Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antimicrobial activity and chemometric modelling of South African propolis

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Keywords
antimicrobial, bactericidal, chemometrics, flavonoid, South African propolis.

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2015/0250: received 11 February 2015, revised 18 June 2015 and accepted 1 July 2015

doi:10.1111/jam.12906

Abstract

Aims: This study reports on the inhibitory and bactericidal properties of 39 South African (SA) propolis samples and three propolis samples from Brazil.
Methods and Results: Ethanolic extracts of propolis (EEP) were prepared and their antimicrobial activities tested using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Some samples displayed substantial antimicrobial activity with MIC and MBC values as low as 6 μg ml^{-1} against *Staphylococcus aureus*. The correlation between liquid chromatography-mass spectrometry (LC-MS) chemical data and the antimicrobial activity of propolis extracts was investigated using multivariate data analysis tools. Orthogonal projections to latent structures (OPLS) models were created for the two Gram-positive bacteria (*Enterococcus faecalis* and *S. aureus*) and *Candida albicans*. Using the S-plot function, it was possible to identify the bioactive constituents in propolis as chrysin, pinocembrin, galangin and pinobanksin-3-*O*-acetate.
Conclusion: The SA propolis samples tested displayed noteworthy antimicrobial activity, favourably comparable to that of the Brazilian comparator and 'gold standard'. The observed antimicrobial activity of SA propolis can possibly be attributed to its flavonoid content.
Significance and Impact of the Study: Based on the good antimicrobial activity observed for SA propolis, this natural resource shows promise and should be considered for development which may contribute to growing the bio-economy in the region.

Introduction

Propolis, a product of the beehive, is a sticky resin which is produced from exudates collected from the buds and flowers from the diverse flora in the vicinity of the beehive. These botanical exudates are masticated by the honeybees (*Apis mellifera*) mixed with salivary enzymes such as β-glycosidase and incorporated with bees wax to form propolis (Castaldo and Capasso 2002; Silva *et al.* 2012). The physical appearance of propolis varies from ochre, brown, green to red and is hard and brittle at temperatures below 0°C but becomes sticky and soft when warm (Lofty 2006; Maraschin *et al.* 2012). Propolis is used by bees to seal off holes in the hive. This offers protection from intruders and prevents putrefaction and infections from spreading throughout the colony (de Groot 2013; Kuropatnicki *et al.* 2013). The use of propolis, as a natural product for medicinal purposes dates as far back as 300 BC (Melliou and Chinou 2004; Ramos and Miranda 2007; El Ashry and Ahmad 2012). Egyptians used propolis for antiputrefactive properties to embalm the deceased (de Groot 2013) and propolis has been mentioned in ancient Egyptian papyri as a treatment for sores and ulcers (Salatino *et al.* 2011; El Ashry and Ahmad 2012). More than 90 years ago, propolis was mixed with petroleum jelly and used during the Anglo-Boer War (South Africa) for wound healing as well as tissue regeneration (Ghisalberti 1979; Ramos and Miranda 2007).

Between the 17th and 20th centuries, propolis became increasingly popular in Europe due to its reported antibacterial activities, leading to its incorporation as an official drug in the British pharmacopoeias of the 17th century (Castaldo and Capasso 2002; de Groot 2013; Wagh 2013).

In modern times, herbalists have recommended propolis for its antibacterial, antiviral and anti-inflammatory properties, all of which, may increase the body's natural defence to infections, as well as for the treatment of duodenal and gastric ulcers (Borrelli *et al.* 2002; Kuropatnicki *et al.* 2013). Propolis has been studied for its antitumor, anti-inflammatory, antimicrobial, antiviral, antiparasitic, antioxidant, anticarcinogenic and antidiabetic properties, hepatoprotective, as well as for cholesterol lowering effects (Banskota *et al.* 2001, 2002; Ramos and Miranda 2007; Sforcin 2007; Viuda-Martos *et al.* 2008; Abu-Mellal *et al.* 2012; El Ashry and Ahmad 2012; da Silva Frozza *et al.* 2013; Siripatrawan *et al.* 2013; Wagh 2013). These properties are now being investigated in greater detail with various studies reporting on the broad-spectrum antimicrobial activity of propolis (Garedew *et al.* 2004; Boyanova *et al.* 2006; Kalogeropoulos *et al.* 2009; Mavri *et al.* 2012; Silva *et al.* 2012; Siripatrawan *et al.* 2013; Campos *et al.* 2014). Globally, research has been dedicated to studying the antimicrobial properties of propolis from various geographical and climatic regions including Colombia, Ethiopia, Italy, Russia, Bulgaria, Greece, Brazil, India, Slovenia, Portugal and Thailand (Garedew *et al.* 2004; Boyanova *et al.* 2006; Kalogeropoulos *et al.* 2009; Righi *et al.* 2011; Choudhari *et al.* 2012; Mavri *et al.* 2012; Silva *et al.* 2012; Siripatrawan *et al.* 2013) to name a few.

