

## Antimicrobial Activity of 3,4-*seco*-Diterpenes Isolated from *Croton blanchetianus* against *Streptococcus mutans* and *Streptococcus parasanguinis*

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In this study, acid and neutral fractions and three diterpenes were isolated from the hexane extracts from roots of three individual specimens of *Croton blanchetianus* Baill., collected from the same geographical site. Moreover, the antimicrobial activity of the extracts, fractions and diterpenes isolated was evaluated against *Streptococcus mutans* and *Streptococcus parasanguinis*, bacteria related with dental caries. The structures of the diterpenes were elucidated mainly by 1D and 2D nuclear magnetic resonance (NMR) spectroscopy, and high-resolution mass spectrometry, such as methyl 12-hydroxy-3,4-*seco*-cleistanta-8,11,13,15,4(18)-pentaene-3-oate; *ent*-3,4-*seco*atisa-4(18),16-dien-3-oic acid and a novel diterpene 3,4-*seco*-beieren-3-oic acid. Regarding to the antimicrobial activity, in general, all extracts, the neutral and acid fractions of the hexane extracts roots of all three specimens, showed antimicrobial activity in concentrations ranging from 500 to 7.8  $\mu\text{g mL}^{-1}$ . In addition, the three diterpenes isolated showed bactericidal and bacteriostatic activity against *S. mutans* and *S. parasanguinis*, suggesting them as alternative agents with the potential to prevent oral diseases.

**Keywords:** *seco*-diterpenes, Euphorbiaceae, *Croton blanchetianus*, oral biofilm

### Introduction

The genus *Croton* (Euphorbiaceae) comprises about 1300 species, which inhabit tropical and subtropical regions around the world. In Brazil, plants belonging to the *Croton* genus are found abundantly in the northeast area. The use of plants belonging to this genus, in folk medicine, includes treatments for cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers and weight loss.<sup>1</sup>

Many species of *Croton* have strong economic potential, especially for the pharmaceutical industry, due to the various secondary metabolites that, according to many studies, are responsible for the therapeutic properties of them.<sup>2</sup> Pirani, a Brazilian botanist, has defined *Croton* as a “problem genus”, mainly because of its large size and taxonomic complexity.<sup>3</sup> According to

The Plant List, *C. micranthus* Müell. Arg.,<sup>4</sup> *C. persicaria* Baill.,<sup>5</sup> and *Oxydectes blanchetiana* (Baill.) Kunté,<sup>6</sup> are synonyms for *C. blanchetianus*, but other Brazilian botanists have reported *C. alagoensis* Müell. Arg. and *C. floribundus* var. *piauhyensis* Rizzini as other synonyms as well.<sup>7-9</sup> In the past, our research group has devoted a large effort on phytochemical studies of the same plant species mistakenly identified, at that time, as *C. sonderianus* Müell. Arg.<sup>7-9</sup> Nowadays, that plant species has been correctly identified as *C. blanchetianus* Baill., and a few reports mention that *C. sonderianus* and *C. blanchetianus* are indeed synonyms.<sup>10,11</sup>

Several biological activities of *Croton* species have been reported, among them: the antimalarial action and low acute and subacute toxicity of the leaf extract of *Croton macrostachyus*,<sup>1</sup> the inhibition of chikungunya virus replication by *Croton mauritianus* leaf extract,<sup>12</sup> the antiulcerogenic action and the low acute and subacute toxicity of *Croton urucurana* extract.<sup>13</sup> Ethanol extracts of the trunks, leaves, and seeds of *Croton tiglium* showed

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some antifungal activities against three dermatophytes. The ethanol extracts of trunk or seed of *C. tiglium* exhibit strong antidermatophytic activities and, thus, could be considered for application on treating skin fungal infections after appropriate processing.<sup>14</sup>

The hydroalcoholic extract from leaves of *C. macrostachyus* demonstrated antimicrobial action against isolated clinical bacteria of interest to the veterinarian.<sup>15</sup> The hydroalcohol, hexane and ethanol extracts from leaves of *Croton doctoris* showed relevant activity against caries-related bacteria: *Actinomyces naeslundii*, *Lactobacillus acidophilus*, *Streptococcus gordonii*, *S. mutans*, *Streptococcus sanguinis*, *Streptococcus sobrinus* and *Streptococcus mitis*. Analysis of the composition of the extract showed that there are large amounts of phenol compounds and diterpenes.<sup>16</sup> *Croton roxburghii* extract demonstrated significant antimicrobial activity against clinical isolates of *Bacillus cereus* and *Shigella flexneri*, strains of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Shigella dysenteriae*, and *Vibrio cholerae*.<sup>17</sup>

The crude polysaccharide fraction, obtained from the hot aqueous extract of *Croton cajucara* leaves, was able to promote gastroprotection. It was able to reduce ethanol-induced gastric ulcers in rats,<sup>18</sup> the antinociceptive and pro-healing activity of *Croton adamantinus* essential oil was seen in rats,<sup>19</sup> the nanoparticles synthesized from the leaf extract of *Croton sparsiflorus* showed significant antimicrobial activity against *S. aureus*, *E. coli* and *Bacillus subtilis*.<sup>20</sup> Diterpenoids from *C. tonkinensis* showed a high antituberculosis activity against *Mycobacterium tuberculosis*,<sup>21</sup> the *ent*-kaurane diterpene of *Croton tonkinensis* showed the induction of apoptosis in colorectal cancer cells,<sup>22</sup> and the diterpenes isolated from *C. cajucara* exhibited an antileishmanial action.<sup>23</sup>

