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Chemical composition and modulation of bacterial drug resistance of the essential oil from leaves of *Croton grewioides*

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ABSTRACT

The essential oil from leaves of *Croton grewioides* Baill was obtained by hydrodistillation using Clevenger apparatus, and its chemical composition was analyzed by GC-MS, where 18 compounds were identified, mostly as monoterpenes (55.56%) and sesquiterpenes (44.44%), in which the major constituent was the α-pinene (47.43%). The essential oil of *Croton grewioides* (EO*Cg*) and its major compound (α-pinene) were evaluated as modulators of antibiotic resistance in strain SA-1199B and IS-58 of *Staphylococcus aureus* that overexpresses efflux protein. The minimum inhibitory concentrations (MICs) of the antibiotics were determined by the microdilution assay in the absence and in the presence of sub-inhibitory concentration of EOCg and α-pinene. Although the EO*Cg* and α-pinene did not indicate relevant antibacterial activity in vitro, they acted as antibiotic resistance modulators, i.e., EOCg in combination with norfloxacin, reducted its MIC, by 64× whereas in combination with tetracycline it was observed a reduction of 4×. Additionally, it was observed a MIC reduction of tetracycline by 32×, when combined with α-pinene. The results suggest that EO*Cg* and α-pinene modulate or even reverse bacterial resistance as a putative efflux pump inhibitor.

1. Introduction

The Euphorbiaceae or spurge family comprises 8000 species distributed throughout the tropical and temperate regions of the world; mainly in Africa and America. The *Croton* genus constitutes about 700 species, many of which have medicinal and toxic properties [1]. A considerable number of folkloric uses have been described for different species in Africa and America and a number of isolated compounds from this genus have shown interesting pharmacological activities. Many of the *Croton* species produce essential oils which are used for medicinal purposes: *C. zehntneri* and *C. cajucara* are commonly used in Brazil to treat gastrointestinal disturbances [2,3].*C. lechleri* is widely used in South American countries against inflammations, gastric ulcers, wound healing and cancer [4]; and *C. nepetaefolius* is used in the folk medicine of Brazil as a stomachic, carminative and intestinal antispasmodic [5]. The composition of the essential oils reported from *Croton* genus shows that the main components are phenylpropanoids, monoterpenes and sesquiterpenes [6–9]. *Croton grewioides*Baill is a shrub of 1.5 m in height, which grows naturally in areas of natural pastures interspersed with woods and rock outcrops. In the savanna, this plant is characterized by its pleasant smell coming mainly from leaves. Previous work with this species, collected in the wild state of Pernambuco-Brazil, have reported the chemical composition of its essential oil and the potent insecticidal activity [10].

The ability of *S. aureus* to acquire resistance to almost all antibiotics is a reason of considerable concern [11]. The mechanisms by which bacteria become resistant are very versatile, being the efflux pump (integral proteins) one of the mechanisms that causes bacterial resistance, which eliminates antimicrobial agents to the extracellular environment. Therefore, the intracellular concentration of the agent remains insufficient to block cell function [12–14]. Modulators of drug resistance by efflux pump inhibitor, also called

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modifiers of antibiotic activity are considered as the most appropriate tools for new antibacterial therapies [15]. Plants produce a large amount of secondary metabolites, and a significant part of this chemical diversity serves to protect it against microbial pathogens. Plant species are a rich source of potent efflux pump inhibitor and several constituents have been identified with that function as such [11,16,17]. An example of these secondary metabolites as a source of modulators of antimicrobial activity is coumarin isopimpinellin that has depleted an ability to modulate antimicrobial activity by reducing the MIC of some antibiotics against strains of *S aureus* carried out by our research group [18]. In this paper we report the chemical composition of the essential oil from *C. grewioides* leaves (EO*Cg*), as well as the evaluation of the EO*Cg* and its major constituent (α-pinene) as modulators of antibiotic resistance using an effluxing strain of *Staphylococcus aureus.*

2. Results and discussion

The essential oil was obtained by hydrodistillation from the leaves of *C. grewioides* with a yield of 0.1% compared to the fresh weight of its botanical material. The total percentage of volatile components identified was of 97.40%, comprising 18 components. The qualitative and quantitative analysis of essential oil was performed by GC-MS. The identification of substances was performed by comparing their mass spectra with the database of the GC-MS (62 Nist. lib.) and Kovats retention index which was obtained by coinjection of the essential oil with a mixture of standard hydrocarbons (C9-C24), applying the equation of Van den Dool & Kratz, 1963 [19]. Monoterpenes (55.56%) and sesquiterpenes (44.44%) were the main groups of chemical constituents isolated, in which, the major compounds were: α-pinene (47.43%), sabinene (12.09%), limonene (7, 98%), bicyclogermacrene (5.96%), *trans*-caryophyllene (5.51%), and germacrene D (4.96%) (Table 1). This chemical composition is consistent with literature data for volatile constituents from *Croton* species [20].

