HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis

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ABSTRACT

The well-known anti-infective properties of propolis are determined by its chemical composition, which in turn is influenced by geographical factors and reflects the botanical diversity in the vicinity of the beehive. Although there are several reports on the anti-infective properties of crude propolis, few are aimed at identifying specific compound(s) responsible for the observed activities. Using South African propolis as an example, the application of high performance thin layer chromatography-bioautography in tandem with mass spectrometry was investigated for the rapid identification of antimicrobial and anti-quorum sensing (anti-QS) compounds. Pinocembrin was found to be responsible for the observed antifungal activity of the propolis against Candida albicans. Three compounds were found to be active against all of the evaluated Gram-positive and Gram-negative bacteria. The identity of the first was confirmed as pinobanksin, one remains unidentified, while the third corresponds to either pinobanksin 3-O-pentanoate or 2-methylbutyrate. The identification of caffeic acid as the anti-QS component was confirmed quantitatively using the violacein inhibitory assay.

1. Introduction

Thin layer and high performance thin layer chromatography (TLC/HPTLC)-bioautography are simple and rapid techniques for the identification of bioactive compounds present in crude extracts. These techniques are particularly suited to the preliminary bioactivity screening of natural products and to bioactivity-guided fractionation and isolation of active components from natural resources (Cheng and Wu, 2013). Many TLC/HPTLC-bioautography methods have been reported for elucidating antibacterial, antifungal, antitumour, antiprotozoal and enzyme-inhibiting compounds (Choma and Grzelak, 2011; Horváth et al., 2013). Antimicrobial bioautographic screening, which directly reveals the presence of antibacterial or antifungal components on a TLC plate, is commonly practised. To date, several antibacterial and antifungal compounds, including anti-quorum sensing (anti-QS) compounds, have been identified from plants and their products using bioautography (Koh et al., 2013; Priya et al., 2013; Zhou et al., 2013). Quorum sensing (QS) has been reported as one of the essential factors that regulate bacterial virulence and pathogenicity (Rutherford and Bassler, 2012). Therefore, the interruption of QS can be an effective strategy to control disease-causing pathogens.

Propolis, a resinous mixture composed of plant exudates, is produced by honeybees to protect the hive against pathogens. In several parts of the world, people have relied on this natural product as an effective treatment for various ailments over many centuries (Sforcin and Bankova, 2013). Propolis displays a wide range of biological properties, including antimicrobial, anti-inflammatory, immunomodulatory, antitumor and antidiabetic activities (Kang et al., 2010; Machado et al., 2012; Campos et al., 2014). These properties are determined by the chemical composition of the propolis, which is complex and highly variable. Regional and seasonal variations in the chemistry are influenced by a range of factors, such as the floral composition in the vicinity of the beehive, the geographic location of the hive, the prevailing illumination, as well as the time of year of collection; the method of propolis collection also affects the composition (Salatino et al., 2011; Tagliacollo and Orsi, 2011).

South Africa is a producer of both honey and propolis for the domestic market. Recently, we reported that propolis from this region is similar in composition to samples from temperate zones i.e. characteristically rich in phenolic acids and flavonols (Kasote et al., 2014). The anti-oxidant, antimicrobial and anti-inflammatory activities of propolis from South Africa have been evaluated

(Garedew et al., 2004; Kumazawa et al., 2004; Du Toit et al., 2009). Although the antimicrobial activities have been extensively studied and can be considered promising, antimicrobial components from South African propolis have not yet been identified.

In the present study, a hyphenated technique comprising HPTLC-bioautography and MS was used for the rapid identification of antimicrobial and anti-QS compounds from South African propolis. A sample originating from Cape Town was selected for the study, based on the fact that it displayed the best antibacterial activity of all the propolis samples screened in a preliminary investigation (data not published).