From a South African (SA) perspective, the antimicrobial investigation of propolis has been somewhat neglected. A study conducted by du Toit *et al.* (2009), briefly investigated the antimicrobial properties of SA propolis, however, the study focused more on the chemical and anti-inflammatory properties, rather than the antimicrobial properties. Kumazawa *et al.* (2004), studied the anti-oxidant and free-radical scavenging properties of SA propolis. Seidel *et al.* (2008) reported on the antimicrobial activity of propolis from different geographical and from distinct climatic zones, which included only one sample from SA. Considering that the antimicrobial efficacy of SA plants has demonstrated promising anti-infective properties (van Vuuren 2008), the probability that propolis derived from SA flora could potentially be an effective antimicrobial is high. Clearly, an in-depth investigation involving propolis from all geographical areas within SA warranted attention.

The chemical composition of propolis is extremely complex and more than 300 constituents have been identified to date (Bosio *et al.* 2000; Sawaya *et al.* 2004; Koru *et al.* 2007; Du Toit *et al.* 2009; Kalogeropoulos *et al.* 2009; Petrova *et al.* 2010; Dias *et al.* 2012; Mavri *et al.* 2012; Siripatrawan *et al.* 2013; Zhang *et al.* 2014). Amongst the constituents in propolis are wax, resins, balsams, essential oils, amino acids, sugars, flavonoids etc. (de Castro 2001; Mohammadzadeh *et al.* 2007; Mavri *et al.* 2012). Brazilian propolis is a highly valued natural product and is of tremendous commercial importance due to its wide range of health benefits (Salatino *et al.* 2011). The green and brown Brazilian propolis are the most common types. Green propolis is rich in prenylated phenyl propanoids, triterpenoids, chlorogenic and benzoic acids, whilst the brown propolis contains mainly flavonoids and terpenes (Sawaya *et al.* 2006; Righi *et al.* 2011). The standardization of propolis has proven to be a challenge due to propolis being chemically complex and diverse (Bankova 2005). Propolis has been reported to be rich in flavonoids, in particular, pinocembrin, galangin and chrysin (Markham *et al.* 1996; Melliou and Chinou 2004; Valencia *et al.* 2012). Propolis from SA has been documented to contain different types of phenolic compounds and may possess some similarities with the poplar propolis found in temperate regions (Kumazawa *et al.* 2004). Limited research has been conducted on the chemical composition of SA propolis. Du Toit *et al.* (2009) reported on the flavonoids as well as a non-flavonoid caffeic acid phenethyl ester (CAPE) present in SA propolis. Zhang *et al.* (2014) reported on the chemical profiles of only five samples obtained from the Kwa-Zulu-Natal province of SA. The study concluded that three samples showed chemical profiles similar to temperate region poplar propolis whilst the other two samples were found to be rich in diterpenoid acids, characteristic of propolis originating from the eastern Mediterranean regions. The objective of this study was to document the antimicrobial properties of SA propolis and provide some correlation with specific chemical constituents in propolis extracts profiled using liquid chromatography-mass spectrometry (LC-MS) that had been identified in our previous study (Kasote *et al.* 2014a,b).

Materials and methods

Sample preparation

Propolis samples ($n = 42$) were sourced in collaboration with the South African Bee Industry Organisation (SABIO). Of these samples, 39 were from SA and three from Brazil. Brazilian samples were included for comparative purposes as it is generally considered to be superior due to the junction of two factors; genetics of Brazilian bees with the diversity of the regional flora. Samples were

kept in a freezer at −4°C to ensure ease of working due to the nature of propolis becoming sticky and difficult to work with when warm. A weighed quantity of macerated propolis was submerged in absolute ethanol. For every 3 g of crude propolis, 10 ml of absolute ethanol was used for extraction (Sawaya *et al.* 2004). Extraction took place in an orbital shaker incubator at 37°C for 7 days. Thereafter, ethanol was syphoned off and the extract was allowed to dry at ambient temperature (Bosio *et al.* 2000; Sawaya *et al.* 2004, 2010 and Scazzocchio *et al.* 2006). The resultant ethanolic extract of propolis (EEP) was dissolved in acetone to a starting concentration of 25 mg ml^{-1}.