Components identified based on IR and GC-MS and listed according to elution order in column DB-5 (30 m).

The MIC of norfloxacin and tetracycline were observed at 64 μg/mL and 32 μg/mL, respectively. Although the EO*Cg* and the α-pinene have not indicated relevant antibacterial activity (MIC = 0,5%) when they were incorporated into the culture medium at subinibitory concentrations (¼ MIC), it was observed a modulation of antibiotic resistance.

In the presence of EO*Cg*, the MIC of norfloxacin and tetracycline was 16 μg/mL (4× reduction) and 0.5 μg/mL (64× reduction), respectively. The α-pinene did not act as a modulator of antibiotic activity for norfloxacin, but modulated the activity of tetracycline resistance, reducing 32× the NorA efflux protein, demonstrated by a MIC of 1 μg/mL (see Table 2).

It is a provocative and plausible idea that other major components of EO*Cg* also act as modulators of drug resistance, once the EOCg demonstrated better results than its major compound, Therefore, experiments have been planned to evaluate this hypothesis in the near future.

In general, antimicrobial resistance alters the antibiotic action through one of the following mechanisms: antimicrobial target modifications (decreasing drug affinity), a decrease in drug absorption, activation of efflux mechanisms to expel the damaging molecule (overexpression of efflux pumps) or global changes in important metabolic pathways through the modulation of regulatory networks [20].

In this context, the antibiotic resistance modulating effects of the extracts or natural compounds from medicinal plants against resistant bacteria have been already reported [21]. Some studies have reported the potentiating effect of essential oils when combined with conventional antimicrobial drugs [22–27], however, it is noteworthy the fact that only a few articles have described the evaluation of essential oil as a modulator of drug resistance by inhibition of efflux pump [28,29], and it is also important to mention that the EO*Cg* exhibited better modulation.

Therefore, the results presented in this article suggest that the essential oil of *C. grewioides* and its major component α-pinene, are a source of potential adjuvants of antibiotics, i.e. as putative efflux pump inhibitors in bacteria, modulating or even reversing the bacterial resistance to antibiotics.

Table 1
Chemical composition of essential oil *C. grewioides*.

Substances	(% relative)	IK exp.	IK lit.*
α-pinene	47,43	931	939
Sabinene	12,09	968	976
β-pinene	1,91	972	980
Mircene	0,70	986	991
α-felandrene	0,61	1001	1005
Limonene	7,98	1023	1031
1,8 cineol	2,21	1025	1033
γ-terpinene	0,61	1052	1062
4-tujanol	0,55	1093	1097
4-terpeneol	0,75	1169	1177
α-copaene	1,29	1368	1376
trans-caryophyllene	5,51	1411	1418
α-humulene	0,61	1444	1454
germacrene D	4,96	1471	1481
bicyclogermacrene	5,96	1487	1494
β-bisabolene	2,99	1499	1509
delta-cadinene	0,46	1513	1524
Spathulenol	0,78	1564	1576
Total identified	97,40		

* Retention indices according to Ref. [31].

3. Materials and methods

3.1. Collection of plant material

The leaves of *C. grewioides* were collected in the city of Serra Branca, located at the micro-region of "Cariri Paraibano" is situated in the Plateau of Borborema in Paraíba, Brazil. This area is characterized by a hot and dry climate and is considered to be among the driest parts of Brazil, having a type of vegetation consisting mainly of xerophytic and deciduous forest [30]. The botanical material was identified by Prof. Dr. Maria de Fatima Agra from the Biotechnology Center (CBiotec/UFPB). A voucher specimen (AGRA et al., 6987) is deposited at the Herbarium Prof. LauroPires Xavier (JPB), Universidade Federal da Paraíba.

3.2. Extraction of the essential oil

The fresh leaves of *C. grewioides* were submitted to

Table 2
Minimum inhibitory concentrations (μg/mL) of antibiotics in the absence and presence of essential oil *C. grewioides* and the α-pinene in a subinibitory concentration.

Substance	MIC of antibiotic (μg/mL)	
	Norfloxacin	Tetracycline
Absence	64	32
Essential Oil of *C. Grewioides*	16 (4×)[a]	0,5 (64×)
α-pinene (47.43%)	64	1 (32×)

[a] Parenthesis the reduction factors of the MIC.