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Antibacterial activity of terpenoids isolated from *Cnidoscolus quercifolius* Pohl (Euphorbiaceae), a Brazilian medicinal plant from Caatinga biome

Raimundo Gonçalves de Oliveira-Júnior^a, Christiane Adrielly Alves Ferraz^a, Michelle Cruz Pontes^a, Noelly Bastos Cavalcante^a, Edigênia Cavalcante da Cruz Araújo^a, Ana Paula de Oliveira^a, Laurent Picot^b, Larissa Araújo Rolim^a, Jackson Roberto Guedes da Silva Almeida^{a,*}

^aDepartment of Pharmacy, Federal University of San Francisco Valley, 56.304-205, Petrolina, Pernambuco, Brazil.

^bFaculty of Science and Technology, UMRi CNRS 7266 LIENSs, University of La Rochelle, La Rochelle, France.

*Correspondence: Jackson R.G.S. Almeida, Universidade Federal do Vale do São Francisco, 56.304-205, Petrolina, PE, Brazil. E-mail: jackson.guedes@univasf.edu.br; Phone/Fax: +55-87-21016862

ABSTRACT

Introduction: *Cnidocolus quercifolius* Pohl (Euphorbiaceae) is a Brazilian medicinal plant, popularly known as “favela”, “faveleira” and “urtiga-branca”. In folk medicine, its leaves and stem barks are used to treat urinary tract infections. In this study, we report the isolation and characterization of a mixture of two lupane-type triterpenes and one bis-*nor*-diterpene from the hexane extract of the stem barks.

Methods: An aliquot of hexane extract was fractionated by means silica gel chromatography column, yielding a mixture of two compounds (1 and 2). Compound 3 was obtained from the chloroform subfraction through preparative thin layer chromatography (TLC). The compounds were identified by comparison of their spectroscopic data to those reported in the literature. The antibacterial activity of these compounds was evaluated by the microdilution method, through determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), using gram-positive (*E. faecalis* and *S. aureus*) and gram-negative (*E. coli*, *K. pneumoniae* and *S. marcescens*) strains.

Results: The chemical constituents were identified as a mixture of lupeol-3 β -*O*-cinnamate (1) and lupeol-3 β -*O*-dihydrocinnamate (2) and, phyllacanthone (3). Compounds 1/2 and 3 showed a bacteriostatic effect ten and twenty times higher (MIC = 0.5 and 0.25 mg.ml⁻¹, respectively) than the hexane extract, respectively (MIC = 5.0 mg.ml⁻¹). The molecules also showed a relevant bactericidal effect, mainly compound 3 (MBC = 0.25 mg.ml⁻¹).

Conclusion: These results suggest that *C. quercifolius* may be considered a promising source of new antibacterial agents. In addition, this investigation supports the medicinal use of this plant for the treatment of urinary tract infections.

Keywords: *Cnidocolus*; medicinal plant; triterpene; diterpene; antibacterial activity.

1. Introduction

The use of antibiotics is widely described in many clinical situations, including in the control of infections during invasive surgeries, in cancer chemotherapy and in the treatment of immune-compromised patients [1]. In recent years, antibiotic resistance has become a serious health problem, leading to the emergence of microorganisms resistant to conventional chemotherapy [2]. In this sense, infectious diseases caused by antibiotic resistant microorganisms have been frequently reported [3,4]. For this reason, the search for new antimicrobial molecules from natural products remains an effective alternative [5-7].

Euphorbiaceae is a pantropical family containing approximately 300 genera and 7000 species, including trees, shrubs and herbs, of which 70 genera and 1000 species occur in Brazil [8]. The neotropical genus *Cnidoscolus* Pohl is represented by 70 species [9,10]. In Brazil, there are 42 *Cnidoscolus* species distributed in different geographical areas, such as Cerrado, Atlantic Forest, Amazon and Caatinga biome [11,12].

