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Phytochemistry of compounds isolated from the leaf-surface extract of *Psiadia punctulata* (DC.) Vatke growing in Saudi Arabia

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ABSTRACT

The surface extract of an accession of *Psiadia punctulata* (DC.) Vatke (Asteraceae) growing in Saudi Arabia was investigated for its phytochemical composition. A bio-guided investigation of the extract led to the isolation of thirteen *ent*-kaurane and trachylobane diterpenes and seventeen compounds previously described, including nine flavonoids and eight diterpenes. Three flavonoids and one *ent*-kaurane diterpene showed antimicrobial activity with MIC100 values ranging from 25 to 150 μg/ml. The extract showed antibacterial activity against *Staphylococcus aureus* (MIC_{100} = 180 μg/ml) and antifungal activity against *Candida albicans* (MIC_0 = 130 μg/ml). The isolated 3′,4′,5,7-tetramethoxyflavone, at a concentration of 40 μg/ml, displayed the ability to reduce biofilm formation of *S. aureus* and *C. albicans* by 50% and 90% respectively.

1. Introduction

Psiadia punctulata (DC.) Vatke (Asteraceae) is a small shrub distributed in Africa and tropical Asia (Xiaoping and Bremer, 1993) and included into the European and Mediterranean genera of Astereae (Greuter, 2003). The leaves, especially in young plants, are covered by a gummy exudate. The species is traditionally used for the treatment of cold, fever, abdominal pain, malaria, skin infection and scabies; it is also used for the removal of ectoparasite from cattle and as analgesic and expectorant (Mahadeo et al., 2018).

Previous phytochemical studies on *P. punctulata* reported the presence of *ent*-kaurane and *ent*-trachylobane diterpenes in apolar extracts of leaf exudates (Juma et al., 2006; Midiwo et al., 1997) as well as the presence of flavones and phenylpropenoids in extracts of leaf exudates and leaves (Abou-Zaid et al., 1991; Juma et al., 2001) as the main components. *P. arabica* Jaub. & Spach. is commonly considered to be synonymous to *P. punctulata* (Abou-Zaid et al., 1991; Flann, 2015); nevertheless, the differences in compounds isolated from the two species (Al-Yahya et al., 1987; El-Domiaty et al., 1993; El-Feraly et al., 1990; Juma et al., 2001, 2006; Midiwo et al., 1997, 2002; Mossa et al., 1992) suggest that they should be considered as two distinct taxa (Juma et al., 2001).

More recent findings about the polyphyly of *Psiadia* Jacq., described as made up of two main species clusters (clade A and B), highlighted the difference between two different accessions of *P. punctulata*, from Southern Africa and from Saudi Arabia, that were defined as two distinct species and placed into two different sub-clades of clade A (Strijk et al., 2012). Mahadeo et al. attribute these differences to ecological adaptations or different geographical location (Mahadeo et al., 2018). The species has been studied for antimicrobial, antitrypanosomal, antileishmanial, antiplasmodial, and cytotoxic activities (Mahadeo et al., 2018). Extracts of the aerial parts of *P. punctulata* collected in Yemen (Mothana et al., 2011) and in Saudi Arabia (Gouda et al., 2014), made with polar solvents showed moderate antimicrobial activity (MIC values ranging from 500 to > 1000 μg/ml) against *Staphylococcus aureus* and *Candida albicans*.

As part of a project searching for new antibacterial compounds, based on the fact that several reports have shown the potentiality of

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plants exudates to be a source of antimicrobial agents (Bisio et al., 2017; Gibbons, 2004), the surface extract of *P. punctulata* from the Arabian region was subjected to a bioassay-oriented fractionation aimed at evaluating its antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Candida albicans*. Moreover, due to the increasing antibiotic resistance, as a consequence of the excessive and often inappropriate use of antibiotics, its antimicrobial activity was evaluated against resistant microbial strains. Minimal Inhibitory Concentration (MIC) values and the ability of active compounds to inhibit biofilm formation were investigated.

2. Results and discussion

The antimicrobial activity of the dichloromethane soluble portion of the surface extract of *P. punctulata* (10.0 g) was tested against a Gram-positive (*S. aureus* A170, a clinically isolated gentamicin resistant strain) and a Gram-negative (*P. aeruginosa* ATCC 27853, ampicillin resistant strain) bacteria as well as the dimorphic fungus *C. albicans*, which is a causal agent of oral and genital opportunistic infections in humans. In these preliminary tests, the crude surface extract showed antibacterial activity against *S. aureus* (MIC_{50} = 100 μg/ml, MIC_{100} = 180 μg/ml), while no activity was detected against *P. aeruginosa*. Moreover, the extract showed antifungal activity against *C. albicans* (MIC_2 = 50 μg/ml and MIC_0 = 130 μg/ml).