2. Results and discussion

Planar chromatography in the form of TLC/HPTLC has proved to be a valuable tool for the analysis of different types of propolis and has been widely used for both qualitative and quantitative studies to determine the chemical composition of the product (Hubicka et al., 2006; Tang et al., 2014). However, as far as could be ascertained, the application of TLC-MS to the analysis of propolis is limited to a single report on the identification of phenolic markers in samples originating from Germany (Bertrams et al., 2013). Antifungal compounds were identified in Chinese propolis using TLC-bioautography after elution of the scrapings from the plate and subsequent analysis by MS (Yang et al., 2011). Pinobanksin, pinocembrin, chrysin and galangin, present in the samples, were found to inhibit the growth of Penicillium digitatum, an important decay pathogen of citrus. In the present study, the advantages of the two techniques were combined by using HPTLC-bioautography in tandem with MS for the rapid identification of antimicrobial and anti-quorum sensing (anti-QS) compounds in South African propolis.

Viewing of the plates, developed with aqueous methanol, under white light and UV light indicated that the separation of the propolis constituents was adequate for further investigations (Fig. 2A–C). Several white zones on the bioautography plates (Fig. 2D–H) indicated the presence of compounds with the ability to inhibit the growth of microbial pathogens. Compound 1 (C1), with an inhibition zone at R_f 0.48 was the only compound with inhibitory activity against the fungus, C. albicans (Fig. 2D). However, this compound appeared to be inactive against the bacteria. Compounds 2 (C2), 3 (C3) and 4 (C4) with R_f values of 0.59, 0.39 and 0.21, respectively, were active against all of the evaluated Gram-negative and Gram-positive bacteria (Fig. 2E–H). The corresponding spots on the untreated plates (Fig. 2A–C) were later eluted, using the TLC-MS interface, for identification.