Cnidoscolus quercifolius Pohl (syn.: *Cnidoscolus phyllacanthus* (Mull. Arg.) Pax & L. Hoffm.) is a Brazilian medicinal plant from Caatinga biome, popularly known as “favela”, “faveleira” and “urtiga-branca”. In folk medicine, its leaves and stem barks are used to treat injury, urinary tract infections and inflammatory processes [13]. Previous pharmacological investigations evaluated the antinociceptive [14], anti-inflammatory [15], antioxidant [16,17], antiproliferative [18,19] and antibacterial [17,20] effects of crude extracts from *C. quercifolius*.

From a phytochemical point of view, lupane-type triterpenes and bis-nor-diterpenes have been reported in *C. quercifolius* [21]. New tricyclic benzocyclohepten diterpenes, called favelins, were isolated from stem barks extracts [22]. New cyclopropane and cyclobutene tetracyclic derivatives, such as favelanone and neofavelanone, respectively, were also described in previous phytochemical studies [23,24]. Apparently, these diterpenes occur exclusively in *C. quercifolius*.

Although *C. quercifolius* extracts have been investigated for their antibacterial potential, there are no reports on the antibacterial activity of compounds isolated from this species.

Considering the popular use of this plant for the treatment of infections, the present study describes the antibacterial activity of terpenoids isolated from the stem barks of *C. quercifolius* against pathogenic strains.

2. Materials and methods

2.1. Plant material

Stem barks of *C. quercifolius* were collected in the city of Petrolina (coordinates 09° 24' 40.97" S and 40° 32' 49.19" W), state of Pernambuco, Brazil, in February 2015. A voucher specimen (n° 19202) has been deposited at the Herbário do Vale do São Francisco (HVASF), of the Universidade Federal do Vale do São Francisco (UNIVASF).

2.2. Extraction and isolation

The dried and pulverized stem barks (534 g) were extracted with hexane (2.0 L, four times), followed by methanol (2.0 L, four times), yielding 8.7 and 18.5 g of hexane and methanol extracts (Hex-Cq and MeOH-Cq, respectively) after removal of the solvents under vacuum. An aliquot of Hex-Cq (7.0 g) was fractionated by chromatography column, using silica gel 60 (Sigma-Aldrich[®], 230-240 mesh) as stationary phase. Hexane, chloroform (CHCl₃), ethyl acetate (AcOEt) and methanol (MeOH) were used as eluents, in increasing order of polarity. In total, 42 fractions (125 ml each) were collected and reunited in 13 different groups (groups A–M), after TLC analysis. Group D (eluted with hexane/CHCl₃ 2.5:7.5, v/v) showed the formation of white crystals (635 mg) soluble in chloroform, which was subsequently identified as a mixture of two compounds (**1** and **2**).

Group F (eluted with CHCl_3) was fractionated by preparative TLC, using hexane/AcOEt (9:1, v/v) as eluent, leading to the isolation of compound **3** (13.7 mg, yellow crystals soluble in chloroform).

2.3. Spectroscopic analysis

NMR spectra (^1H , ^{13}C , DEPT-135, COSY, HSQC and HMBC) were recorded on a Bruker Ascend[®] (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer, at 25 °C and with CDCl_3 as solvent (internal standard 0.05% TMS, Tedia[®], Brazil). Chemical shifts are given in ppm values (δ) and coupling constants (J) in Hz. In addition, retention factor (R_f) was calculated for each compound, through TLC analysis, using the same eluent (hexane/AcOEt, 9.5:0.5, v/v). TLC plates were visualized in UV chamber (254 and 365 nm) and, subsequently, revealed with 1% sulfuric vanillin solution.

2.4. Antibacterial activity

2.4.1. Bacteria strains assay

Reference bacterial strains were obtained from National Institute of Quality Control in Health (INCQS/FIOCRUZ, Brazil). The pathogenic microorganisms used were *Enterococcus faecalis* (ATCC[®] 19433), *Escherichia coli* (ATCC[®] 25922), *Klebsiella pneumoniae* (ATCC[®] 13883), *Serratia marcescens* (ATCC[®] 13880) and *Staphylococcus aureus* (ATCC[®] 25923).