*C. blanchetianus* Baill., popularly known as “marmeleiro preto”, is found in the Brazilian semi-arid region. There are botanical synonyms like *Croton sonderianus* Müll. Arg. and *Croton alagoensis* Müll. Arg.<sup>3,11,24</sup> Distributed at the Caatinga biome, it can be found in several places, with relatively low height and diameters and a significant number of representatives. It is widely used by rural populations, mainly for the production of firewood and the construction of small corral to house animals.<sup>25</sup>

Studies have shown that isolated compounds and the essential oil of *C. blanchetianus* present antinociceptive activity<sup>26</sup> and insecticidal activity against *Aedes aegypti*.<sup>27</sup> The hydroalcoholic extract of *C. blanchetianus* bark showed acaricidal activity,<sup>28</sup> antibacterial and antifungal properties.<sup>7-9</sup> In addition, the essential oil has been found useful to inhibit the growth of pathogenic microorganisms in foods.<sup>29</sup> Considering that extracts from *C. blanchetianus*

were able to inhibit the growth of microorganisms, the objective of this study was to isolate and characterize the compounds responsible for the antimicrobial action on bacteria related to the development of dental caries.

## Experimental

Infrared (IR) spectra were recorded on a PerkinElmer spectrometer, Spectrum 100 FTIR, equipped with universal attenuated total reflectance accessory (UATR). <sup>1</sup>H and <sup>13</sup>C, 1D and 2D nuclear magnetic resonance (NMR) spectra were obtained either on a Bruker Avance DRX-500 (500 MHz to <sup>1</sup>H and 125 MHz to <sup>13</sup>C) or Avance DPX-300 spectrometers (300 MHz to <sup>1</sup>H and 75 MHz to <sup>13</sup>C). High resolution mass spectra (HRMS) were performed on an LCMS-IT-TOF (225-07100-34, Shimadzu), equipped with electrospray ionization (ESI) source either on positive or negative mode.

The separation, and compounds purification was carried out on a Waters HPLC, consisting of a binary pump (Waters-1525) and a PDA UV detector (Waters-2996), using flow rates of 1 mL min<sup>-1</sup> for analytical column (4.6 × 250 mm, 5 μm) and 4.72 mL min<sup>-1</sup> for semi-preparative column (10 × 250 mm, 5 μm). In the gravitational adsorption chromatography, silica gel 60 (70-230 mesh, Vetec) was used, while Sephadex LH-20 (Pharmacia) was used in the chromatographic fractionation by molecular exclusion. Thin layer chromatography (TLC) was performed on precoated silica gel aluminum sheets (5-40 mesh, Merck) with fluorescence indicator in the range of 254 nm (F254). The substances were revealed by spraying with a vanillin-perchloric acid/EtOH solution, followed by heating in an oven (ca. 100 °C) for 5 min.

### Sample preparation, strains and culture conditions

For the antimicrobial assays, solutions containing extracts, fractions and diterpenes isolated from *C. blanchetianus*, were prepared in culture medium BHI (brain heart infusion) broth with 8% dimethyl sulfoxide (DMSO). The microorganisms used in this study were *S. mutans* (UA159) and *S. parasanguinis* (ATCC903). Bacteria were cultured from a stock culture in BHI broth containing 20% glycerol. An aliquot of 20 μL of each of the bacterium was inoculated into Petri dishes containing BHI agar medium and incubated at 37 °C for 24 h with 5% CO<sub>2</sub>. Next, isolated colonies were inoculated into 10 mL of sterile BHI broth and incubated under the same conditions previously mentioned. After antimicrobial assays, the bacterial culture was adjusted to a concentration of 2 × 10<sup>6</sup> colony-forming unit (CFU) mL<sup>-1</sup>.

### Minimum inhibitory concentration and minimum bactericidal concentration determination

The minimum inhibitory concentration (MIC) was determined by microdilution tests on polystyrene plates, standardized according to the NCCLS (present CLSI), approved standard M7-A6,<sup>30</sup> with modifications. In the 96-well polystyrene plates, the substance was serially diluted until the obtained concentrations were ranging from 125 to 1.8  $\mu\text{g mL}^{-1}$ . One hundred microliters of each bacterial suspension was added to the plate containing the substances, resulting in a final volume of 200  $\mu\text{L}$  per well. The plates were then incubated for 24 h at 37 °C in an atmosphere containing 5%  $\text{CO}_2$ . After 24 h, the evaluation of bacterial growth was determined by visualizing the turbidity of the culture medium. The presence of turbidity meant that the substance did not inhibit microbial growth. In addition, the bacterial growth was measured by determining the optical density at 620 nm by using a microplate reader (SpectraMax i3 Multi-Mode microplate reader). In this way, the MIC was considered as the lowest concentration showing complete inhibition of visible bacterial growth. The negative control consisted of 8% DMSO in BHI culture medium and chlorhexidine gluconate was used as positive control.

Regarding minimum bactericidal concentration (MBC) determination, 10  $\mu\text{L}$  of the bacterial suspension in the MIC were incubated in Petri dishes containing BHI agar. The plates were maintained at 37 °C and 5%  $\text{CO}_2$  for 24 h and, after this time, bacterial growth was evaluated. MBC was considered the lowest concentration that no colony growth was observed.