The extract was subsequently separated by column chromatography on silica gel, yielding nine fractions (A-I). The fractions were evaluated for their antimicrobial activity at doses ranging from 20 to 100 μg/ml. Among the nine fractions analysed, fractions A, B and I were inactive against all the species tested, while six fractions (C−H) were active against *C. albicans* and the Gram-positive strain, but not against the Gram-negative strain (Supporting Information S54). Fractions C-H were then separated by RP-HPLC to yield thirteen compounds (**1–13**) along with known diterpenes (**14–21**) and flavones (**22–30**) (Fig. 1).

Compound **1** was obtained as an amorphous white powder. Its molecular formula $C_{20}H_{34}O_3$, equating to four double bond equivalents, was deduced from HRESIMS (*m/z* 323.2581 $[M+H]^+$, calcd. for 322.2508). The ^{1}H NMR spectrum displayed signals due to four tertiary methyl groups at δ_H 0.81 (3H, s), 0.99 (3H, s), 1.26 (3H, s) 1.34 (3H, s), and two hydroxymethine groups at δ_H 3.15 (1H, dd, *J* = 11.7 and 4.4 Hz) and 3.92 (1H, m). The ^{13}C NMR data (Table 1) revealed the presence of 20 carbon signals, indicative of four methyls; seven methylenes; three methines; four quaternary carbons, one of which was oxygenated; and two hydroxymethines. A comparison between these carbon resonances and those of the related *ent*-kaurane-3α,16α-diol suggested that compound **1** possesses the same skeleton (Tori et al., 1993). Analysis of 1D-TOCSY, COSY and HSQC experiments of **1** showed the presence of the same partial structure of rings A, B and D as those of *ent*-kaurane-3α,16α-diol, but indicated that ring C was the point of difference between the two molecules due to the presence of the 12-hydroxy group in **1**. The presence of one additional oxygenated methine at C-12 was confirmed by ^{1}H-^{1}H COSY and 1D TOCSY correlations H-9/H_2-11/H-12 and H-12/H-13/H_2-14 as well as HMBC correlations H_2-11/C-13, H_2-14/C-12 (Fig. 2). The relative configuration of **1** was found to be the same as that of *ent*-kaurane-3α,16α-diol (Tori et al., 1993) on the basis of the coupling constant values of the H-3 axial signal at δ_H 3.15 (1H, dd, *J* = 11.7 and 4.4 Hz) and by the analysis of the 1D ROESY spectra: selective irradiation of H-3 produced significant enhancement of the proton signal at δ_H 0.79 (H-5) and 0.99 (H_3-18). Furthermore, upon irradiation of H-12 (δ_H 3.92), a ROE response with H-13 (δ_H 1.96) and H-17 (δ_H 1.34) was observed. Thus, **1** was characterised as *ent*-kauran-3α,12α,16α-triol.

Compound **2** was obtained as a colourless powder. The positive HRESIMS gave a quasi-molecular ion peak at *m/z* 339.2531, in accordance with the molecular formula $C_{20}H_{34}O_4$ and four indices of hydrogen deficiency. The ^{1}H and ^{13}C NMR data (Table 1) of compound **2** showed marked similarities with compound **1**, except for the resonances of an additional oxymethylene group (δ_H 3.58; 3.65) and the absence of the signal for one methyl group (δ_H 1.34). The HMBC cross-peaks of signals at ppm 3.58 (1H, d, *J* = 13.0 Hz) and 3.65 (1H, d, *J* = 13.0 Hz) with C-13, C-15 and C-16 suggested that the hydroxy group was located at C-17. The β-orientation of CH_2OH-17 was elucidated by comparison between the ^{13}C NMR data and those of the literature (Ge et al., 2008; Zhang et al., 2011) and on the basis of the 1D ROESY correlation between H-12 and H-17. Thus, the structure of **2** was defined as *ent*-kauran-3α,12α,16α,17-tetraol.