Preparation of cultures

Two Gram-positive bacteria; *S. aureus* (American Type Culture Collection (ATCC) 25923) and *Enterococcus faecalis* (ATCC 29212), two Gram-negative bacteria; *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), and two yeasts; *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 14116) were used as test pathogens. All micro-organisms were cultured in Tryptone Soya broth (TSB) (Oxoid, UK) and kept viable by sub-culturing. Streak plates were prepared to ensure purity of the cultures. Bacteria were incubated at 37°C for 24 h and yeasts were incubated at 37°C for 48 h. A waiver for the use of these micro-organisms was granted by the University of the Witwatersrand Human Research Ethics Committee (Reference W-CJ-131026-1).

Minimum inhibitory concentration (MIC) assay

MIC assays were performed according to the National Committee for Clinical Laboratory Standards (2009). This assay was used to determine the antimicrobial activity of the propolis samples. A volume of 100 μl sterilized distilled water was placed into each well of the micro-titre plate. A volume of 100 μl of each sample was introduced into the first column of the micro-titre plate and the serial doubling dilution technique was employed. Negative controls of acetone only (to ensure that the solvent itself was not exerting the observed antimicrobial effect) were prepared to 25 mg ml^{-1}. Positive controls (conventional antimicrobials) of ciprofloxacin (0·01 mg ml^{-1}) or amphotericin B (0·1 mg ml^{-1}) were used to confirm microbial susceptibility. A culture control of bacteria and yeast, respectively, was included to ensure that the broth was capable of supporting microbial growth. All assays were undertaken in duplicate or in triplicate. The wells of the micro-titre plates were inoculated with an approximate inoculum of 1×10^6 colony forming units (CFU) ml^{-1} of the micro-organism to be tested, previously prepared as a 0·5 McFarland's standard. Plates were sealed and incubated at 37°C for 24 h for bacterial species and at 37°C for 48 h for the yeasts. After incubation, 40 μl (0·4 mg ml^{-1}) of the indicator *p*-Iodonitrotetrazolium violet solution (INT) was added to the inoculated wells. This indicator turns pink in the presence of microbial growth. Once an observable colour change was noticed within the culture control column, the plate was analysed and the MIC values recorded appropriately as the lowest concentration of propolis that inhibited the growth of the test micro-organism (van Vuuren *et al.* 2010).

Minimum bactericidal concentration (MBC) assay

The MBC is the lowest concentration of propolis that kills off a micro-organism after subculturing onto an agar plate (Andrews 2001). Once MIC assays were recorded, an MBC assay was undertaken by streaking (culture/sample mix) out of wells where inhibition was apparent, onto a sub-divided Tryptone Soya agar plate (TSA) (Oxoid). The plates were incubated at 37°C for 24 and 48 h for bacteria and yeasts respectively. Results were recorded as the lowest concentrations of propolis where no growth of the micro-organism was observed.

Chemometric data analysis to correlate LC-MS profiles with activity

In an attempt to correlate antimicrobial activity to specific chemical constituents in propolis extracts, a metabolomics approach was applied. Using Simca-P + 13·0 (Umetrics, Umeå, Sweden) CHEMOMETRICS software, multivariate data analysis tools were applied on a data set consisting of both LC-MS profiles of 42 propolis extracts (previously compiled, Kasote *et al.* 2014a,b) and the corresponding MIC values. Sample preparation and LC-MS analysing parameters were referred from Kasote *et al.* (2014a,b). The propolis extracts were assigned to classes based on the level of antimicrobial activity observed. For the purpose of biomarker identification, extracts with MICs $\leq$ 500 μg ml^{-1} were considered to have good activity and were assigned to class 1. Conversely, extracts with MICs $\geq$ 500 μg ml^{-1} were considered to be less active and assigned to class 2. A dummy *Y*-variable was assigned to this classification to allow the orthogonal projections to latent structures (OPLS) model to be constructed. Initially, principal component analysis (PCA) was performed on the X-data (LC-MS) to observe chemical variation within propolis extracts. The X-data were pareto scaled to reduce the relative importance of larger values by decreasing large-fold changes more than the smaller changes while maintaining the data structure partially