### Plant material

On February 2012, in the locality of Garapa, located in the municipality of Acarape, CE, Brazil, the leaves and inflorescences of eight individual specimens of *C. blanchetianus* were collected. After, it was analyzed the <sup>1</sup>H NMR spectra of leaf and inflorescences essential oils of these eight specimens of *C. blanchetianus*, showing different chemical profiles. These differences were used as criteria for the selection of three specimens, designated CB1, CB4 and CB8, for the study. On April of the same year, roots, heartwood and trunk bark of the three specimens of *C. blanchetianus* were harvested.

The authentication of the plant material was carried out by MSc Maria Lenise Guedes of the Instituto de Biologia (Universidade Federal da Bahia). The voucher specimens (No. 53043, 53045 and 53047) are deposited in the Herbarium Prisco Bezerra (EAC) of the Universidade Federal do Ceará.

### Extraction and isolation

The extracts of hexane from roots (RCB), heartwood (WCB) and trunk bark (BCB) from three specimens of *C. blanchetianus* were produced. 100.0 g of the entire roots, heartwood or trunk bark of CB1, CB4 and CB8, after drying and crushing, were individually subjected to maceration in hexane for 24 h ( $3 \times 0.5$  L). The hexane solutions were pooled, and then evaporated under reduced pressure to obtain the hexane extracts from roots (RCB1 = 4.44 g, RCB4 = 7.09 g and RCB8 = 5.36 g), heartwood (WCB1 = 2.36 g, WCB4 = 3.80 g and WCB8 = 2.94 g), and trunk bark (BCB1 = 8.42 g, BCB4 = 8.91 g and BCB8 = 6.82 g). From the initial results of the microbiological tests performed with these extracts (Table 1), it was decided to perform the phytochemical analysis of the root extracts.

The procedure was then repeated with 2.5 kg of roots by maceration in hexane for 72 h ( $3 \times 7.0$  L). The hexane solutions were evaporated under reduced pressure to yield the respective extracts RCB1 (73.13 g, 2.93%), RCB4 (116.08 g, 4.64%), and RCB8 (75.13 g, 3.00%).

10.0 g of RCB1 were solubilized in  $\text{CH}_2\text{Cl}_2$ , adsorbed onto 100.0 g of silica gel, added to a column ( $8.0 \times 50.0$  cm) of 40.0 g of silica gel. 18 fractions (A1-A18) of 225 mL were collected by elution with: hexane (A1-A3), hexane/ $\text{CH}_2\text{Cl}_2$  1:1 (A4-A8),  $\text{CH}_2\text{Cl}_2$  (A9-A13), EtOAc (A14-A15), and finally MeOH (A16-A18). After concentration and TLC analysis, similar fractions were pooled together. 680.0 mg of fraction A9 were solubilized in  $\text{CH}_2\text{Cl}_2$ , adsorbed on 1.0 g silica gel and chromatographed on a column ( $3.0 \times 50.0$  cm) of 10.0 g silica gel. Fractions were collected with the following volumes: B1-B53, 10 mL; and B54-B61, 50 mL. The eluents used were: hexane (B1-B8), hexane/ $\text{CH}_2\text{Cl}_2$  25% (B9-B27), hexane/ $\text{CH}_2\text{Cl}_2$  1:1 (B28-B56),  $\text{CH}_2\text{Cl}_2$  (B57), EtOAc (B58) and MeOH (B59-B61). After rotaevaporation under reduced pressure and TLC analysis, similar fractions B34-B37 (104.2 mg) were pooled, resulting in a colorless resin named ICB1.

<sup>1</sup>H NMR analysis of ICB1 revealed its carboxylic acid character, then another approach was designed. 10.0 g of RCB1 were dissolved in 100 mL of 2% KOH/MeOH solution, stirred at room temperature (ca. 25 °C) for 4 h and then rotaevaporated under vacuum to half of its volume (50 mL). Moreover, 100 mL of distilled  $\text{H}_2\text{O}$  was added and then extracted with  $\text{CH}_2\text{Cl}_2$  (100, 50 and 50 mL). The final solution of all organic phases together was washed with distilled  $\text{H}_2\text{O}$  ( $3 \times 50$  mL), dried with anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and rotaevaporated under vacuum, providing a viscous liquid material called NFCB1 (2.64 g). The hydromethanol phase was acidified with aqueous HCl

**Table 1.** MIC and MBC values of the hexane extracts from roots, heartwood and trunk bark of CB1, CB4 and CB8 on *S. mutans* and *S. parasanguinis*

Hexane extract	<i>S. mutans</i>		<i>S. parasanguinis</i>	
	MIC / ( $\mu\text{g mL}^{-1}$ )	MBC / ( $\mu\text{g mL}^{-1}$ )	MIC / ( $\mu\text{g mL}^{-1}$ )	MBC / ( $\mu\text{g mL}^{-1}$ )
RCB1	15.6	250	31.2	62.5
WCB1	62.5	–	62.5	125
BCB1	31.2	–	62.5	125
RCB4	31.2	250	31.2	250
WCB4	250	–	62.5	250
BCB4	62.5	–	62.5	–
RCB8	31.2	–	31.2	–
WCB8	250	–	125	250
BCB8	31.2	62.5	62.5	250

RCBx: *C. blanchetianus* roots; WCBx: *C. blanchetianus* heartwood; BCBx: *C. blanchetianus* trunk bark; x = 1 for specimen 1, x = 4 for specimen 4, and x = 8 for specimen 8. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

(5%) to pH 1, and then extracted with  $\text{CH}_2\text{Cl}_2$  (100, 50 and 50 mL). After pooled, the organic phases gave a solution which was washed with distilled  $\text{H}_2\text{O}$  ( $3 \times 50$  mL) until pH 7, dried with anhydrous  $\text{Na}_2\text{SO}_4$  and rotaevaporated under vacuum to give the acidic fraction AFCB1 (6.52 g).

