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Modulation of the norfloxacin resistance in *Staphylococcus aureus* by *Cordia verbenaceae* DC

Edinardo F.F. Matias[1], Karla K. A. Santos[1], Vivyanne S. Falcão-Silva[2], José P. Siqueira-Júnior[2], José G. M. Costa[3,4] & Henrique D.M. Coutinho[1]

[1]Laboratório de Microbiologia e Biologia Molecular, Universidade Regional do Cariri, Crato (CE), Brasil, [2]Laboratório de Genética de Microrganismos, Universidade Federal da Paraíba, João Pessoa (PB), Brasil, [3]Laboratório de Pesquisa em Produtos Naturais, Universidade Regional o Cariri, Crato (CE), Brasil & [4]Faculdade Leão Sampaio, Juazeiro do Norte (CE), Brasil

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***Background & objectives*: Several chemical compounds isolated from natural sources have antibacterial activity and some enhance the antibacterial activity of antibiotics reversing the natural resistance of bacteria to certain antibiotics. In this study, the hexane and methanol extract of *Cordia verbenaceae* were assessed for antibacterial activity alone and combinated with norfloxacin against the *Staphylococcus aureus* strain SA1199B.**

***Methods*: The minimum inhibitory concentration (MIC) of extracts was assayed using microdilution assay and the modulatory activity was evaluated using plate diffusion assay.**

***Results*: The MIC observed varied between 256 to >1024 µg/ml. However, the antibiotic activity of norfloxacin was enhanced in the presence of subinhibitory concentrations of hexane extract of *C. verbenaceae* (HECV).**

***Interpretations & conclusions*: Our results indicate that *Cordia verbenaceae DC.* can be a source of plant derived products with antibiotic modifying activity.**

Key words Antibacterial activity - antibiotics - *Cordia verbenaceae* DC - hexane extract - modulation of resistance - *Staphylococcus aureus*

Some species of *Staphylococcus* are frequently recognized as agents of opportunistic infections[1,2]. *S. aureus, S. epidermidis, S. saprophyticus* and *S. haemolyticus* are the most important species responsible for human infections. *S. aureus* represents the main agent of purulent infections (*e.g.* osteomyelitis, furuncle, carbuncle, abscess, myocarditis, endocarditis, pneumonia, shunt-associated meningitis, bacterial arthritis)[3,4].

There has been scientific interest in chemical and pharmacological investigations of the biological properties of medicinal plants[5-8]. Medicinal plants

have been the source of many medications that are now applied in clinical practice. The use of extracts as antimicrobial agents demonstrated a low risk to the resistance development because these are complex mixtures[9]. Many plants have been evaluated not only for direct antimicrobial activity but also as resistance-modifying agents. Various chemical compounds, synthetic or from natural sources, have direct activity against many species of bacteria, enhancing the activity of a specific antibiotic, reversing the natural resistance of bacteria to specific antibiotics, causing the elimination of plasmids and inhibiting the active efflux of antibiotics through the plasma membrane[1,5].

The genus *Cordia* is present in all tropical and subtropical zones worldwide[10]. *Cordia verbenaceae* DC is distributed in all regions of Brazil[11]. The bulk extract of aerial parts is used in folk medicine as a topical anti-inflammatory product and several studies demonstrated a phototoxic, antibacterial and modulatory effect of antibiotic activity[12-14]. The anti-inflammatory activity observed is thought to be due the flavonol artemetine[15].

The aim of this study was to verify the phytochemical composition of the methanol and hexane extracts of *C. verbenacea* and determine the modulatory antibiotic activity of these extracts against the norfloxacin in *S. aureus.*

Material & Methods

Bacterial strains: The bacterial strains utilized were the clinical isolate *S. aureus* 358 (SA358) and the strains *S. aureus* ATCC25923 (SA-ATCC25923) and *S. aureus* 1199B (SA1199B). The strain 1199B overexpress the *norA* gene, encoding the NorA efflux protein, the main protein of the efflux pump responsible by the extrusion of norfloxacin and other biocides[16]. All isolates were maintained on slants with heart infusion agar (HIA, Difco Laboratories Ltd., USA). Before the assay, the cells were grown overnight at 37ºC in brain heart infusion broth (BHI, Difco, USA).

Plant material: Leaves of *C. verbenacea* DC. were collected in the county of Crato, Ceará, Brazil. The plant material was identified and voucher specimens were deposited in the Herbario Prisco Bezerra of Universidade Federal do Ceará - UFC, as Nº. 044171.

Preparation of methanol and hexane extracts of C. verbenaceae DC.: For the preparation of the extracts, leaves were collected and submersed in two reservoirs with methanol or hexane for 72 h. The extract was filtered and concentrated under vacuum using a rotary evaporator (model Q-344B - Quimis, Brazil) and an ultrathermal bath (model Q-214M2 - Quimis, Brazil)[1]. Overall, 31.4 g of leaves yielded 1.74 g of methanol extract (MECV) and 107.47 g leaves yielded 1.72 g of hexane extract (HECV). The extracts solutions utilized in the tests were dissolved using DMSO (Dimethyl Sulphoxide) to a concentration of 10 mg/ml and diluted with distilled water to obtain a final concentration of 1024 µg/ml.

Phytochemical tests: The phytochemical tests to detect the presence of heterosides, saponins, tannins, flavonoids, steroids, triterpenes, cumarins, quinones, organic acids and alkaloids were performed according to the methods described by Matos[17]. The tests were based on the visual observation of colour changes and observations of precipitates after the addition of specific reagents (Table I).