Compound **3**, obtained as an amorphous white powder, was assigned the molecular formula $C_{20}H_{32}O_3$ by its HRESIMS *m/z* 321.2422 $[M+H]^+$ (calcd. for 320.2351). This information along with the ^{13}C NMR data, which sorted 20 carbons into two methyls; eight methylenes, one of which had an sp^2 carbon; three methines; four quaternary carbons, one of which was an sp^2 carbon; two hydroxymethines; and one hydroxymethylene, led to the determination of five indices of hydrogen deficiency and a *ent*-kaurane nucleus for **3**. The ^{1}H and ^{13}C NMR data (Table 1) were quite similar to those reported for *ent*-kaur-16(17)-en-6,19-dihydroxy-2-one (**14**) (Almutairi et al., 2014); the point of difference was the chemical shift of protons and carbons of ring A. The structure of **3** differed from propsiadin at C-2, where the oxo group was replaced by an oxymethine group (δ_C 64.3; δ_H 3.83). This was verified by the ^{1}H-^{1}H COSY cross-peaks H_2-1/H-2 and H-2/H_2-3 and HMBC correlations H_2-1/C-2, H_2-1/C-20 and H_2-3/C-1, H_2-3/C-19. The *J* values (br ddd, $J_{(H\text{-}2ax/H\text{-}1ax)}$ = 13.6 Hz, $J_{(H\text{-}2ax/H\text{-}3ax)}$ = 12.6 Hz, $J_{(H\text{-}2ax/H\text{-}3eq)}$ = 4.1 Hz) suggested an α-axial orientation of H-2, which was confirmed by a ROE enhancement of the signals at δ_H 1.21 (H_3-20), 3.52 and 3.97 (H_2-19) upon irradiation at δ_H 3.83. The relative position of the H-6 was inferred by its *J* values (ddd, $J_{(H\text{-}6ax/H\text{-}7ax)}$ = 11.0 Hz, $J_{(H\text{-}6ax/H\text{-}5)}$ = 11.2 Hz, $J_{(H\text{-}6ax/H\text{-}7eq)}$ = 3.6 Hz) and by the 1D ROESY correlation between H-6 (δ_H 4.08) and H_3-20 (δ_H 1.21). From the above information and literature data, the structure of compound **3** was described as *ent*-kaur-16(17)-en-2β,6β,19-triol.

Compound **4**, obtained as an amorphous white powder, showed a molecular formula of $C_{20}H_{32}O_4$, according to a $[M+H]^+$ ion at *m/z* 337.2373 (calcd. for 336.2301) in its HRESIMS, indicative of five indices of hydrogen deficiency. The NMR data of **4** (Table 1) showed a high degree of similarity with compound **3**. The signals observed in the ^{1}H and ^{13}C NMR spectrum at δ_H 3.62, 4.03 (overlapped signal) and δ_C 65.4 suggested the presence of an additional oxymethyl group. This group was located at C-18, on the basis of the proton and carbon chemical shifts of A-ring and HMBC correlations H-3/C-18, H-5/C-18, H_2-18/C-4, H_2-18/C-19 and H_2-19/C-18. Therefore, the structure of **4** was identified as *ent*-kaur-16(17)-en-2β,6β,18,19-tetraol.

Compound **5**, obtained as a white powder, gave a molecular formula of $C_{20}H_{32}O_5$ according to a $[M+H]^+$ ion at *m/z* 353.2331 (calcd. for 352.2250) in its HRESIMS spectrum, requiring five degrees of unsaturation. The ^{1}H and ^{13}C NMR features of **5** (Table 2) were closely related to those of *ent*-kauran-16α,17,19-trihydroxy-2-one (Chen et al., 2010). A comparison between the NMR spectral data of compound **5** and those of *ent*-kauran-16α,17,19-trihydroxy-2-one demonstrated these compounds to be identical in the A, C, D-rings, but different in the B-ring portion. Infact, hydrogen and carbon NMR signals due to the atoms of the B-ring were somewhat shifted. Particularly, the methylene moiety at position 6 in *ent*-kauran-16α,17,19-trihydroxy-2-one was replaced by an oxymethine in **5**. This was confirmed by the downfield shifting of C-6 (δ_C 68.7), C-5 (δ_C59.6) and C-7 (δ_C51.3); the upfield shift of C-4 (δ_C 44.7 ppm); and the 1D TOCSY spectrum showing the H-5/H-6/H_2-7 spin system. The β OH-6 was defined by the observation of the H-6 coupling constants [δ_H 4.04 ddd (*J* = 12.0, 11.0, 3.7 Hz)], typical of an axial proton. The 1D ROESY confirmed the relative configuration. Accordingly, compound **5** was identified as *ent*-kauran-6β,16α,17,19-tetrahydroxy-2-one.

Compound **6** was isolated as an amorphous white powder. Its molecular formula was determined as $C_{20}H_{34}O_4$ by the positive HRESIMS signal at *m/z*339.2529 (calcd. for 338.2457) and accounted for four