This procedure was repeated for RCB4, resulting in 2.77 g of NFCB4 and 6.85 g of AFCB4, as well as for RCB8, resulting in 5.22 g of NFCB8 and 3.64 g of AFCB8.

The non acidic fractions NFCB1, NFCB4 and NFCB8 were individually solubilized in  $\text{CH}_2\text{Cl}_2$  and pre-adsorbed on silica gel, corresponding to 10 times the mass of each fraction. Twelve fractions of 250 mL were collected for NFCB1 and NFCB4, and 18 fractions for NFCB8 (see Table S1, Supplementary Information (SI) section). This procedure was repeated for the AFCB1, AFCB4 and AFCB8 acid fractions. 11 fractions of 250 mL were collected for each column ( $8.0 \times 50.0$  cm) with 70.0 g of silica gel (Table S2, SI section). 200.0 mg of the NFCB4 fraction were subjected to HPLC (high performance liquid chromatography) on a semi-preparative silica gel column with hexane/EtOAc (30% v/v) as the isocratic mobile phase. 20.1 mg of a yellow resin designated ICB4 (retention time ( $t_R$ ) = 4.42 min) was obtained.

3.64 g of AFCB8 were adsorbed onto silica gel (37.0 g) packed over a column ( $5.0 \times 50.0$  cm) of 25.0 g silica gel. Eleven fractions (D1-D11) of 250 mL were collected by elution with: hexane (D1), hexane/ $\text{CH}_2\text{Cl}_2$  1:1 (D2-D3),  $\text{CH}_2\text{Cl}_2$  (D4-D6),  $\text{CH}_2\text{Cl}_2$ /EtOAc 25% (D7-D8), EtOAc (C9), EtOAc/MeOH 1:1 (D10) and MeOH (D11). 370 mg of D4 were solubilized in MeOH and chromatographed on a column ( $4.0 \times 50.0$  cm) of 27.0 cm of Sephadex LH-20 dextran gel. The first fraction (D1) was collected after elution of 100 mL of MeOH, using a mean flow of 12 drops  $\text{min}^{-1}$ , and then twenty fractions

(E1-E20) of 8.0 mL were collected. The E10 fraction after rotaevaporation and TLC analysis resulted in 113.0 mg of a colorless resin named ICB8.

## Results and Discussion

The compound ICB1, a colorless resin displayed an HRESIMS ion at  $m/z$  303.2321  $[\text{M} + \text{H}]^+$  (calcd. for  $\text{C}_{20}\text{H}_{31}\text{O}_2$ , 303.2324) or 301.2157  $[\text{M} - \text{H}]^-$  (calcd. for  $\text{C}_{20}\text{H}_{29}\text{O}_2$ , 301.2168) in its HRESIMS spectra,  $[\alpha]_D^{21} +18.2^\circ$  ( $c$  0.11,  $\text{CHCl}_3$ ). Its IR spectrum showed characteristic absorptions of O–H stretching of carboxylic acid ( $3400$  to  $2400$   $\text{cm}^{-1}$ ) and also C=O stretching at  $1705$   $\text{cm}^{-1}$ . It also showed absorptions at  $1635$   $\text{cm}^{-1}$  of C=C stretching and  $745$   $\text{cm}^{-1}$  of out-of-plane =C–H bending. The  $^1\text{H}$  NMR spectrum of compound ICB1 (Table 2) revealed the presence of protons of a *cis* and of a terminal carbon-carbon double bonds at  $\delta_H$  5.72 (d,  $J$  5.0 Hz, H-15), 5.49 (d,  $J$  5.0 Hz, H-16), 4.87 (s, H-18a) and 4.68 (s, H-18b). Three singlets related to methyl protons at  $\delta_H$  0.77 (H-20), 1.01 (H-17) and 1.75 (H-19) were also observed. The latter one is characteristic of an isopropenyl group, while the other two seem to be attached to quaternary carbons. The  $^{13}\text{C}$  NMR-BB (broad-band) spectrum of compound ICB1 (Table 2) presented 20 spectral lines with the signal at  $\delta_C$  180.8 (C-3) referring to an acid carboxyl, the signals at  $\delta_C$  147.7 (C-4), 113.8 (C-18) and 23.9 (C-19) corresponding to the isopropenyl group, as well as two other olefinic carbons at  $\delta_C$  134.8 (C-15) and 137.0 (C-16). Comparison of the BB and distortionless enhancement of polarization transfer (DEPT)  $135^\circ$   $^{13}\text{C}$  NMR spectra permitted to identify the hydrogenation pattern of each carbon of compound ICB1, confirming its molecular formula  $\text{C}_{20}\text{H}_{30}\text{O}_2$ , indicating 6 double bond equivalents (DBE's),