2.4.2. Determination of minimum inhibitory concentration (MIC)

Antibacterial activity was evaluated by the microdilution method, as recommended by National Committee for Clinical Laboratory Standards [25]. Stock solutions of extracts (Hex-Cq and MeOH-Cq, 20 mg.ml⁻¹) and isolated compounds (**1/2** and **3**, 2 mg.ml⁻¹) were prepared in 2%

DMSO (v/v). Subsequently, 100 μl of this solution was added to microplates containing 100 μl of Müller-Hinton broth, in order to obtain final concentrations of 10 – 0.16 $\text{mg}\cdot\text{ml}^{-1}$ (for extracts) and 1 – 0.008 $\text{mg}\cdot\text{ml}^{-1}$ (for isolated compounds). Gentamicin was used as positive control (final concentrations of 1.6 – 0.012 $\text{mg}\cdot\text{ml}^{-1}$). Wells were reserved for sterility control of the broth (negative control). Finally, an inoculum containing 5×10^5 CFU. ml^{-1} (0.5 in McFarland scale) was added to each well and microplates were maintained under aerobic conditions (37 °C, during 18-24 hours). After incubation, 10 μl of 2% 2,3,5-triphenyl-tetrazolium (CTT) solution (v/v) were added to each well to detect the color change of the CTT (colorless) to red, reflecting the bacterial metabolism active. MIC was defined as the lowest concentration of extracts or compounds that visibly inhibited the bacterial growth [in three independent experiments](#).

2.4.3. Determination of minimum bactericidal concentration (MBC)

To determine MBC, an aliquot (10 μl) was withdrawn from each well containing extracts or isolated compounds and transferred to Petri dishes containing agar Müller-Hinton. Plates were incubated for 24 h in the same conditions previously described. The absence of bacterial colony for a given concentration indicates that it was able to kill 99.9% or more of bacterial inoculum used. MBC was defined as the lowest concentration of extracts or compounds that visibly inhibited the bacterial colony production [in three independent experiments](#).

3. Results

3.1. Structural characterization of isolated compounds

TLC analysis revealed a good degree of purity for the isolated compounds. Compound **3** showed fluorescence at 365 nm, suggesting the presence of an aromatic ring in its structure. In

addition, TLC analysis using sulfuric vanillin revealed the presence of purple spots, indicating that these compounds were terpenes (Fig. 1).

After careful NMR (1D and 2D) analysis, as well as comparison with spectral data reported in the literature [18,21], we identified compounds **1** and **2** as lupeol-3 β -*O*-cinnamate and lupeol-3 β -*O*-dihydrocinnamate, respectively. The presence of a lupeol unit in both molecules was recognized by chemical shifts and multiplicity of all ^1H and ^{13}C NMR signals. ^1H NMR spectra showed two broad singlet signals for two ethylenic protons (H-29, δ 4.70 and 4.58), and two singlet signal attributed to methyl groups (H-30, δ 1.69 for compound **1**, and δ 1.68 for compound **2**), compatible for lupeol units. ^{13}C NMR spectra displayed signals at δ 172.8 and 166.9, indicating the presence of an ester function for both compounds. Two signals for carbinolic protons (H-3) at δ 4.63 (dd, $J = 10.4$ and 5.2 Hz) and 4.48 (dd, $J = 10.4$ and 5.6 Hz) suggested that the site of esterification was at C-3 (A ring of the basic skeleton of lupeol). ^1H NMR spectra also indicated a cinnamyl group for compound **1** through the signals at δ 7.28-7.53 (m, H-2'/6', H-3'/5' and H-4'), 7.67 (d, $J = 16$ Hz, H-7') and 6.45 (d, $J = 16$ Hz, H-8'). A 7',8'-dihydrocinnamyl group corresponding to the major component **2** was indicated by the signals δ 2.96 (t, H-7') and 2.63 (t, H-8') [21]. The intensities (revealed integration) of the signals at δ 7.67 (H-7' for compound **1**) and 2.96 (H-7' for compound **2**) were used to estimate the relative proportions of them in the mixture: 25% of **1** (approximately 159 mg) and 75% of **2** (approximately 476 mg).