intact (Eriksson *et al.* 1999). An OPLS model was constructed following PCA. This is a supervised classification algorithm that investigates chemical variations (predictive) within the X-matrix (LC-MS data) that are correlated to the predefined classes (dummy *Y*-variable). Additionally, the algorithm further identifies variation that is uncorrelated (orthogonal) to the *Y*-variable. Score scatter plots were used to evaluate the (dis)similarities among the propolis extracts by observing clustering patterns. An S-plot was used to identify putative biomarkers (retention time-mass pairs) that are associated with the active and less-active samples. The identification of the corresponding compounds was performed using MS fragment comparisons, library database searches and literature review.

Results

The percentage yield, average MIC and MBC results of the antimicrobial studies undertaken on the individual propolis samples as well as the average activity are reported in Table 1. Most samples with higher yields were noted to display better antimicrobial activity with the exception of an outlier (Western Cape 2), which had a low yield of 7·27% but displayed strong antimicrobial activity against *Staph. aureus* and *C. neoformans*. The inhibitory activity of EEP against all pathogens tested ranged from 6 to 1563 μg ml^{-1}. Propolis extracts where MIC values are 125–500 μg ml^{-1} are considered as having moderate activity and propolis extracts where MIC values are >500 μg ml^{-1} are considered as having weak activity (Alencar *et al.* 2007; Velazquez *et al.* 2007; Seidel *et al.* 2008). According to this criteria defined herewith, 28 propolis extracts displayed noteworthy activity (MIC $\leq$ 125 μg ml^{-1}) against *Staph. aureus*. Three propolis extracts displayed noteworthy activity (MIC $\leq$ 125 μg ml^{-1}) against *Ent. faecalis*. Twenty-nine samples displayed noteworthy activity when tested against *C. neoformans*. Propolis originating from Honeydew, Edenvale, and the Southern suburbs of Cape Town, Gauteng, and Western Cape regions of SA displayed noteworthy inhibition against *Staph. aureus* with average MICs as low as 6 μg ml^{-1}. More than half of SA propolis samples, when tested against Gram-positive *Staph. aureus,* displayed average MIC values lower than the Brazilian propolis extracts. Of the SA samples tested, 28% showed better inhibition than the Brazilian comparator when tested against the Gram-negative *Ps. aeruginosa* strain. Furthermore, when tested against the yeast species *C. neoformans,* 74% of the SA propolis samples displayed greater inhibition with average MIC values (12 μg ml^{-1}) lower than that of the Brazilian samples. The average antimicrobial activity ranged from 155 to 1496 μg ml^{-1} (Table 1). To compare against all three Brazilian samples, the average activity of all three samples was determined. Of the 39 SA propolis samples tested, it was found that 56% displayed better antimicrobial activity (i.e. against all six pathogens tested) than the three Brazilian comparators.

The MBC assays undertaken demonstrated that propolis possessed noteworthy ($\leq$125 μg ml^{-1}) cidal activity against some pathogens (Table 1). Propolis samples obtained from Honeydew (Gauteng), Edenvale (Gauteng) and the Southern suburbs of Cape Town (Western Cape), displayed MBCs as low as 6 μg ml^{-1} against *Staph. aureus*. The correlation of inhibitory activity to cidal activity against *Staph. aureus* was 67%, *Ent. faecalis* 41%, *C. albicans* 41% and *C. neoformans* 67%.

