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Ent-trachyloban-19-oic acid isolated from *Iostephane heterophylla* as a promising antibacterial agent against *Streptococcus mutans* biofilms

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ABSTRACT

From the roots of *Iostephane heterophylla*, six known compounds, namely, *ent*-trachyloban-19-oic acid (**1**), the mixture of *ent*-kaur-16-en-19-oic acid (**2**) and *ent*-beyer-15-en-19-oic acid (**3**), xanthorrhizol (**4**), 16α-hydroxy-*ent*-kaurane (**5**) and 16α-hydroxy-*ent*-kaur-11-en-19-oic acid (**6**) were isolated using a bioassay-guided fractionation method. The known compounds (**1**–**6**) were identified by comparison of their spectroscopic data with reported values in the literature. In an attempt to increase the resultant antimicrobial activity of **1** and **4**, a series of reactions was performed on *ent*-trachyloban-19-oic acid (**1**) and xanthorrhizol (**4**), to obtain derivatives **1a**, **1b**, and **4a**–**4d**. All the isolated compounds (**1**–**6**) and the derivatives **1a**, **1b**, and **4a**–**4d** were evaluated for their antimicrobial activity against two oral pathogens, *Streptococcus mutans* and *Porphyromonas gingivalis* associated with caries and periodontal disease, respectively. Compounds **1**, **1b**, **2** + **3**, **4** and **4d** inhibited the growth of *S. mutans* with concentrations ranging from 4.1 μg/mL to 70.5 μg/mL. No significant activity was found on *P. gingivalis* except for **4** with an MIC of 6.8 μg/mL. The ability of **1**, **1b**, **2** + **3**, **4** and **4d** to inhibit biofilm formation by *S. mutans* was evaluated. It was found that **1**, **1b**, **4** and **4d** interfered with the establishment of *S. mutans* biofilms, inhibiting their development at 32.5, 125.0, 14.1 and 24.4 μg/mL, respectively.

1. Introduction

Iostephane heterophylla (Cav.) Benth Hemsl is a traditional medicinal plant that grows in the pine–oak–juniper forested mountains of western, central and southern Mexico where is commonly known as "*tecpahtli*", "*bauji*", "*corsoner*", "liga", "tlalpopolote" and "raíz del manso" [1, 2]. The roots of *I. heterophylla* are widely commercialized species in Mexico for the treatment of bacterial infections, in fresh wounds and sores in the form of a poultice, to alleviate rheumatism and arthritis by rubbing an alcoholic tincture or aqueous extract on the joints, and to make tea for diabetes, gastrointestinal complaints and liver ailments [2]. Previous phytochemical investigation on *I. heterophylla* roots resulted in the isolation and identification of several diterpenoids, bisabolenes and coumarins [3–5]. The traditional uses of *I. heterophylla* suggested the presence of antibacterial agents. Furthermore, a preliminary test revealed that the $CHCl_3$ extract prepared from the roots of the plant showed MIC values of 77 and 105 μg/mL for *Streptococcus mutans* and *Porphyromonas gingivalis*, respectively. Accordingly, in our continuous efforts to investigate Mexican plants widely used to treat oral diseases [6], the aim of the present investigation was to isolate and characterize the antibacterial principles against oral pathogens from *I. heterophylla*. Following a bioassay-guided isolation procedure, *ent*-trachyloban-19-oic acid (**1**), the mixture of *ent*-kaur-16-en-19-oic acid (**2**) and *ent*-beyer-

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15-en-19-oic acid (**3**), xanthorrhizol (**4**), 16α-hydroxy-*ent*-kaurane (**5**) and 16α-hydroxy-*ent*-kaur-11-en-19-oic acid (**6**) were isolated. The structures of the known compounds (**1**–**6**) were determined by comparison of their spectroscopic data with reported values in the literature [3, 7, 8].

The antibacterial activity of compounds **1**–**6** and derivatives **1a**, **1b**, and **4a**–**4d** was evaluated against *S. mutans* and *P. gingivalis* quantitatively. The compounds *ent*-trachyloban-19-oic acid (**1**), *ent*-trachyloban-19-ol (**1b**), xanthorrhizol (**4**) and 12,13-dihydroxanthorrhizol (**4d**) also interfered with the establishment of *S. mutans* biofilms, inhibiting their development at concentrations ranging from 14.1 to 125.0 μg/mL.

