria	Origin	Resistance
Escherichia coli 27	Surgical Wound	Ast, Ax, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Chlo, Im, Kan, Szt, Tet, Tob
Staphylococcus aureus 358	Surgical Wound	Oxa, Gen, Tob, Ami, Kan, Neo, Para, But, Sis, Net
Pseudomonas aeruginosa 03	Urine culture	Cpm, Ctz, Im, Cip, Ptz, Lev, Mer, Ami

Ast - Aztreonan; Ax – Amoxicillin; Amp - Ampicillin; Ami - Amikacin; Amox – Amoxicillin; Ca - Cefadroxil; Cfc - Cefaclor; Cf - Cephalothin; Caz -Ceftazidime, Cip - Ciprofloxacin; Chlo - Chloramphenicol; Im - Imipenem; Kan - Kanamycin; Szt – Sulphametrim; Tet - Tetracycline; Tob -Tobramycin; Oxa - Oxacillin; Gen - Gentamicin; Neo - Neomycin; Para – Paramomicin - But - Butirosin; Sis - Sisomicin Net – Netilmicin, Cpm – Cefepime, Ctz – ceftazidime, Ptz - Piperacilin-tazobactam, Lev - levofloxacin; Mer - Meropenen.

Santos and a voucher specimen was deposited in the Prof. Vasconcelos Sobrinho Herbarium at the Universidade Federal Rural de Pernambuco under the number 48,718. Stem bark from at least three individual samples was collected to cover intra-species variations relating to the quality and quantity of secondary metabolites. The samples were dried at room temperature for 7 to 15 days and then pulverized in a Wiley mill with a standard 20 mesh particle screen.

Microorganism

The bacterial strains used in this study were *E. coli* (EC27) *S. aureus* (SA358) and *P. aeruginosa* (PA03). All the strains were clinical isolates with a profile of multi-drug resistance, as shown in Table 1. The strains were maintained in a tilted Brain-Heart Infusion agar (BHI, Difco Laboratories Ltda.) under refrigeration. Before conducting the test, the cells were cultured for 24 h at 37°C in BHI (BHI, Difco Laboratories Ltda).

Extract preparation

The extract was obtained by maceration of powdered stem bark of *C. tapia* L. in ethanol (70%). Seven successive extractions were carried out at 24-h intervals. The eluent was filtered through filter paper and 100 mL of fresh solvent was added to the solid residue. The total liquid extract was dried by using a rotary evaporator at temperatures below 40°C and under reduced pressure. The extraction product was used to prepare the solutions at 10 mg/mL, dissolved in dimethyl sulfoxide (DMSO) and then diluted with distilled water to a concentration of 1024 µg/mL.

Phytochemistry

Different classes of secondary metabolites were evaluated with regard to their presence/absence in the extract. Tests for phenols, tannins, flavonoids, steroids, triterpenes, alkaloids, coumarins and saponins were carried out using the methodology described by Matos (1997). The assays were based on the formation of color or precipitation after addition of reagents specific to each class of secondary metabolite.

Antimicrobial activity assay

The method developed by Javadpour et al. (1996) assessed the antibiotic activity of hydroalcoholic extract of *C. tapia* L. against *E. coli* (EC27), *S. aureus* (SA358) and *P. aeruginosa* (PA03). Bacterial inoculants for each strain were suspended in a 3.8% BHI broth and grown in a bacteriological incubator for 24 h at 37°C. After growth, each suspension was diluted to 10% BHI at a concentration of 1 x 10^6 CFU/mL and distributed in 96-well plates as follows: 100 µL of bacterial suspension per 100 µL of extract at different concentrations, to a final concentration of 5 x 10^5 CFU/mL. The extract solution previously prepared at a concentration of 1024 µg/mL was used to perform serial dilutions until concentrations in the range of 512-8 µg/mL were obtained. All assays were carried out in triplicate and the plates were incubated in a bacteriological incubator at 35 ± 2°C for 24 h.

Modulatory activity assay

To assess antibiotic resistance modulatory activity, the minimum inhibitory concentration (MIC) for each antibiotic was determined, as described in the previous section, in the presence and absence of the extract at a sub-inhibitory concentration (MIC/8) (Coutinho et al., 2008). For each clinically isolated strain, three antibiotics were used: Amikacin, Neomycin and Gentamicin, at final concentrations ranging from 2500 - 1 µg/mL. The colorimetric method of adding resazurin was used to evaluate antibacterial activity. After incubation, 25 µL of a 0.01% aqueous resazurin solution was added to each well. In the presence of bacterial growth, resazurin changes color from blue to pink. The minimum inhibitory concentration was determined to be the last dilution where there was no cell growth.