The correlation between chemical data and antimicrobial activity was investigated using multivariate data analysis tools. Three OPLS models were created for the two Gram-positive bacteria (*Ent. faecalis* and *Staph. aureus*) and *C. albicans* where higher antimicrobial activities were observed. For illustration purposes, a detailed explanation of the model developed for *Ent. faecalis* is provided while a summary of the model statistics and the biomarkers identified for all three micro-organisms is provided in Table 2. Figure 1 shows the OPLS scores scatter plot of propolis extracts discrimanting between the active class (red) and poorly active class (blue) along the predictive component (tp1) with a 10% chemical variation (R^2Xp = 0·10) being attributed to this classification. Pareto scaling provided maximum, clean separation of the data compared to other scaling methods. The separation demonstrates that almost 50% of extracts are highly active while the other 50% are poorly active against *Ent. faecalis*. Although a general conclusion could not be drawn on the influence of geographical locality to antimicrobial activity, a few scenarios dominated from the plot where all the propolis from Brazil and Lydiana (Pretoria) demonstrated low activity while propolis from Cape Town, Edenvale and Western Cape showed consistently high activity against *Ent. faecalis*. To investigate the bioactive markers within the propolis extracts, an S-plot of covariance and correlation was constructed for the *X*-variables (Fig. 2). The points in the S-plot represent the LC-MS retention times (min) while the corresponding mass_scan pairs were identified as secondary observations in the data set. The extreme ends of the S- plot show variables of high magnitude and high reliability in the differentiation of the two propolis classes. The highlighted variables in the left bottom quadrant (red) are the biomarkers that are correlated to high antimicrobial activity of the propolis extracts while the top right (blue) show low activity. Using the filtered retention times (min) in combination with the mass_scan number

Table 1 Average MIC and MBC values (μg ml^{-1}) of South African propolis against six pathogens

Source of sample	% Yield	*S. aureus* (ATCC 25923)		*E. faecalis* (ATCC 29212)		*E. coli* (ATCC 25922)		*P. aeruginosa* (ATCC 27853)		*C. albicans* (ATCC 10231)		*C. neoformans** (ATCC 14116)		Average activity*
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	
Eastern Cape province														
Baviaanskloof-PE	16·72	1563	1563	1563	1563	781	1563	391	>6250	781	781	391	391	912
Free State province														
Bloemfontein	55·67	49	49	391	521	1563	>6250	195	6250	391	521	98	98	448
Gauteng province														
Beaulieu-Midrand	33·31	24	24	195	195	1563	>6250	391	>6250	195	293	49	49	403
Devon-Sedibeng area	35·08	49	49	391	391	781	>6250	391	3125	195	293	98	98	318
Edenvale 1	56·45	24	24	391	586	781	>6250	195	>6250	391	586	98	98	313
Edenvale 2	71·25	6	9	195	488	781	>6250	195	6250	391	391	49	49	270
Honeydew	51·08	6	6	49	49	781	2604	391	1563	195	456	12	49	239
Johannesburg 1	59·50	49	49	195	586	781	>6250	391	3125	391	391	49	65	309
Johannesburg 2	49·44	195	456	1563	6250	781	>6250	391	>6250	781	781	24	49	623
Johannesburg 3	58·44	24	37	391	2344	1563	>6250	195	>6250	781	781	49	65	501
Lakeside/Westlake	27·05	98	98	781	2604	1563	>6250	195	>6250	391	521	49	49	513
Lydiana Gardens 1-Pretoria	33·18	98	98	1563	1563	781	1563	391	>6250	781	1953	195	195	635
Lydiana Gardens 2-Pretoria	30·35	24	37	781	1172	1563	>6250	391	6250	781	1953	49	49	598
Northern Pretoria	15·75	391	391	781	1172	1563	>6250	391	>6250	781	781	98	260	668
President Park-Midrand	49·67	49	49	391	391	781	3125	391	1563	195	391	24	24	305
Pretoria 1	37·12	24	37	781	781	781	>6250	391	>6250	781	781	195	195	492
Pretoria 2	46·85	24	24	781	781	781	>6250	391	3125	391	391	98	98	411
Springs	37·00	24	41	195	195	1563	>6250	391	1563	195	260	49	65	403
Walkerville (Vereeniging)	40·87	98	98	195	195	781	>6250	391	1563	195	391	98	98	293
Wilgerivier-Bronkhorstspruit	27·01	49	98	391	391	781	>6250	391	>6250	391	586	98	98	350
Kwa-Zulu Natal province														
Kwa-Zulu Natal 1	28·16	195	195	781	781	781	1563	391	3125	391	586	195	195	456
Northern Cape province														
Douglas	39·17	49	65	391	2344	1563	>6250	195	6250	391	521	98	98	448
Northern Cape 1	37·00	195	586	781	3646	781	>6250	391	>6250	391	651	98	130	440
Northern Cape 2	19·64	24	24	195	195	781	1563	195	3125	195	293	24	24	236
Orange River	16·76	1563	1563	1563	2344	1563	>6250	391	>6250	391	521	98	130	928
North–West province														
Christiana 1	35·01	49	74	98	147	781	>6250	391	>6250	195	260	98	98	269
Christiana 2	32·56	49	49	195	195	781	>6250	195	>6250	195	260	98	98	252
Mooinooi	41·67	1563	3125	1563	6250	1563	>6250	781	3125	3125	3125	391	391	1496
North West	15·87	391	391	1563	1563	1563	>6250	391	>6250	781	781	195	195	814
Western Cape province														
Beaufort West	17·73	1563	>6250	1563	1563	781	>6250	781	>6250	1563	1172	195	391	1074
Botrivier	51·58	195	195	781	2084	1563	>6250	391	>6250	781	1042	98	195	635
Graafwater	28·86	391	651	1563	3907	1563	>6250	781	>6250	781	781	195	260	879
Outeniqua Mountains, Oudtshoorn	45·12	12	12	195	293	1563	6250	391	>6250	781	781	98	130	507
Somerset West	39·38	391	521	1563	6250	1563	>6250	391	>6250	391	781	195	195	749
Southern Suburbs-Cape Town 1	30·41	6	6	98	98	781	1563	195	2604	195	488	24	24	217
Southern Suburbs-Cape Town 2	48·05	24	24	195	195	391	1563	195	>6250	98	147	24	24	155
Southern Suburbs-Cape Town 3	42·78	24	49	195	195	1563	>6250	391	>6250	98	147	49	49	387
Western Cape 1	74·43	195	228	781	3125	781	>6250	195	>6250	781	781	195	130	488
Western Cape 2	7·27	24	24	195	195	781	3125	195	3125	195	195	49	49	240
South America														
Brazil 1	28·24	98	195	781	1953	781	>6250	391	3125	781	781	195	195	505
Brazil 2	40·39	98	147	781	781	781	>6250	391	>6250	195	651	195	195	407
Brazil 3	46·50	195	260	781	781	781	>6250	391	>6250	781	781	24	41	492