from which three were related to three rings, two carbon-carbon double bonds and a carboxyl. The  $^1\text{H}, ^1\text{H}$ -COSY (correlation spectroscopy) spectrum analysis evidenced the correlations for the olefinic protons at  $\delta_{\text{H}}$  5.72 (d,  $J$  5.0 Hz, H-15) and 5.49 (d,  $J$  5.0 Hz, H-16). The presence of the isopropenyl group was confirmed through the correlation of the protons of the methyl carbon at  $\delta_{\text{H}}$  1.75 (s, H-19) with the protons of the methyldiene carbon at  $\delta_{\text{H}}$  4.87 (s, H-18a) and  $\delta_{\text{H}}$  4.68 (s, H-18b). The two dimensional  $^1\text{H}, ^{13}\text{C}$ -HMBC (heteronuclear multiple bond correlation) spectrum revealed the presence of the isopropenyl group attached to carbon C-5, which was confirmed through the correlations of the terminal vinyl protons at  $\delta_{\text{H}}$  4.87 (s, H-18a) and 4.68 (s, H-18b) with the carbons at  $\delta_{\text{C}}$  147.7 (C-4), 51.3 (C-5) and 23.9 (C-19). The presence of the C-17 methyl linked to carbon C-13 was confirmed through the correlations observed for the protons of C-17 at  $\delta_{\text{H}}$  1.01 (s, 3H), with the carbons at  $\delta_{\text{C}}$  44.1 (C-13), 61.3 (C-14), 33.3 (C-12), and 137.0 (C-16). The *cis* olefinic system (C-15, C-16) was ratified through the HMBC correlations of the protons at  $\delta_{\text{H}}$  5.72 (d,  $J$  5.0 Hz, H-15) and 5.49 (d,  $J$  5.0 Hz, H-16) with the carbons at  $\delta_{\text{C}}$  44.1 (C-13), 61.3 (C-14) and 49.1 (C-8). The relative stereochemistry was defined based on the  $^1\text{H}, ^1\text{H}$ -NOESY (nuclear Overhauser effect spectroscopy) spectrum by the dipolar coupling correlation of the proton at  $\delta_{\text{H}}$  1.99 (H-5) with the proton at  $\delta_{\text{H}}$  1.14 (H-9). The correlations for the protons of methyl C-20 at  $\delta_{\text{H}}$  0.77 (s, 3H) with the protons at  $\delta_{\text{H}}$  5.72 (d,  $J$  5.0 Hz, H-15), 1.81 (d,  $J$  12.8 Hz, H-11a) and 4.68 (s, H-18b) allowed the determination of the relative configurations at the stereogenic centers C-8, C-10 and C-13. It is possible to conclude that the compound ICB1 is the unknown diterpene 3,4-*seco*-beieren-3-oic acid (Figure 1). Compound ICB1 is the “3,4-*seco*” counterpart of the *ent*-Beyer-15-en-18-oic acid, previously isolated from *C. sonderianus*.<sup>31</sup>

The  $^1\text{H}$  NMR spectrum of compound ICB4 (Table 2) revealed the presence of olefinic protons of a vinyl moiety at  $\delta_{\text{H}}$  6.73 (dd,  $J$  11.4, 18.0 Hz, H-15), 5.57 (dd,  $J$  2.0, 11.4 Hz, H-16a), and 5.26 (dd,  $J$  2.0, 18.0 Hz, H-16b), in addition to signals at 4.99 (s, H-18a) and 4.84 (s, H-18b), characteristic of the methyldiene protons of a terminal carbon-carbon double bond, as observed for ICB4. There were also three singlets related to methyl protons at  $\delta_{\text{H}}$  1.24 (s, H-20), 1.77 (s, H-19) and 2.52 (s, H-17), and another singlet related to a methoxyl at  $\delta_{\text{H}}$  3.56. The  $^{13}\text{C}$  NMR-BB spectrum of ICB4 (Table 2) presented 21 spectral lines, from which the signal at  $\delta_{\text{C}}$  174.8 (C-3) was assigned to an ester carbonyl and the signals at  $\delta_{\text{C}}$  155.8 (C-12), 139.7 (C-14), 142.0 (C-9), 126.3 (C-8), 121.6 (C-13), and 112.5 (C-11), to the carbons of a penta-substituted benzene ring. In addition, four olefinic carbons at  $\delta_{\text{C}}$  147.9 (C-4) and 114.9 (C-18) of the terminal

double bond, 137.0 (C-15) and 119.9 (C-16), characteristic of the vinyl moiety, were also characterized. Comparison of the BB and the DEPT  $135^\circ$   $^{13}\text{C}$  NMR spectra permitted to identify the hydrogenation pattern of ICB4, confirming the molecular formula  $\text{C}_{21}\text{H}_{28}\text{O}_3$ , presenting 6 DBE's. Its IR spectrum showed an intense absorption characteristic of C=O at  $1730\text{ cm}^{-1}$ , compatible with an ester carbonyl. A broad band centered at  $3420\text{ cm}^{-1}$ , characteristic of O-H; an absorption at  $3050\text{ cm}^{-1}$ , characteristic of benzene C-H stretching; besides absorptions at 1600 and  $1480\text{ cm}^{-1}$  for the skeletal bands; at  $1650\text{ cm}^{-1}$ , with a shoulder, of the C=C stretching of alkenes, were also observed. Comparison of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR-BB spectra with data from the literature,<sup>4</sup> allowed to identify compound ICB4 as the methyl 12-hydroxy-3,4-*seco*-cleistanta-8,11,13,15,4(18)-pentaene-3-oate, a diterpene, previously isolated from *C. sonderianus* (Figure 1).<sup>4</sup>