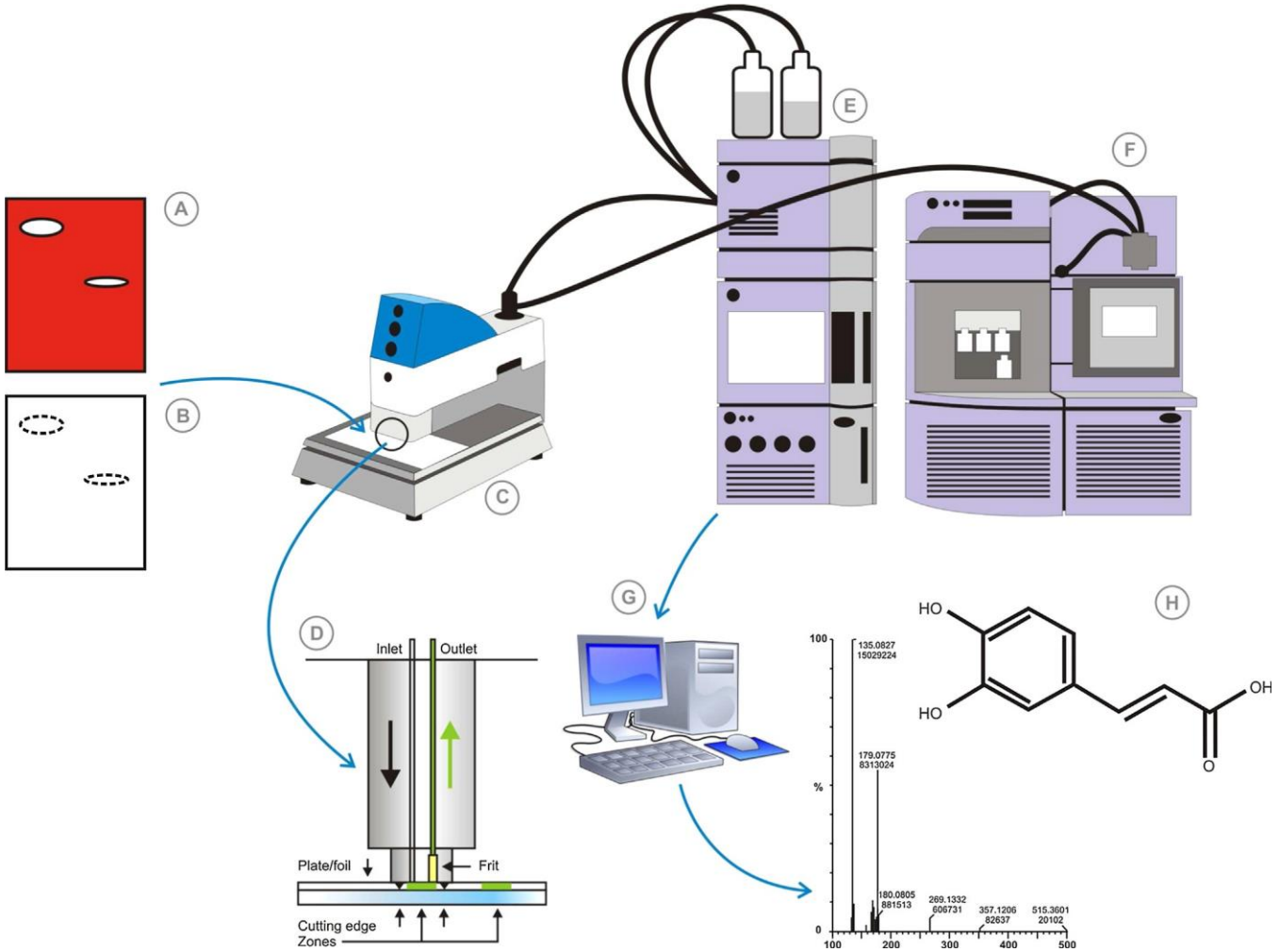

Fig. 1. Workflow diagram illustrating the hyphenated high performance thin layer chromatography-bioautography-mass spectrometry (HPTLC-bioautography-MS) technique used to identify antimicrobial compounds in propolis. (A) Bioautography HPTLC plate, (B) reference HPTLC plate, (C) TLC interface, (D) flowpump enabling the elution of target compounds from the plate, (E) UPLC pump, (F) time-of-flight mass spectrometer, (G) data analysis, and (H) mass spectral data allowing identification of compounds.