^1H NMR spectra for compound **3** displayed two singlets at δ 7.62 (H-1) and 6.59 (H-4), attributed to two protons *para*-related, and another singlet signal at 6.29 (H-11), attributed to a vinyl hydrogen. ^{13}C NMR spectra showed a signal at δ 201.5, attributed to a ketone group, suggesting the presence of an acetophenone unit. ^1H NMR spectra also revealed signals for two methyl groups attached to a quaternary sp^3 carbon [δ 0.75 (s, H-16) and 1.12 (s, H-17)], and one methyl group attached to a sp^2 carbon [δ 2.18 (s, H-18)]. The identification of a methoxy group [δ 3.87 (s, H-19)] led to the molecular formula $\text{C}_{18}\text{H}_{21}\text{O}$ for the basic skeleton, compatible for a bis-*nor*-diterpene. Homonuclear and heteronuclear shift correlations (^1H - ^1H COSY and ^{13}C - ^1H HMBC, respectively)

confirmed the substitution pattern of the aromatic ring and all ^1H and ^{13}C assignments. These data allowed the unambiguous structural characterization of compound **3** as the bis-*nor*-diterpene phyllacanthone [21]. All the spectra data obtained for each compound are shown in the supplementary material.

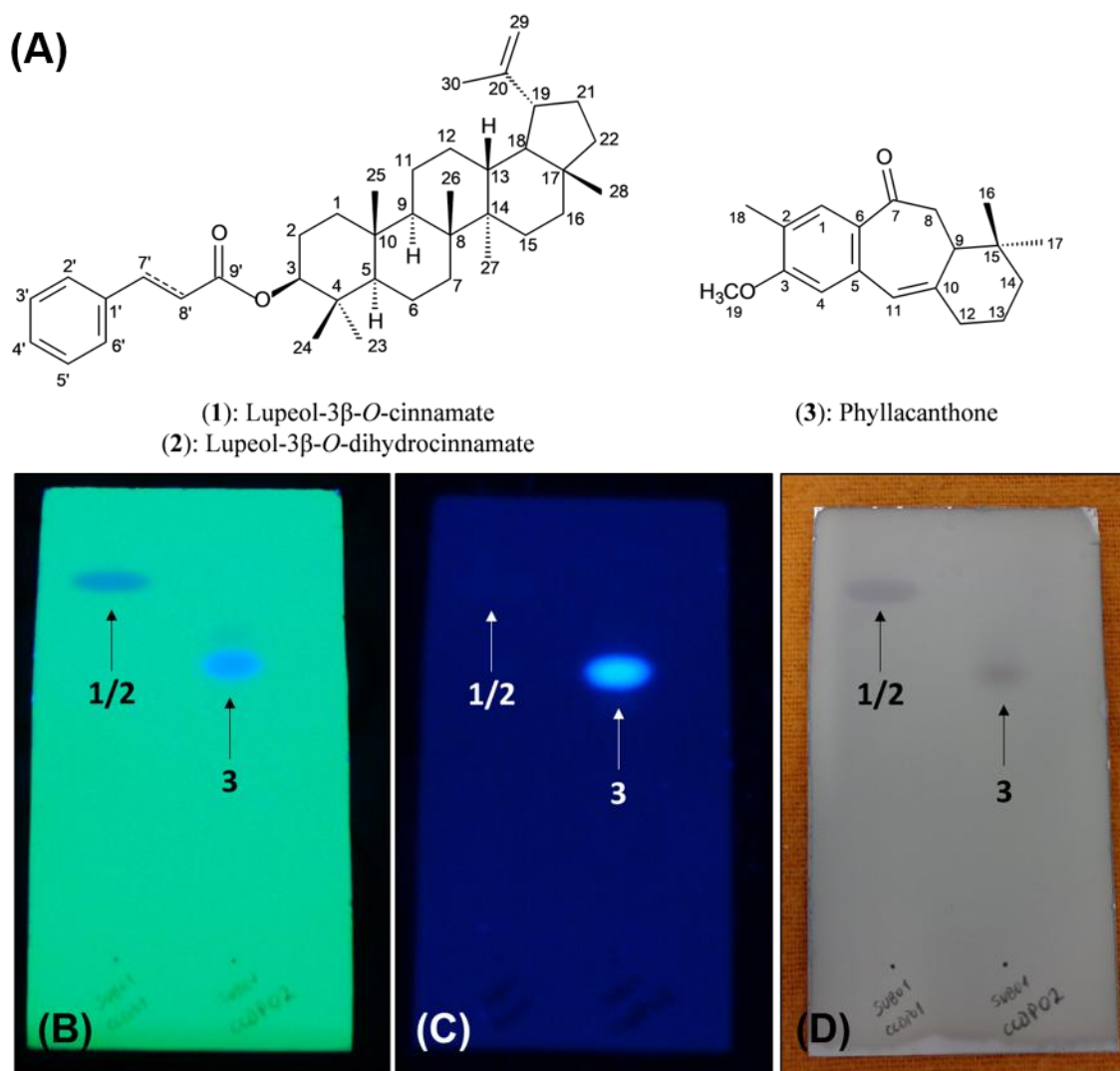


Fig. 1. Chemical structures (A) and analytical TLC plates (eluent: hexane-ethyl acetate, 95:5) of compounds **1/2** and **3**, visualized in an UV radiation chamber at 254 (B) and 365 (C) nm, and after staining with 1% sulfuric vanillin (D).

3.2. Antibacterial activity