Compound ICB8 showed a  $^{13}\text{C}$  NMR-BB spectrum with 20 spectral lines (Table 2). Comparison of the BB and DEPT  $135^\circ$   $^{13}\text{C}$  NMR spectra permitted to establish the hydrogenation pattern of each carbon atom, as 2 methyls, 8 methylenes and 2 methyldienes, and 5 non-hydrogenated carbons, with the molecular formula  $\text{C}_{20}\text{H}_{30}\text{O}_2$ , indicating 6 DBE's. The  $^1\text{H}$  NMR spectrum of ICB8 (Table 2) showed signals at  $\delta_{\text{H}}$  4.96 (s, H-18a), 4.89 (s, H-17a), 4.87 (s, H-18b) and 4.72 (s, H-17b) corresponding to terminal double bond protons, and 2 singlets for two methyl at  $\delta_{\text{H}}$  1.81 (s, H-19) and 0.95 (s, H-20). Its IR spectrum showed characteristic absorptions for O-H stretching of carboxylic acid ( $3400$  to  $2400\text{ cm}^{-1}$ ), and an intense absorption characteristic of C=O carboxyl stretching at  $1705\text{ cm}^{-1}$ . In addition, it showed absorptions at  $1639\text{ cm}^{-1}$  of the C=C stretching of alkenes and the correspondent out-of-plane bending at  $755\text{ cm}^{-1}$ . The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data (Table 2), suggested the structure of a tetracyclic diterpene with an *ent*-atisane backbone. According to the analysis of the spectral data obtained, and comparison with the literature data, it was possible to suggest that compound ICB8 was indeed the *ent*-3,4-*seco*atisa-4(18),16-dien-3-oic acid, previously isolated from *Excoecaria agallocha* L.<sup>32</sup> (Figure 1), isolated for the first time from *C. blanchetianus*.

#### Antimicrobial activity of extracts, fractions and diterpenes

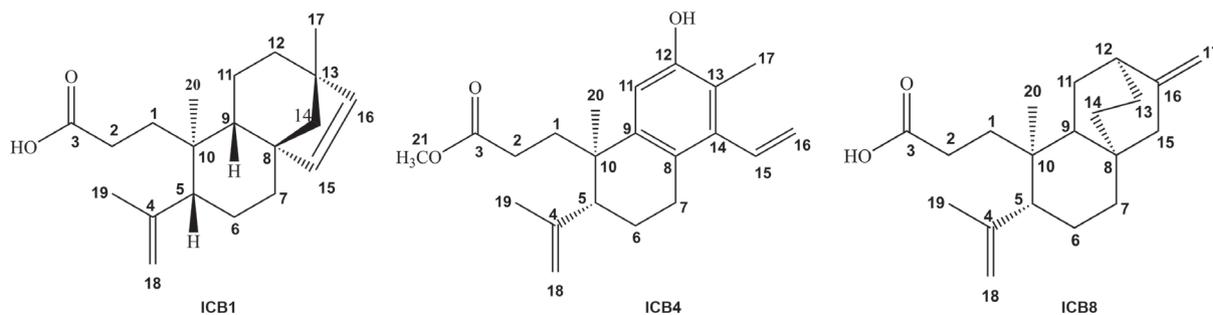
The results of the antimicrobial assays performed with the hexane extracts from trunk bark, heartwood and roots of the three specimens of *C. blanchetianus* (CB1, CB4 and CB8) are shown in Table 1.

According to the results obtained, all extracts tested inhibited the bacterial growth. The RCB1, RCB4 and BCB8

**Table 2.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for compounds ICB1, ICB4 and ICB8 isolated from the hexane extract from roots of the three individual specimens of *Croton blanchetianus*

C	ICB1 <sup>a</sup>		ICB4 <sup>b</sup>		ICB8 <sup>b</sup>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult., <i>J</i> in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult., <i>J</i> in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult., <i>J</i> in Hz)
1	36.4	1.57 (m, Ha) 1.36 (m, Hb)	36.1	2.22 (m, 2H)	34.9	1.95 (c, Ha) 1.79 (c, Hb)
2	28.6	2.39 (m, Ha) 2.30 (m, Hb)	30.3	2.45 (m, Ha) 2.16 (m, Hb)	29.9	2.57 (m, Ha) 2.44 (m, Hb)
3	180.8	–	174.8	–	176.9	–
4	147.7	–	147.8	–	148.3	–
5	51.3	1.99 (brd, 12.0, H)	47.3	2.49 (m, H)	51.2	2.07 (brd, 11.5, H)
6	20.8	1.43 (m, Ha) 1.33 (m, Hb)	25.8	1.83 (td, 3.9, 11.5, Ha) 1.77 (c, Hb)	25.4	1.72 (c, Ha) 1.31 (c, Hb)
7	32.6	1.55 (m, 2H)	29.2	2.81 (dt, 3.9, 15.0, Ha) 2.56 (c, Hb)	38.6	1.29 (brd, 11.0, Ha) 1.11 (brt, 11.0, Hb)
8	49.1	–	126.3	–	33.9	–
9	44.1	1.14 (d, 11.8, H)	142.0	–	44.4	1.39 (c, H)
10	39.3	–	41.7	–	40.3	–
11	26.2	1.81 (d, 11.8, Ha) 1.40 (m, Hb)	112.5	7.20 (s, H)	28.8	1.89 (m, Ha) 1.42 (m, Hb)
12	33.3	1.31 (m, Ha) 1.26 (m, Hb)	155.8	–	37.1	2.23 (brs, H)
13	43.8	–	121.6	–	27.7	1.60 (m, Ha) 1.51 (m, Hb)
14	61.3	1.49 (d, 9.2, Ha) 1.04 (d, 9.2, Hb)	139.7	–	28.9	1.62 (m, Ha) 0.97 (c, Hb)
15	134.8	5.72 (d, 5.0, H)	137.0	6.73 (dd, 11.4, 18.0, H)	48.7	2.03 (brd, 13.8, Ha) 1.92 (brd, 13.8, Hb)
16	137.0	5.49 (d, 5.0, H)	119.9	5.57 (dd, 2.0, 11.4, Ha) 5.26 (dd, 2.0, 18.0, Hb)	152.7	–
17	25.0	1.01 (s, 3H)	14.3	2.52 (s, 3H)	105.7	4.89 (s, Ha) 4.72 (s, Hb)
18	113.8	4.87 (s, Ha) 4.68 (s, Hb)	114.9	4.99 (s, Ha) 4.84 (s, Hb)	114.0	4.96 (s, Ha) 4.87 (s, Hb)
19	23.9	1.75 (s, 3H)	23.5	1.77 (s, 3H)	24.3	1.81 (s, 3H)
20	18.7	0.77 (s, 3H)	28.6	1.24 (s, 3H)	18.3	0.95 (s, 3H)
O-CH <sub>3</sub>	–	–	51.8	3.56 (s, 3H)	–	–