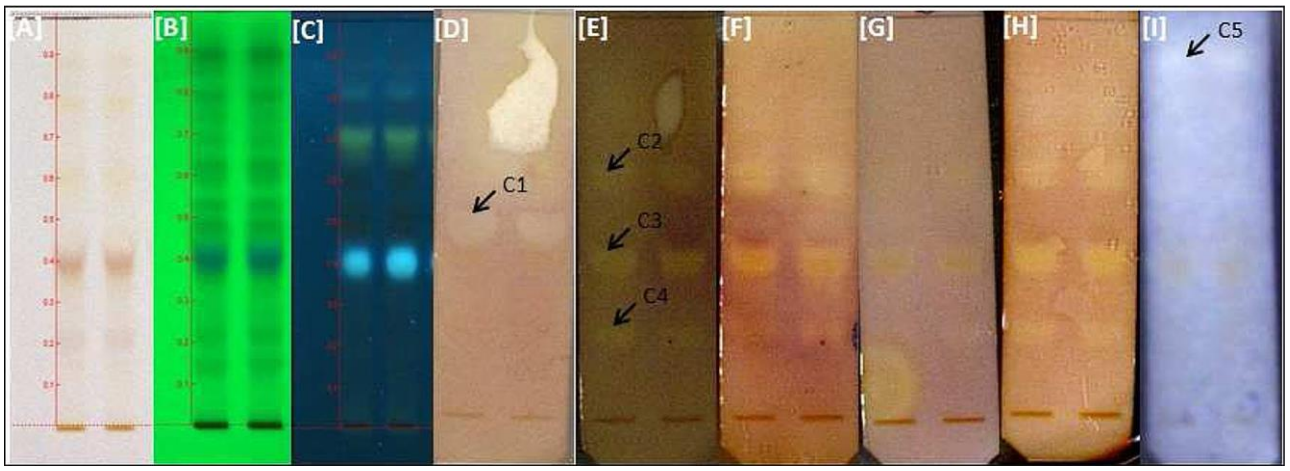

Fig. 2. Bioautography chromatograms of a methanol extract of South African propolis: developed HPTLC plate (A) under white light, (B) under UV 254 nm, (C) under UV 366 nm, (D) against C. albicans, (E) against E. faecalis (F) against S. aureus, (G) against E. coli, (H) against M. catarrhalis, and (I) against C. violaceum.

A compound (C5) in the propolis extract with anti-QS potential against the bacterial strain Chromobacterium violaceum was evident at R_f 0.89 (Fig. 2I). The ability of C5 to inhibit the QS-related production of violacein by C. violaceum indicates the potential of the compound to interfere with bacterial signalling, thereby reducing the virulence of the pathogen.

Five compounds with antimicrobial activity, observed as inhibition zones on the bioautograms, were targeted for identification using HPTLC-MS. The mass spectra, representing each of the zones, are presented in Fig. 3. The mass-to-charge ratios (m/z) of the most intense ions are listed in Table 1. Compound 1 (C1), with antifungal activity against C. albicans, yielded the most intense ion at m/z 255 (Fig. 3C1) which, according to literature, corresponds to the compound pinocembrin (Pellati et al., 2011; Falcã o et al., 2012). A less intense signal at m/z 313 is probably due to the formation of an acetonitrile-water adduct. The presence of another small peak at m/z 213 ($[M-H-C_2H_2O]^-$) supports the identification of C1 as pinocembrin (Fig. 4). The compound, present in propolis from China, was previously reported to inhibit the in vitro growth of several fungal pathogens, including C. albicans and Penicillium italicum (Yang et al., 2011).

Intense ions corresponding to m/z 271 and 325 are prominent in the mass spectrum of C2 (Fig. 3C2). This compound was active against all the bacterial strains evaluated. The ion at m/z 271 corresponds to the parent ion of pinobanksin (Pellati et al., 2011) (Fig. 4). Adduct formation could possibly account for the presence of the ion fragment at m/z 325 ($[M-H-3H_2O]^-$). Compound C3, with the most intense ion fragment at m/z 325 (Fig. 3C3), could not be identified. No match was found between this base peak and those previously reported in literature for compounds in propolis. Further investigations aimed at identifying C3 are warranted. The identity of C4 remains uncertain, since the most intense ion fragment at m/z 355 (Fig. 3C4) corresponds to both pinobanksin-3-O-pentanoate and 2-methylbutyrate (Kasote et al., 2014).

Compound 5 was identified with certainty as caffeic acid (Fig. 4), the only compound observed to have anti-QS activity. The most intense mass fragment at m/z 179 (Fig. 3C5) corresponds to that in the spectrum of the pure compound. In addition, after simultaneous HPTLC analysis, the R_f value of the standard and the compound in the propolis extract corresponded. Currently there is only one report on the anti-QS activity of a propolis sample from a temperate region of Argentina (Alvarez et al., 2012). However, none of the compounds responsible for the observed activity were identified.

In our previous study, 15 phenolic acids and flavonols, including pinocembrin, pinobanksin, caffeic acid, were identified in South African propolis samples using ultra-performance liquid chromatography coupled to photodiode detector-quadrupole/time-of-flight mass spectrometry (UPLC-PDA-qToF-MS) (Kasote et al., 2014). Although efficient for compound identification, this technique does not provide any link between the bioactivity of the sample and the responsible components.