(*Continued*)

Table 1 (*Continued*)

Source of sample	% Yield	*S. aureus* (ATCC 25923) MIC	MBC	*E. faecalis* (ATCC 29212) MIC	MBC	*E. coli* (ATCC 25922) MIC	MBC	*P. aeruginosa* (ATCC 27853) MIC	MBC	*C. albicans* (ATCC 10231) MIC	MBC	*C. neoformans** (ATCC 14116) MIC	MBC	Average activity*
Positive control†		0·313		0·625		0·02		0·313		6·25		0·78		
Negative control		>6250		>6250		>6250		>6250		>6250		>6250		
Culture control		>6250		>6250		>6250		>6250		>6250		>6250		

*Average across all six pathogens tested excluding values where no end point was attained.
†Ciprofloxacin for bacteria and Amphotericin B for yeasts.

Table 2 Model statistics and identified biomarkers contributing to good activity of propolis extracts for selected micro-organisms

Organism	Model statistics	Retention time (min) (Biomarkers)	Mass	Identity
Enterococcus faecalis	A = 1 + 4; R^2Xp = 0·10; R^2X_{cum} = 0·58; Q^2_{cum} = 0·48	5·8661	255·066	Chrysin
		6·2039	257·082	Pinocembrin
		6·3759	271·061	Galangin
Staphylococcus aureus	A = 1 + 2; R^2Xp = 0·07; R^2X_{cum} = 0·58; Q^2_{cum} = 0·29	6·2039	257·082	Pinocembrin
		6·4064	301·072	Galangin
		6·6038	227·072	Pinobanksin-3-*O*-acetate
Candida albicans	A = 1 + 1; R^2Xp = 0·20; R^2X_{cum} = 0·37; Q^2_{cum} = 0·42	5·8661	255·066	Chrysin
		6·2065	257·082	Pinocembrin
		6·3759	271·061	Galangin