The MIC values determined for the samples are presented in Table 1. Cq-Hex and Cq-MeOH displayed weak antibacterial activity. However, compounds **1/2** and **3** showed MIC values ten and twenty times lower (0.5 and 0.25 mg.ml⁻¹, respectively) than those verified for both extracts (5.0 mg.ml⁻¹) against all bacteria strains. Furthermore, the isolated compounds demonstrated MBC values between 0.25 and 0.5 mg.ml⁻¹, suggesting that these molecules also exert a bactericidal effect (Table 2). In this case, compound **3** was more effective than gentamicin against *E. faecalis* and *E. coli*.

Table 1. Minimum inhibitory concentration (MIC, mg.ml⁻¹) of extracts (Hex-Cq and MeOH-Cq) and isolated compounds (**1/2** and **3**) from *C. quercifolius*.

Microorganism	MIC (mg.ml ⁻¹)				
	Cq-Hex	Cq-MeOH	1/2	3	Gentamicin
<i>E. faecalis</i>	5.0	5.0	0.5	0.25	0.40
<i>E. coli</i>	5.0	5.0	0.5	0.25	*
<i>K. pneumoniae</i>	5.0	5.0	0.5	0.25	0.05
<i>S. marcescens</i>	5.0	5.0	0.5	0.25	*
<i>S. aureus</i>	5.0	5.0	0.5	0.25	0.025

* Absence of bacterial growth in all tested concentrations (n=3).

Table 2. Minimum bactericidal concentration (MBC, mg.ml⁻¹) of extracts (Hex-Cq and MeOH-Cq) and isolated compounds (**1/2** and **3**) from *C. quercifolius*.

Microorganism	MBC (mg.ml ⁻¹)				
	Cq-Hex	Cq-MeOH	1/2	3	Gentamicin
<i>E. faecalis</i>	> 10	> 10	0.5	0.25	0.40
<i>E. coli</i>	> 10	> 10	0.5	0.25	0.40
<i>K. pneumoniae</i>	> 10	> 10	0.5	0.25	0.05
<i>S. marcescens</i>	> 10	> 10	0.5	0.25	0.025
<i>S. aureus</i>	> 10	> 10	0.5	0.25	0.025

> 10 indicates bacterial growth in all tested concentrations (n=3).