<sup>a</sup>125 MHz, CDCl<sub>3</sub>; <sup>b</sup>125 MHz, C<sub>5</sub>D<sub>5</sub>N; C: number of carbons in the compounds. ICBx: isolated from *C. blanchetianus*, x = 1 for specimen 1, x = 4 for specimen 4, x = 8 for specimen 8.

**Figure 1.** Structures of the *seco*-diterpenes isolated from the hexane extract from roots of the three individual specimens of *C. blanchetianus*.

**Table 3.** MIC and MBC values of the acid fractions of CB1, CB4 and CB8 against *S. mutans* and *S. parasanguinis*

Acid fraction	<i>S. mutans</i>		<i>S. parasanguinis</i>	
	MIC / ( $\mu\text{g mL}^{-1}$ )	MBC / ( $\mu\text{g mL}^{-1}$ )	MIC / ( $\mu\text{g mL}^{-1}$ )	MBC / ( $\mu\text{g mL}^{-1}$ )
AFCB1-HD2	31.2	62.5	31.2	125
AFCB1-D1	31.2	125	31.2	125
AFCB1-D3	250	–	250	–
AFCB1-DA1	–	–	–	–
AFCB1-AM	125	–	125	–
AFCB4-HD2	62.5	250	62.5	125
AFCB4-D1	62.5	125	62.5	125
AFCB4-D3	–	–	500	–
AFCB4-DA1	–	–	–	–
AFCB4-AM	500	–	500	–
AFCB8-HD1	7.8	7.8	15.6	15.6
AFCB8-D1	15.6	15.6	31.2	31.2
AFCB8-DA1	250	–	–	–
AFCB8-A	250	–	500	–
AFCB8-AM	500	–	250	500

AFCBx: acid fractions of *C. blanchetianus*, x = 1 for specimen 1, x = 4 for specimen 4, and x = 8 for specimen 8; HD: hexane/ $\text{CH}_2\text{Cl}_2$  50%; D:  $\text{CH}_2\text{Cl}_2$ ; DA:  $\text{CH}_2\text{Cl}_2/\text{EtOAc}$  25%; AM:  $\text{EtOAc}/\text{MeOH}$ ; HD1: first fraction collected using hexane/ $\text{CH}_2\text{Cl}_2$  50%; HD2: second fraction collected using hexane/ $\text{CH}_2\text{Cl}_2$  50%; D1: first fraction collected using  $\text{CH}_2\text{Cl}_2$ ; D3: third fraction collected using  $\text{CH}_2\text{Cl}_2$ ; DA1: first fraction collected using  $\text{CH}_2\text{Cl}_2/\text{EtOAc}$  25%. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

extracts presented a bactericidal and bacteriostatic effect for both bacteria.

Considering the lowest inhibitory concentrations of the microbial growth, the hexane extracts from the roots were selected to perform the fractionation. In order to identify the compounds responsible for the antimicrobial action, the hexane extracts from roots were separated in acid and neutral fractions and then submitted to antibacterial assay.

Acid and neutral fractions showed antimicrobial activity, however, the acid fractions presented the lowest values of concentration with effective action. The antibacterial results for all acid fractions are presented in Table 3.

Fraction AFCB8 showed a more effective bactericidal and bacteriostatic effect, with the lowest concentrations, 7.8 and 15.6  $\mu\text{g mL}^{-1}$ , against both bacteria. The antibacterial results for the neutral fractions are presented in Table S3 (SI section).

After several fractionation steps, three diterpenes (ICB1, ICB4 and ICB8) were isolated and their antibacterial activity was determined (Table 4).