To authenticate the HPTLC-bioautography-MS findings, the anti-QS activity of caffeic acid was quantified, using the violacein inhibitory assay. More than 90% inhibition of violacein production, with respect to the controls, was observed at a concentration of 0.5 mg/mL (Fig. 5). Inhibition was found to be concentration dependent. The percentage inhibition of violacein production was determined as 71%, 63% and 54% at 0.250, 0.125 and 0.062 mg/mL, respectively. These results are consistent with a previous report that indicated an inhibition of more than 70% violacein production by C. violaceum, following exposure to caffeic acid and other phenolic compounds at a concentration of 1 mg/mL (Borges et al., 2014). Since the anti-QS inhibition zone on the bioautography plate (Fig. 2I) is poorly visible, this implies a low concentration of caffeic acid in the propolis sample.

This is the first report on the use of HPTLC-bioautography in tandem with MS to assign specific compounds responsible for the antimicrobial and anti-quorum sensing (anti-QS) potential of propolis. The hyphenated TLC/HPTLC-MS technique allows unknown compounds from crude natural product extracts to be identified without tedious sample clean-up. In addition, key structural information allows for the tentative, and in many cases, the definite identification of metabolites in a very short time. In spite of the obvious advantages, the technique has not been embraced for drug discovery from natural sources.

3. Experimental

3.1. Microbial strains, media and culture conditions

A yeast strain (Candida albicans, ATCC 10231), Gram-positive bacteria (Enterococcus faecalis, ATCC 29212; Staphylococcus aureus, ATCC 126000), and Gram-negative bacteria (Escherichia coli, ATCC 8739; Moraxella catarrhalis, ATCC 23246) were used for the bioautography studies. All micro-organisms were initially grown in Tryptone Soya Broth (TSB), whereafter the pure cultures were