Tabe I. Phytochemical prospection of methanol and hexane extracts of *C. verbenaceae* DC

Metabolites / Extracts	Phenols	Tannin pyrogallates	Tannin phlobaphenes	Anthocyanins	Anthocyanidins	Flavones	Flavonols	Xanthones	Chalcones	Aurones	Flavononols	Leucoanthocyanidins	Catechins	Flavonones	Alkaloids	Terpenes
MECV	-	-	+	-	-	+	+	+	+	+	+	+	+	+	-	+
HECV	-	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+

(+) presence; (-) absence; MECV, Methanol extract of *Cordia verbenaceae*; HECC-hexane extract of *C. verbenaceae*

Drugs: Norfloxacin was purchased from Bayer S.A., Brazil, and all the other drugs were purchased from Sigma Chemical Co., USA.

Antibacterial test (MIC): MIC (minimal inhibitory concentration) of extracts was determined using the microdilution assay[18]. Bacterial inoculum (100 µl) was suspended in BHI 10 per cent to a final concentration of 10^5 cfu/ml and distributed in 96-well microtiter plates. Each well received 100 µl of each extract solution. The final concentrations of the extracts varied between 512-8 µg/ml using two-fold serial dilutions[18]. MICs were recorded as the lowest concentrations of the extracts required to inhibit growth. The same method were used to evaluate the MIC of norfloxacin, with a drug concentration ranging between 2.5 to 0.0012 mg/ml[18]. The plates were incubated for 24 h at 37 °C.

Modulation assay: To evaluate the modulatory antibiotic activity of the extracts, the MIC of antibiotic was determined in the presence or absence of MECV and HECV using a sub-inhibitory concentration (MIC/8 = 32 µg/ml). The modulation assay was realized following the plate modulation method, as described by Stavri *et al*[19]. All experiments were performed in triplicate.

Results & Discussion

Methanol extract of *C. verbenaceae* DC showed MIC of ≥1024 µg/ml for both strains SA 358 and SA-ATCC 25923. Hexane extract gene ≥1024 µg/ml MIC for SA358 and 256 µg/ml for SA-ATCC 25923 strain. The results demonstrated that HECV was more effective against the assayed bacteria.

Table II shows the modulatory antibiotic activity of the extracts when associated with norfloxacin. When the extracts were incorporated into the growth medium with a sub-inhibitory concentration of 32 µg/ml, an enhancement of 50 per cent in the inhibition zone was observed as compared with the antibiotic alone.

Table II. Enhancement of the inhibition zone by *C. verbenaceae*

S. aureus 1199B (mm ± SD*)		
	Norfloxacin	Variation (%)
No treatment	8 ± 0	ND
DMSO	8 ± 0	0
MECV (32 mg/ml)	8.5 ± 0.5	6.25
HECV (32 mg/ml)	12 ± 0	50

MECV, Methanol extract of *C. verbenaceae*; HECC, Hexane extract of *C. verbenaceae*; SD, Standard deviation; ND, not determined

With the increase of the bacterial resistance to antibiotics, the use of natural products from plants could represent an interesting alternative[1]. Some plant extracts and phytochemicals are known to have antimicrobial properties. Several studies have been conducted in different countries, demonstrating the efficacy of this approach[20]. Many plants have been evaluated not only for direct antimicrobial activity but also as resistance modifying agents[21].

The antibacterial activity of *C. verbenacea* was demonstrated against Gram- positive and -negative bacteria[14], as well as the phototoxic and modifying antibiotic activity against aminoglycoside, another class of antibiotics that inhibits the protein synthesis[12,13]. These activities can be related with the presence of phytocompounds with low polarity as tannins, flavonoids and terpenes. These compounds are usually found in higher concentrations on apolar fractions or extracts, as the hexane extract of *C. verbenacea*. That could be the reason for a better antibacterial and modulatory activity of HECV, with lower MIC values and a better enhancement of norfloxacin activity.

About the tannins, the antimicrobial properties appear to be associated with the hydrolysis of an ester bond with gallic acid, thereby serving as a mechanism of natural defence against microbial infections. The tannins, epicatechin and catechin from *Vaccinium vitisidaea* L. demonstrated a strong antimicrobial activity against bacteria and fungi[22]. Flavonoids are synthesized by plants in response to microbial infection[23] and are effective against a broad range of microorganisms. The activity is probably due to their capacity to form complexes with extracellular soluble proteins associated with the bacterial cell wall. Some lipophilic flavonoids can also cause rupture of the plasma membrane of microorganisms[24]. Terpenes occur in the form of diterpenes, triterpenes, tetraterpenes as well as hemi- and sesquiterpenes. Terpenenes or terpenoids are active against bacteria[25,26].

The mechanisms by which extracts can inhibit the growth of microorganisms are varied, and can be due to the hydrophobic nature of some components. Due to this fact, these can show a higher interaction with the lipid bilayer of the cell membrane, affecting the respiratory chain and production of energy[27] or even enhancing the permeability of the cell membrane

to antibiotics[28,29]. The interference with bacterial enzyme systems can also be a potential mechanism of action[30]. These mechanisms of action may be due to combination of antibiotic with extract at a sub-inhibitory concentration applied directly to the culture medium[1,5]. This synergy approach may lead to the development of a new generation of phytopharmaceuticals[31].

In conclusion, the results indicated that *C. verbenacea* DC. could serve as a source of plant-derived natural products that modify antibiotic resistance for use against multidrug-resistant bacteria.

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Reprint requests: Dr Henrique Douglas Melo Coutinho, Laboratório de Microbiologia e Biologia Molecular - LMBM;
Departamento de Química Biológica - DQB, Universidade Regional do Cariri - URCA,
Rua Cel. Antonio Luis 1161, Pimenta 63105-000, Crato (CE), Brasil
e-mail: hdmcoutinho@gmail.com