4. Discussion

Compounds **1** and **2** have previously been described in *C. quercifolius* [18,21]. In fact, these compounds are frequently obtained as a mixture, due to its structural similarity. This mixture has also been reported in *C. vitifolius* [26]. Other lupane-type triterpenes were reported in *C. quercifolius*, such as lupeol [21] and lupeol-3 β -*O*-nanoate [18]. Compound **3** is a bis-nor-diterpene commonly found in *C. quercifolius* [18,21,27]. Also known as faveline-methyl-ether, phyllacanthone (**3**) was one of the first isolated substances from this plant. To date, there are no reports of other species that present this chemical constituent in its composition, being considered of exclusive occurrence in *C. quercifolius*. A recent study demonstrated the cytotoxic potential of phyllacanthone (**3**) against human pro-myelocytic leukemia (HL-60), human lung carcinoma (NCI-H292) and human breast adenocarcinoma (MCF-7) cells [18]. However, these compounds have not been sufficiently investigated for their biological activities.

In this paper, we evaluated the antibacterial activity of extracts (Hex-Cq and MeOH-Cq) and isolated compounds (**1/2** and **3**) against pathogenic bacteria strains. In general, compounds **1/2** and **3** were effective against all the microorganisms used. Interestingly, compound **3** was more effective

than the standard drug (gentamicin) against *E. faecalis* and *E. coli* strains. Although it is present in the human gastrointestinal microbiota, *E. faecalis* is considered an opportunistic pathogen associated with hospital-acquired wound, bloodstream, and urinary tract infections [28]. Similarly, *E. coli* is also among the most frequent pathogens found in urinary tract infections. These microorganisms are able to persist in the host for long periods through formation of biofilm that prevents phagocytosis by immune system cells [29]. In addition, both microorganisms have developed multidrug resistance mechanisms in recent years, making it difficult to control associated infections [30,31]. In this context, the search for bioactive molecules that can treat *E. faecalis* and *E. coli* infections remains a current challenge.

Terpenoids are admittedly used in the treatment of bacterial infections. Due to their lipophilic character, terpenoids can easily interact with the bacterial wall, interfering with the biosynthesis of its components. Furthermore, these compounds penetrate the bacterial cell and may also interfere with protein synthesis and DNA replication and repair mechanisms. In recent years, terpenoids have shown ability to enhance the effect of conventional antibacterial drugs. This has been an extremely useful strategy in controlling antibiotic resistance. These molecules have modulated the effect of antibacterial agents either by promoting direct cytotoxicity or by increasing their residence time in the cell, ensuring greater pharmacological activity at lower concentrations [32]. In this sense, compounds isolated from *C. quercifolius* may play an important role not only in the control of bacterial infections, especially those caused by *E. faecalis* and *E. coli*, but also in the control of multidrug resistance mechanisms. *In vivo* studies should be performed to determine the toxicity of these molecules, as well as their pharmacokinetic properties, associated or not with other antibacterial agents.

Peixoto-Sobrinho et al. [20] have reported the antibacterial activity of four *Cnidioscolus* species (*C. infestus* Pax and K. Hoffman, *C. pubescens* Pohl, *C. urens* (L.) Arthur and *C. quercifolius*) using the agar diffusion method. In this study, *C. quercifolius* extracts were the most bioactive, showing a relevant bacteriostatic effect against *S. aureus*. However, this was a

preliminary study conducted with crude extracts. In the present paper, we report the isolation of antibacterial compounds from *C. quercifolius* extracts for the first time. We believe that these compounds are responsible, at least in part, for the antibacterial potential observed for the extracts. These findings suggest that this plant may be considered a promising source of new antibacterial agents and further studies should be performed in order to characterize their mode of action.

5. Conclusions

In this phytochemical investigation, we report the isolation and characterization of three terpenoids from the stem barks of *C. quercifolius*: lupeol-3 β -*O*-cinnamate (**1**), lupeol-3 β -*O*-dihydrocinnamate and phyllacanthone (**3**). The isolated substances showed significant antibacterial activity, mainly compound **3**. These results indicate that *C. quercifolius* has antibacterial potential, supporting its use in folk medicine in the treatment of urinary tract infections.

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Conflict of interests

All authors declare that there is no conflict of interest for the publication of this manuscript.

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