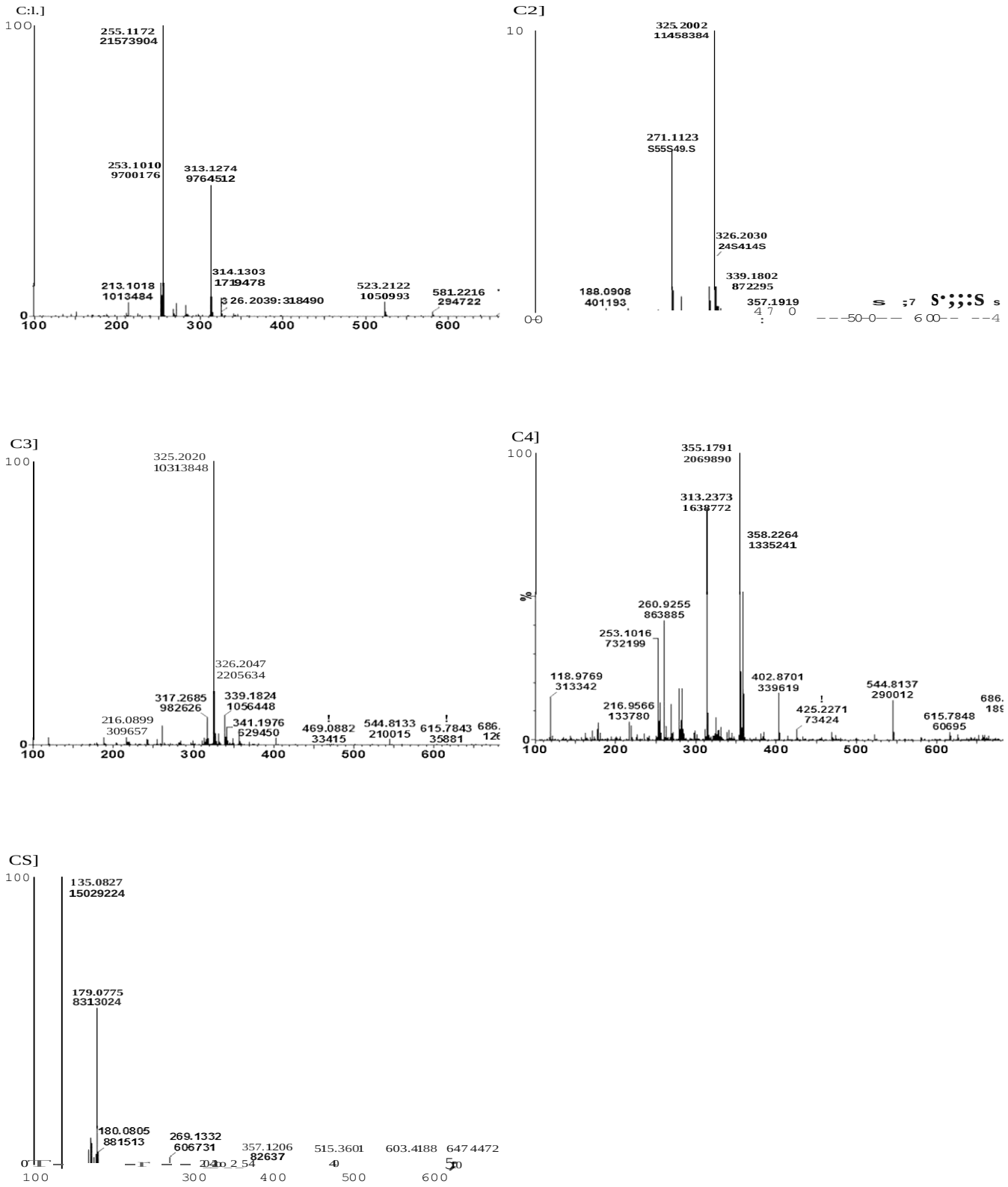

Fig. 3. Mass spectra of compounds 1-5 (Cl-CS), obtained through the use ofToF-MS.

maintained on Tryptone Soya Agar (TSA). The isolates were sub-cultured and incubated at 37 0C for their respective incubation periods, prior to the bioautography assays. For the anti-QS assay, wild-type bioreporter bacterial strain C. *violaceum* (ATCC 12472) was routinely cultured in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 0.5% NaCI, per 100 mL distilled water) at 30 0C for 24 h with shaking at 220 rpm. All the chemicals and media were purchased from Oxoid, England.

Table 1
Antimicrobial and anti-quorum sensing compounds identified from South African propolis using hyphenated HPTLC-ToFMS.

Inhibition zone	Pseudomolecular ion $(m/z)^-$	Compound identified
C1	255	Pinocembrin[a]
C2	271	Pinobanksin[a]
C3	325	Not identified
C4	355	Pinobanksin-3-O-pentanoate or 2-methylbutyrate[a]
C5	179	Caffeic acid[a,b]

[a] Identity confirmed by comparison with literature data (Pellati et al., 2011; Falcão et al., 2012; Kasote et al., 2014)
[b] Identity confirmed from standard.

3.2. Propolis sample preparation

An extract of the selected sample was prepared by shaking 100 mg of the propolis together with 10.0 mL of methanol (AR grade; SMM Chemicals, South Africa) for 24 h at 37 8C on a platform shaker (Labcon, South Africa). The final suspension was filtered through a 0.22 mm syringe filter (Clarinert™, USA).

3.3. HPTLC analysis

A CAMAG (Switzerland) semi-automated HPTLC system was used for separation of the propolis extract. The sample was applied to silica gel pre-coated aluminium plates (DC-Fertigfolien ALUGRAM® Xtra SIL G/UV$_{254}$, Germany) by the automated TLC Sampler 4, fitted with a 25 mL Hamilton microsyringe and connected to a nitrogen supply. Aliquots (10 ml) of the extract were applied as 8 mm bands, 5 mm from the lower edge. Thereafter, each plate was transferred to the ADC2 development chamber, consisting of a glass twin-trough chamber (20 cm × 10 cm) with metal lids. Plates were developed with methanol:water (60:40, v/v) to a migration distance of 70 mm in the pre-saturated chamber. The developed plates were dried using the CAMAG TLC plate heater III. Images were subsequently captured, under white and ultraviolet light (254 and 365 nm), with the Reprostar 3 documentation device and stored using winCATS version 1.4.4.6337 planar chromatography manager software.