The structures of the known compounds (**1**–**6**) and the derivatives (**1a**, **1b**, and **4a**–**4d**) were determined by comparison of their spectroscopic data with reported values in the literature [3, 7–11].

2. Experimental

2.1. General procedures

NMR were recorded on a Varian VNMRS spectrometer operating at 400 MHz. EIMS was obtained in a positive mode in a Thermo Electron DFS spectrometer. CC was performed using silica gel (300–400 mesh). TLC was carried out by using silica gel plates prepared with GF254 (Merck); spots were visualized by UV light or 1% sulfuric vanillin.

2.2. Plant material

Fresh roots of *I. heterophylla* were purchased in Mercado de Sonora of Mexico City. One of the authors (R. Bye), authenticated the plant material (R. Bye and E. Linares 17986). A voucher specimen is deposited in the Ethnobotanical Collection in the Institute of Biology, National Autonomous University of Mexico, Mexico City.

2.3. Extraction and isolation

The air dried and powdered roots (2.8 kg) were extracted with $CHCl_3$ by maceration, for up to 1 week. The resultant extract was concentrated *in vacuo* at 40 °C. A portion of $CHCl_3$-soluble extract (313 g) was subjected to silica gel vacuum column chromatography (VLC) and eluted with a gradient mixture of *n*-hexane-EtOAc (10:0→0:10) to give twenty pooled fractions. Fractions F-02 and F-05 were active when tested against *S. mutans* and *P. gingivalis* (Table 1). The most active fraction F-02 was chromatographed over a silica gel column and eluted with *n*-hexane-EtOAc to yield **1** (4.6 g). Fraction F-05, eluted with *n*-hexane-EtOAc (9:1), was chromatographed over a silica gel column, using a mixture of *n*-hexane-EtOAc (9:1) as solvent system, to give a mixture of **2** and **3** (100 mg) and **4** (120.2 mg). From inactive fractions F-08 and F-20 compounds **5** and **6** were respectively isolated.

2.4. Preparation of derivatives

2.4.1. Preparation of ent-trachyloban-19-oic methyl ester (**1a**)

Ent-trachyloban-19-oic acid (20.0 mg) was treated with ethereal diazomethane to yield *ent*-trachyloban-19-oic methyl ester (**1a**, 19.5 mg). Compound **1a** exhibited spectroscopic (^{1}H NMR, ^{13}C NMR and EIMS) data comparable to published values [3].

Table 1
MIC and MBC values of compounds isolated from *Iostephane heterophylla* and its derivatives against *Streptococcus mutans* and *Porphyromonas gingivalis*.

Compound	Minimum inhibitory concentration (μg/mL)[a]		Minimum bactericidal concentration (μg/mL)[a]	
	S. mutans	*P. gingivalis*	*S. mutans*	*P. gingivalis*
$CHCl_3$ extract	77.0	105.0	308.0	720.0
F-02	37.6	154.1	150.4	432.8
F-05	64.1	187.2	512.8	697.6
1	8.9	57.6	35.6	70.8
1a	>1000	>1000	>1000	>1000
1b	70.5	125	250	1000
2 + **3**	34.4	95.3	340.4	362.4
4	4.1	6.8	8.2	18.2
4a	>1000	>1000	>1000	>1000
4b + **4c**	>1000	>1000	>1000	>1000
4d	13.6	138.9	270.3	466.8
5	>1000	>1000	>1000	>1000
6	125	>1000	>1000	>1000
CHX[b]	1.2	3.2	10.8	12.8

[a] Values represent the average obtained from a minimum of three experiments.
[b] CHX: chlorhexidine gluconate.

2.4.2. Preparation of ent-trachyloban-19-ol (**1b**)

To a stirring solution of $LiAlH_4$ (7.9 mmol) in anhydrous THF (5 mL) at 4 °C was added the *ent*-trachyloban-19-oic methyl ester (**1a**) (1, 1.654 mmol) in 15 mL of THF drop wise over a period of 1 h. The solution was stirred at refluxing temperature under N_2 for 12 h. The solution was cooled to 4 °C by ice bath, and 1 mL of 1 N NaOH followed by H_2O (2 mL) was slowly added to the solution to quench the reaction. The solution was stirred at 23 °C for 1 h and then filtered to remove the solid material. The solution was concentrated. Purification by TLC (hexanes/acetone 95:5) gave the *ent*-trachyloban-19-ol (**1b**). This compound exhibited spectroscopic (^{1}H NMR, ^{13}C NMR and EIMS) data comparable to published values [9–11].