RESULTS

Phytochemical analysis of crude hydroalcoholic extract of *C. tapia* (CECT) showed the presence of phenols of the flavone, flavonol and xanthone types. In addition to these, alkaloids were also found. These data are shown in Table 2, with the presence or absence of each metabolite under analysis indicated by + or -, respectively.

The minimum inhibitory concentration of the CECT against all strains used in this study cannot be determined because there was bacterial growth at all concentrations ≥1024 µg/mL. The results for antibiotic resistance modulatory activity are presented in Table 3.

Table 2. Phytochemical analysis of a hydroalcoholic crude extract of stem bark of *C. tapia* L..

	Metabolite																		
Extract	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
CECT	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-

The presence (+) or absence (-) of the different metabolites is indicated. 1- Phenols; 2- hydrolyzable tannin; 3- condensed tannin; 4- anthocyanins; 5- anthocyanidins; 6- flavones; 7- flavonols; 8 - xanthones; 9- chalcones; 10 - aurones; 11 - flavononols; 12- leukoanthocyanidins; 13- catechins; 14- flavonones; 15- cumarines, 16- free steroids, 17 - pentacyclic triterpenoids, 18- alkaloids; 19- saponins.

Table 3. Modulatory activity of antibiotic activity of a crude extract *C. tapia* L. against multi-resistant strains (MIC at μg/mL).

Antibiotic	*Escherichia coli* 27			*Staphylococcus aureus* 358			*Pseudomonas aeruginosa* 03		
	Ant.	Ant. + CECT	Reduction (%)	Ant.	Ant. + CECT	Reduction (%)	Ant.	Ant. + CECT	Reduction (%)
Amikacin	78.12	78.12	0	312.5	39.06	87.5	312.5	312.5	0
Neomycin	156.25	625	- 400	156.25	156.25	0	312.5	312.5	0
Gentamicin	312.5	312.5	0	312.5	78.12	75.0	312.5	78.12	75.0

Ant.- Antibiotic, CECT- crude extract of *C. tapia*.

Of particular interest is the synergistic activity with Amikacin against SA358, in which the MIC decreased from 312.5 to 39.06 μg/mL, which represents a reduction of approximately 87.5% in the concentration needed to inhibit microbial growth. Another important result was obtained for gentamicin against SA358 and PA03. The MIC for both strains decreased from 312.5 to 78.12 μg/mL, a reduction of approximately 75%. It is also worth highlighting the antagonistic activity when the extract was combined with Neomycin and then exposed to EC27, where the MIC increased from 156.25 to 625 μg/mL, reducing the power of the antibiotic by 400% in terms of the concentration required to prevent bacterial prolife-ration.

DISCUSSION

No study to date, to our knowledge, has evaluated the antimicrobial activity of a hydroalcoholic extract of *C. tapia*. However, Salvat et al. (2001) tested the methanolic crude extract of the species in question and found an MIC of 500 μg/mL against *P. aeruginosa* ATCC strains, considering active extracts to be those that obtained an MIC of less than or equal to 500 μg/mL. The present study, using a hydroalcoholic extract, did not find this level of activity. This difference in activity may be explained by the fact that the samples in the two studies were collected from different regions. Although both

studies were conducted on the same continent, Gobbo-Neto and Lopes (2007) have reported that the quantity and quality of secondary metabolites can be influenced by the climate, altitude, soil and radiation levels. Even within the same collection area, slight differences, such as the habitat where each specimen is collected, can produce statistically significant differences in results. This has been demonstrated by Araújo et al. (2012) in a model for the species *Spondias tuberosa* that investigated the tannin levels and the antioxidant activity of sequestrated free radicals. It should also be pointed out that Salvat et al. (2001) did not use clinically isolated multi-resistant strains but rather standard sensitivity ATCC strains, which normally exhibit a higher degree of susceptibility to substances with antibiotic potential.

The evaluation of phytochemical compounds conduc-ted by this study did not find a wide variety of secondary compound classes, as observed in previous studies by the same team. These compounds can be classified broadly into phenols and alkaloids. Patil et al. (2010), in a study of the pharmacognosy of *C. Tapia* leaves, also identified the presence of phenols and alkaloids and other metabolics such as steroids and glycosides. Phenols are substances commonly associated with antimicrobial activity in various plant species, as observed by Machado et al. (2003), who found poly-phenols isolated from the extract of *Punica granatum* to be responsible for activity against methicillin-resistant *S. aureus* strains. Although the present study detected the

presence of phenols in the sample tested, the antimicrobial activity of the crude hydroalcoholic extract of *C. tapia* against the three strains tested (*E. coli*; *S. aureus*; *P. aeruginosa*) was not of clinical importance, because the MIC was ≥ 1024 µg/mL, a concentration considered impractical for therapeutic applications.