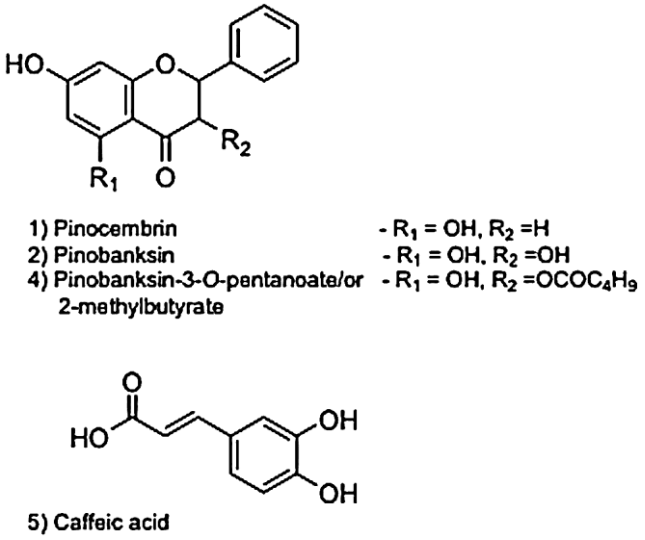

Fig. 4. Chemical structures of antimicrobial and anti-quorum sensing compounds identified from South African propolis.

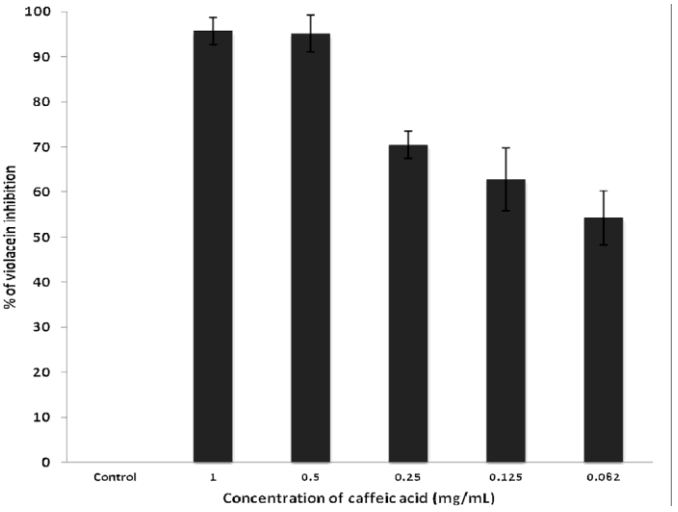

Fig. 5. Percentage violacein inhibition as determined for different concentrations of standard caffeic acid. Bar graphs represent the mean of three independent readings ± standard deviation.

3.4. HPTLC bioautography

The method reported by Valgas et al. (2007) with some modifications, was used for HPTLC bioautography. Briefly, the TLC plates prepared as described were placed face-up on a layer of TSA, set in sterile glass plates. An additional layer of TSA (incubated overnight), containing freshly grown bacterial or fungal cells at an inoculum size of 1×10^6 colony forming units (CFU)/mL, was spread thinly over the TLC plate. The plates were subsequently incubated at 37 8C for 24–48 h to allow microbial growth. After incubation, the bioautographs were sprayed with 0.4 mg/mL of p-iodonitrotetrazolium violet solution (INT). The plates were incubated for a further 4–6 h at room temperature, until a red colour developed to indicate microbial growth. Zones of inhibition were correlated with the spots visible on the reference HPTLC plate under UV/visible light.