2.4.3. Preparation of 1-O-acetylxanthorrizhol (**4a**)

To a stirred solution of **4** (15 mg) in pyridine (0.1 mL), 0.15 mL of acetic anhydride was added. When TLC indicated complete consumption of starting material, the reaction solution was diluted with EtOAc (3 mL), washed with 1 N HCl (3×3 mL), saturated $NaHCO_3$ solution (3×4 mL), and water (3×3 mL), then dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness to yield an acetyl derivative (**4a**, 10 mg). Compound **4a** exhibited spectroscopic data (^{1}H NMR, ^{13}C NMR and EIMS) comparable to published values [7, 8].

2.4.4. Preparation of (12R/12S)-12,13-epoxy-xanthorrhizols (**4b** + **4c**)

To a suspension of *m*-chloroperbenzoic acid (23.32 mg), $NaHCO_3$ (5 mg), and $CHC1_3$ (2.5 mL), a solution of xanthorrhizol (15.0 mg) in $CHC1_3$ (1 mL) was dropwise added and stirred, keeping the reaction at 5 °C. After 1 h, 50% sodium sulfite in water (2.5 mL) was added and 30 min later, water (3×1 mL) was added. The organic layer was separated and

washed with 5% aqueous sodium carbonate (3×2.5 mL). The aqueous layers were extracted with CH_2Cl_2 (3×2.5 mL) and the organic solution dried with Na_2SO_4. The organic residue was chromatographed (Si Gel, using *n*-hexane-ethyl acetate gradient elution system) to obtain a 1:l mixture of **4b** + **4c** (50% yield). Compounds **4b** and **4c** exhibited spectroscopic (^{1}H NMR, ^{13}C NMR and EIMS) data comparable to published values [3, 8].

*2.4.5. Preparation of the 12,13-dihydroxanthorrhizol (**4d**)*

A solution of xanthorrhizol (**4**) (19 mg) dissolved in ethyl acetate (2 mL) was hydrogenated using 10% Pd/C (5 mg), as catalyst. The reaction was monitored by TLC (petroleum ether/ethyl acetate 85:15), and the reaction product was purified by preparative chromatography as a yellowish oil. Compound **4d** exhibited spectroscopic (^{1}H NMR, ^{13}C NMR and EIMS) data comparable to published values [7].

2.5. Determination of MIC and MBC

The *in vitro* antibacterial activity of compounds **1**–**6** was determined against *S. mutans* and *P. gingivalis* according to the National Committee of Clinical Laboratory Standards (NCCLS) recommended minimum inhibitory concentration (MIC) protocol with modifications [6, 9, 10]. Briefly, 2-fold dilution series were made from all tested antibacterial agents starting from 2000 μg/mL in a 96-well plate. *S. mutans* strain ATCC 10499 was grown at 37 °C under aerobic conditions in brain heart infusion (BHI) broth media (Becton Dickinson, Sparks, MD). An aliquot of 20 μL of bacterial suspension at a concentration of 10^6 colony-forming units/mL was added to 180 μL of antibacterial dilution. Chlorhexidine (0.12%) was used as positive control and the untreated suspension as negative control. The MIC was defined as the lowest concentration of the test agent that had restricted growth to a level<0.05 at 660 nm after incubation at 37 °C for 16–24 h. For the determination of MBC, an aliquot of 50 μL of all the incubated test samples was subcultured on BHI agar supplemented with 5% of defibrinated sheep blood. MBC was defined as the lowest concentration that allows no growth on the agar.

2.6. In vitro biofilm formation, treatment and quantification

The formation of *S. mutans* biofilms was studied in commercially available presterilized, flat-bottom 96-well microtiter plates by the method described previously [12, 13] with modifications. The plates were conditioned with 200 μL of artificial saliva solution. The plates were then incubated at room temperature for 2 h with gentle shaking and air-dried after removal of the excess artificial saliva solution. The growth of biofilm formation was initiated by addition of 175 μL of BHI broth supplemented with 3% (w/v) of sucrose to each well of a 96-well microtiter plate. A 96-well microtiter plate containing 175 μL of BHI broth supplemented with sucrose per well was inoculated with 25 μL of a previously prepared inoculum cell suspension at a density of 2×10^5 cells/mL. The final density of inoculum in each well of a 96-well microtiter plate was 2.5×10^4 cells/mL. The microtiter plates were then incubated at 37 °C during 24 h for biofilm development. After the biofilm growth in the presence of 0, 5, 10, 50 and 200 μg/mL of the test compounds, the content of each well was removed.