ICB1 presented the same MIC and MBC values against *S. mutans* (410  $\mu\text{M}$ ), while against *S. parasanguinis* showed values of 206 and 410  $\mu\text{M}$ , respectively. The AFCB1 fraction, from which ICB1 was obtained, presented a four-fold inhibitory effect of ICB1 against *S. mutans* and

**Table 4.** MIC and MBC values of ICB1, ICB4, ICB8 diterpenes and chlorhexidine gluconate (positive control) against *S. mutans* and *S. parasanguinis*

Compound	<i>S. mutans</i>		<i>S. parasanguinis</i>	
	MIC / $\mu\text{M}$	MBC / $\mu\text{M}$	MIC / $\mu\text{M}$	MBC / $\mu\text{M}$
ICB1	410	410	206	410
ICB4	95	95	23.7	23.7
ICB8	103	103	103	206
Chlorhexidine	2.1	18.4	1.1	2.1

ICBx: isolated from *C. blanchetianus*, x = 1 for specimen 1, x = 4 for specimen 4, x = 8 for specimen 8. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

two-fold increase against *S. parasanguinis*. However, MBC values remained the same for both bacteria.

Regarding to ICB4, the compound was obtained from the neutral fraction NFCB4-D2. ICB4 presented MIC and MBC values of 95  $\mu\text{M}$  against *S. mutans*, and 23.7  $\mu\text{M}$  against *S. parasanguinis*. ICB4 showed lower MIC values compared to NFCB4-D2. Moreover, NFCB4-D2 showed no bactericidal activity.

ICB8, isolated from the acid fraction AFCB8-D1, showed MIC and MBC values of 103  $\mu\text{M}$  against *S. mutans*. On the other hand, AFCB8-D1 showed MIC and MBC values lower compared to ICB8. The MIC

values of AFCB8-D1 and ICB8 remained the same against *S. parasanguinis*, while MBC values of AFCB8-D1 were lower. Chlorhexidine gluconate was used as a positive control, presenting values ranging from 1.1 to 18.4  $\mu\text{M}$ .

In this study, it was observed that all the hexane extracts from trunk bark, heartwood and roots of *C. blanchetianus* presented antimicrobial activity against *S. mutans* and *S. parasanguinis*, with concentrations ranging from 250 to 15.6  $\mu\text{g mL}^{-1}$ . Our results corroborate with the study of Silva *et al.*,<sup>33</sup> where *C. sonderianus* Mull. ethanol extract inhibited dental caries-related bacteria growth, including *S. mitis* ATCC 9811. The authors emphasize the possibility of using this extract for oral pathologies treatment.<sup>34</sup> The root hexane extract of *C. sonderianus* showed antibacterial activity against *B. subtilis*, *S. aureus* and *Mycobacterium smegmatis*, and antifungal activity against *Candida albicans*, *Trichophyton mentagrophytes*, and *Helminthosporium* sp. The reported action was attributed to the diterpenes hardwickiic acid and 3,4-secotrachylobanoic acid.<sup>34</sup> The EtOAc extract from *C. macrostachyus* trunk bark inhibited the growth of *Salmonella typhi*, *E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Listeria monocytogenes*, with MIC values ranging from 125 to 250  $\text{mg mL}^{-1}$ , being considered as a promising antimicrobial agent against important human pathogens.<sup>35</sup>

In this work, acid and neutral fractions were separated and it was observed that the acid fractions presented antimicrobial activity in smaller concentrations when compared to the neutral fractions (see Table S3, SI section). A previous study<sup>31</sup> with the acid and neutral fractions of *C. sonderianus* showed that acid fraction had better antimicrobial activity against *B. subtilis*, *S. aureus* and *Pseudomonas aeruginosa* when compared to neutral fractions. Moreover, the authors isolated the compound *ent*-Beyer-15-en-18-oic acid that presented antimicrobial activity against *B. subtilis*.<sup>31</sup>

*Croton* species have been widely studied in relation to their volatile and non-volatile constituents. Many species are producers of a large number of substances belonging to the alkaloids, phenylpropanoids and terpenoids classes.<sup>36</sup> The diterpenes isolated from *C. blanchetianus* showed clearly antimicrobial activity against *S. mutans* and *S. parasanguinis*. The diterpene class exhibits significant antimicrobial activity against Gram-negative, Gram-positive bacteria and yeasts.<sup>37,38</sup> In addition, it is suggested that diterpenes interact in a non-specific way with the bacterial cell membrane, causing disruption of its structure.<sup>38</sup> A casbane diterpene isolated from *Croton nepetifolius* Baill. showed potential antibacterial activity against several streptococci species related to caries, with MIC values ranging from 250 to 62.5  $\mu\text{g mL}^{-1}$ .

In addition, the ability to inhibit the formation of oral biofilms has been verified, therefore, being considered by the authors as a promising molecule on oral pathogens related to dental caries.<sup>39</sup> Thus, the diterpenes isolated in this work have large potential for microbial therapy, however, toxicity tests are still required to determine the therapeutic index allowing the safety use.

## Conclusions

In summary, in this study three 3,4-*seco*-diterpenes were isolated from the hexane extracts of roots of three individual specimens of *C. blanchetianus* Baill., including a novel diterpene 3,4-*seco*-beieren-3-oic acid (ICB1). Furthermore, the acid and neutral fractions, and the isolated compounds, showed a potential activity against two oral streptococci.

## Supplementary Information

Supplementary data of the compounds ICB1, ICB4 and ICB8 (NMR, IR and HRMS spectra, Figures S1-S25) and Tables S1-S3 are available free of charge at <http://jbs.sbq.org.br> as PDF file.

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