To detect the anti-QS compounds on the TLC plate, a layer of LB agar containing C. violaceum was poured over the developed TLC plate. After overnight incubation at 30 8C, a purple coloured background, reflecting quorum-sensing by the bacteria, was observed on the plate. In contrast, the presence of pale turbid zones indicated the presence of compounds in the propolis extract responsible for anti-QS activity. The zones were measured and correlated with the spots observed on the reference HPTLC plate under UV/visible light. Images of all the bioautography plates were captured under white light.

3.5. HPTLC-MS analysis

A workflow diagram, representing the hyphenated HPTLC-MS analysis, is presented in Fig. 1. Utilising a CAMAG TLC-MS-Interface (oval elution head, 4 × 2 mm, Muttenz, Switzerland), spots corresponding to those with activity on the bioautography plates (Fig. 1A) were eluted from the untreated reference HPTLC plates (Fig. 1B). The HPTLC-MS interface (Fig. 1C) is fitted with a flowpump (Fig. 1D) of which the inlet is connected to an UPLC pump (Fig. 1E; Waters ACQUITY™ UPLC™ system; Waters Corp., Milford, MA, USA), while the outlet is attached to a Micromass-LCT Premier time-of-flight (ToF) mass spectrometer (Fig. 1F). Acetonitrile (HPLC grade, Romil Ltd, Cambridge, UK) was used as eluent at a flow rate of 0.1 mL/min to remove the target compounds from the silica gel plate (run time: 1 min). Following electrospray ionisation (ESI) of the eluate, mass spectra were acquired in the negative

mode. Conditions for the ESI source were: capillary voltage, 3.4 kV; sampling cone, 40 V; source temperature, 100 8C; and desolvation temperature, 500 8C. Nitrogen served as the desolvation gas at a flow rate of 600 L/h. MassLynx version 4.0 software (Waters) was used for instrumental control and data acquisition (Fig. 1G). Identification of compounds was carried out by comparing the most intense mass fragments (Fig. 1H) to literature data and with those of a caffeic acid standard (AR grade; Sigma Aldrich).

3.6. Anti-QS assay of caffeic acid

After identification of caffeic acid as a QS inhibitor, the anti-QS potential of the pure compound was quantitatively assessed by measuring its ability to inhibit violacein production as described previously by Chenia (2013). Caffeic acid was added in triplicate to 5.00 mL of LB-broth in test-tubes to provide final caffeic acid concentrations of 1.00; 0.500; 0.250; 0.125 and 0.062 mg/mL, respectively. C. violaceum culture (5×10^6 CFU/mL) was added to each tube (100 mL), where after they were placed in a shaker incubator at 150 rpm and 30 8C for 24 h. After incubation, 1.00 mL aliquots were transferred to eppendorf tubes and centrifuged at 8000 rpm for 10 min, to precipitate the insoluble violacein. The supernatant was discarded and the pellet was resuspended in 1.00 mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich). To remove the bacterial cells, the tubes were again centrifuged under the same conditions. The supernatants were individually transferred to the wells of a 96-well Elisa plate. Absorbance measurements were taken at 585 nm using a UV-visible microplate reader (Biotek ELx800 UV-Vis Universal). The mean absorbance and standard deviations for each replicate set were determined. The percentage of violacein inhibition was calculated by using the formula:

$$\text{Percentage of violacein inhibition} = \left[\frac{\text{control OD585 nm} - \text{test OD585 nm}}{\text{control OD585 nm}}\right] \times 100$$

where, OD represents the optical density of the appropriate solution.

4. Conclusions

This study confirms that HPTLC-bioautography in tandem with MS is a powerful tool for the rapid identification of bioactive compounds from crude natural products. Our research group has previously reported that South African propolis samples are characteristically rich in phenolic acids and flavonols. The current study confirms the role of specific phenolic compounds, including pinocembrin and pinobanksin, in the antifungal and antibacterial properties of propolis from this region. Caffeic acid was the only anti-QS component detected in the sample. The overall findings of this study will help to establish methods based on HPTLC-bioautography-MS for the rapid identification of unknown bioactive compounds in propolis.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

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