Although the extract did not seem to exhibit clinically significant activity against the tested microorganisms, antibiotic modulatory activity was observed with some combinations. This concurs with the findings of Morais-Braga et al. (2013), who tested the crude hydroalcoholic extract of *Lygodium venustum* against these same strains. In that particular case, the extract tested was considered inactive with an MIC ≥ 1024 µg/mL. However, when tested in combination with antibiotic aminoglycosides, modulatory action exhibited a pronounced decrease of 99% in MIC. An evaluation of the antimicrobial activity of a methanol and hexane extract of *Ocimum gratissimum*, a plant species common in the semi-arid regions of Northeastern Brazil, against *S. aureus* did not reveal clinically significant activity (MIC ≥ 1024 µg/mL). However, in combination with Neomycin, there was significant synergism, decreasing the MIC of the antibiotic from 317 to 2.2 µg/mL (Matias et al., 2011). These findings reinforce the idea that one should never overlook the potential modulatory effect of an extract based solely upon its antimicrobial activity, as it has been demonstrated that, even when an extract is considered inactive against certain strains, it may present important synergism when used in combination with antibiotics frequently used in medical practice.

The combination with the antibiotic Neomycin against EC27 exhibited antagonistic activity of 400%. Antibiotic resistance can occur by way of three main mechanisms of action: modification of the binding site with a reduction in drug-receptor interaction, modification or destruction of the antibiotic molecule by enzyme action, and efflux of the antibiotic by way of preventing the ingress or expulsion of the same molecules which have penetrated the microorganism. A plant extract or isolated substance, natural or synthetic, may interfere with any of these bacterial resistance mechanisms, producing an antagonistic modulating effect (Wagner and Ulrich-Merzenich, 2009).

The phenols found in the extract investigated by the present study were classified as flavones and flavonols, which may explain their modulatory activity, since, according to Cowan (1999), these compounds are able to create a complex in the bacterial cell wall, causing the expression of membrane receptors that alter the profile of resistance to a particular antibiotic. Another mechanism of action for the modulatory activity of antibiotics through polyphenols has been described by Samoilova et al. (2013) using strains of *E. coli*. The researchers concluded that these compounds may reduce the bactericidal action of Ciprofloxacin and Ampicillin and increase bacterial sensitivity to Kanamycin by reduced or

increased oxidative stress induced by the antibiotic. This shows that the crude extract may have synergistic or antagonistic effects depending on the strain and associated antibiotic being tested.

Alkaloids are widely known for their activity in the central nervous system but this class of secondary metabolites is also acquiring importance in microbiology. Xing et al. (2012) evaluated the activity of the alkaloid harmaline against clinically isolated strains of S. *aureus* in combination with the antibiotic chlorhexidine. Their study found that harmaline is a relatively weak substance when confronted with strains tested in cells in suspension as well as in biofilms. However, when combined with chlorhexidine, it showed a significant synergistic effect against almost all clinical isolates tested. Another alkaloid whose antimicrobial activity has been assessed is Berberine, a derivative of isoquinoline. Berberine in fact shows activity both when used alone and in combination with chlorhexidine against a mixture of oral biofilm forming micro-organisms (Xie et al., 2012).

As far as we are aware, no study of the modulatory antibiotic activity of this plant species or even of the Capparaceae family can be found in the literature available to date.

It is worth highlighting one of the most useful results presented in this study: *C. tapia* crude extract in combination with the antibiotic Amikacin against *S. aureus* produced an MIC reduction of 87%. This plant species is thus a good candidate for future studies, such as extract fractionation. The same can be said of other extract types and plant parts of *C. tapia*.

Conclusion

The present study has extended phytochemical knowledge of *C. tapia,* and identified the presence of phenols and alkaloids in the hydroalcoholic extract of its stem bark. It has further demonstrated the modulatory antibiotic activity of this species, although it does not exhibit this activity when used alone. These findings also reveal the need to expand studies aiming to combine plant extracts with commercial antibiotics to combat microbial multi-drug resistance.

Conflict of interests

The authors did not declare any conflict of interest.

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