Biofilm formation was quantified by a crystal violet assay [14]. Briefly, the biofilm-coated wells of microtiter plates as described above for biofilm formation were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were washed twice with 200 μL of 50 mM PBS (pH = 7.0) and air dried for 45 min, and 200 μL of absolute ethyl alcohol was transferred to each well, in order to fix the adherent cells, and allowed to contact during 15 min. Then, each well washed was stained with 100 μL of 0.4% aqueous crystal violet solution for 45 min. Afterwards, each well was washed twice with 200 μL of sterile distilled water and immediately de-stained with 200 μL of 90% ethanol. After 45 min of de-staining, 100 μL of de-staining solution was transferred to a new well and the amount of the crystal violet stain in the de-staining solution was measured with a tunable microplate reader at 595 nm. The activity of the tested compounds was expressed as the percentage of the absorbance of biofilm treated compared with the control (untreated).

3. Results and discussion

In this study, the *in vitro* antimicrobial activity of the $CHCl_3$ extract and isolated compounds from *I. heterophylla* against *S. mutans* and *P. gingivalis* was investigated. *S. mutans*, a Gram-positive facultative anaerobic coccus is commonly acknowledged as the main bacteria responsible for the formation of the dental plaque and dental caries and *P. gingivalis*, the Gram-negative anaerobic oral bacterium most commonly associated with gum diseases [6]. In traditional Mexican medicine the hot water extract of *I. heterophylla* has been used for the treatment of oral cavity infections. In our data, 77 and 105 μg/mL of the $CHCl_3$ extract showed antibacterial activity against *S. mutans* and *P. gingivalis*. This result supports the scientific rationale that native inhabitants used the extract for the treatment of dental diseases. Purification of the active fractions F-02 and F-05 (Table 1) led to the isolation of *ent*-trachyloban-19-oic acid (**1**), the mixture of *ent*-kaur-16-en-19-oic acid (**2**) and *ent*-beyer-15-en-19-oic acid (**3**), and xanthorrhizol (**4**). From the inactive fractions F-08 and F-20, 16α-hydroxy-*ent*-kaurane (**5**) and 16α-hydroxy-*ent*-kaur-11-en-19-oic acid (**6**) were isolated (Fig. 1). The structures of the known compounds were identified by their spectroscopic data (^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC, HMBC, UV and IR) and by comparison with published values [3, 7, 8].

The selected microorganisms were predictive of potential applications against human diseases caused by bacteria. The results in Table 1 indicate that the extract from *I. heterophylla* inhibited the growth of *S. mutans* and *P. gingivalis* with MIC values of 77 and 105 μg/mL, respectively. Among the evaluated metabolites, compounds **1**, the mixture of **2** + **3** and **4** displayed the highest antibacterial activity against *S. mutans* and *P. gingivalis* with MICs ranging from 4.1 to 95.3 μg/mL. The MBC values of the tested compounds shown in Table 1 were higher (four to ten times) than the MIC values, ranging from 8.2 to 362.4 μg/mL. Concerning *S. mutans*, compounds **1**, and **4** also interfered with the establishment of its biofilms,

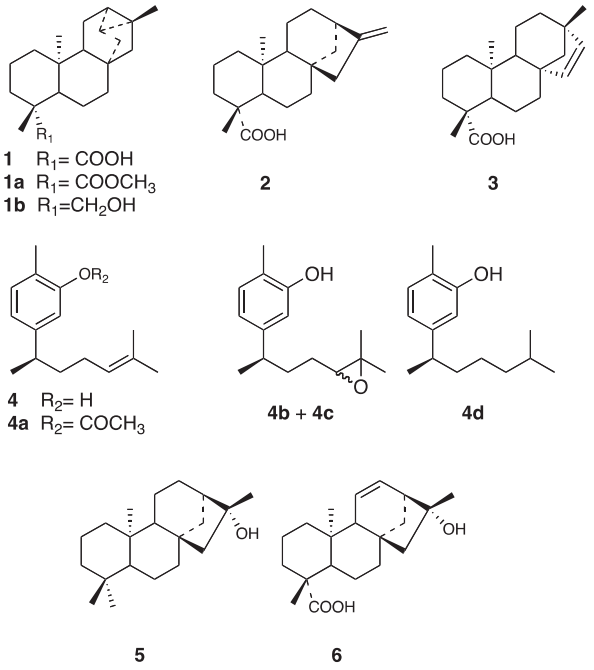

Fig. 1. Isolated compounds from *I. heterophylla* and *ent*-trachyloban-19-oic acid (**1**) and xanthorrhizol (**4**) derivatives.

inhibiting their development at concentrations of 32.5 and 14.1 μg/mL, respectively.

Regarding with the degree of activity of the tested compounds, it can be noted that the MIC values varied from 77 to 105 μg/mL, 4.1 to the concentration limit of 1000 μg/mL, and 1.2 to 3.2 μg/mL for the crude extract, pure compounds and reference antibiotic, respectively. The MIC and MBC values obtained with compounds **1**, **1b**, the mixture of **2** + **3** and **4** were less potent than that for chlorhexidine on the corresponding microorganisms, being the most active xanthorrhizol (**4**) (Table 1). The diterpenes **5** (16α-hydroxy-*ent*-kaurane) and **6** (16α-hydroxy-*ent*-kaur-11-en-19-oic acid) were inactive whereas the mixture of **2** + **3** showed significant activity (34.4 μg/mL) against *S. mutans*. Even though the diterpene trachylobanoic acid (**1**) was less active than sesquiterpene xanthorrhizol (**4**), it showed MIC of 8.9 μg/mL and was more active than the mixture of kaurenoic acids **2** + **3**. Recently, Urzúa et al. [15] have established that a lipophilic decalin ring system, with a strategically positioned hydrogen-bond-donor group (HBD; hydrophilic group), is very important for the antimicrobial activity displayed by diterpenes. Moreover, the authors pointed out that a second HBD introduced in the decalin ring system led to a reduction in or suppression of the activity. Two reasons may explain these observations. The first, the presence of two HBDs decreases the lipophilicity of the hydrophobic moiety, hindering its interaction with the bacterial membrane. A second explanation, the intramolecular HBD group interactions compete with intermolecular hydrogen-bonds between each HBD and the cell membrane. A careful observation of the results in Table 1 reveals that compounds **1**, **1b**, **2** and **3**, which contain HBD at C-19, display MIC and MBC values lower than that of compound **5**, which has no HBD in its chemical structure. In fact, the presence of two HBDs in the decalin ring system of compound **6** really decreases the antimicrobial activity displayed by these diterpenes.

To find out if activity could be increased in the two most active terpenoids (**1** and **4**), derivatives **1a**, **1b**, and **4a**–**4d** were obtained. As Table 1 shows, the acidic moiety in trachylobanoic acid (**1**) is essential to keep antimicrobial activity, meanwhile blocking of phenol group and double bond C12-13 in xanthorrhizol, also leads to loss of activity but hydrogenation of this bond keeps activity but at a lesser extent. Compounds **1b** and **4d** also interfered with the establishment of *S. mutans* biofilms, inhibiting their development at 125.0 and 24.4 μg/mL, respectively.

Earlier *in vitro* studies have shown that *ent*-trachyloban-19-oic acid (**1**) has antimicrobial activity against methicillin-resistant *Staphylococcus aureus* and *Mycobacterium smegmatis* [16]. This diterpene inhibits also larval development of *Homeosoma electullum* (sunflower moth) and the three Lepidoptera species *Heliotis virscens*, *Heliotis zea*, and *Pectinophera gossypiella* (pink bollworm) [17]. Xanthorrhizol (**4**) has been isolated from Zingiberaceae [18, 19] and Asteraceae families of higher plants [7]. Several pharmacological properties of xanthorrhizol (**4**) have been demonstrated, including antibacterial, anti-biofilm, antifungal, cytotoxic and as inhibitor of the tonic contraction of rat uterus [20–23].

In conclusion, the compounds isolated from *I. heterophylla* are effective antibacterial agents and represent an alternative to commonly used compounds to treat oral infections. The results in this study confirmed that the roots of *I. heterophylla* have a significant antimicrobial activity against oral pathogens and justifies its ethnomedical use as such. The roots are also safe for consumption as traditional remedy, as toxicity tests have demonstrated [24].

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