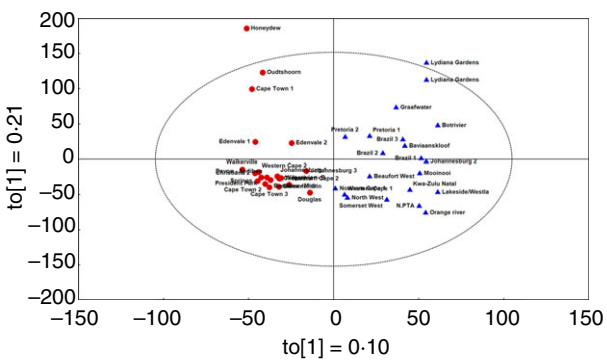

Figure 1 OPLS scores plot showing separation of active propolis (●) and poorly active propolis (▲) extracts against *Enterococcus faecalis*.

combinations, it was possible to identify the active constituents in propolis for the three models (Table 2). Table 2 lists the active constituents in propolis to be chrysin, pinocembrin, galangin and pinobanksin-3-*O*-acetate. Figure 3 is the Ultra Performance Liquid Chromatography (UPLC-ESI^+-MS) chromatograph of the methanol propolis extract showing the identified biomarkers and the corresponding structures. Three of the active compounds have been identified as major compounds in propolis extracts so it is plausible to predict that extracts containing higher levels chrysin, pinocembrin, galangin are more likely to possess higher antimicrobial activity compared to the others.

Discussion

Propolis from regions in Argentina have been reported to exhibit better Gram-positive than Gram-negative activity with MICs as low as 15·30 $\mu g\ ml^{-1}$ against *Staph. aureus* (Nieva Moreno *et al.* 1999). The antimicrobial activity of propolis from the regions of Anatolia, Turkey, reported low MICs against *Staph. aureus* (8–16 $\mu g\ ml^{-1}$), *Ps. aeruginosa* (32–256 $\mu g\ ml^{-1}$), *E. coli* (16–128 $\mu g\ ml^{-1)}$ and *C. albicans* (4–32 $\mu g\ ml^{-1}$) (Uzel *et al.* 2005). In another study conducted on Brazilian red propolis from Maceió and the state of Algoas, the growth of *Staph. aureus* was reported to be inhibited at very low concentrations (exact concentration is not reported). MICs of 256 $\mu g\ ml^{-1}$ were reported against *Ps. aeruginosa* and *C. albicans* and 512 $\mu g\ ml^{-1}$against *Ent. faecalis* and *E. coli* (Righi *et al.* 2011). Propolis from Oman was found to display MICs ranging from 42 to 169 $\mu g\ ml^{-1}$ against *Staph. aureus* and 169–356 $\mu g\ ml^{-1}$ for *E. coli* (Popova *et al.* 2013).

In alignment with previous studies on the antimicrobial properties of propolis (Nieva Moreno *et al.* 1999; Uzel *et al.* 2005; Righi *et al.* 2011; Popova *et al.* 2013), this study demonstrated that SA propolis displays greater inhibitory efficacy. Predominant cidal activity against Gram-positive bacteria and yeasts were observed. Three SA propolis samples displayed noteworthy activities as low as MICs of 6 μg ml^{-1} (Table 1). It was also noted that the majority of these whole extracts, when tested against Gram-positive *Staph. aureus,* and the yeast species *C. neoformans*, demonstrated activities comparable to antimicrobial activities displayed by isolated bioactive compounds reported previously in literature (van Vuuren 2008), thus suggesting a natural product with superior activity.

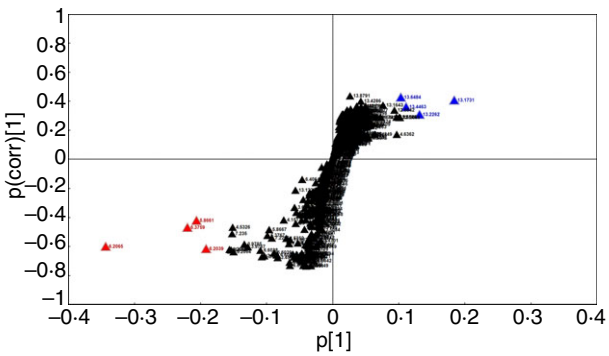

Figure 2 An S-plot showing putative biomakers that have been identified as contributing significantly to good activity (▲ left bottom quadrant) and poor activity (▲ top right quadrant) against *Enterococcus faecalis*.

The chemical composition of propolis is largely dependent on the climate, bee species and regional flora (Markham *et al.* 1996; Righi *et al.* 2011). In Europe and in the more temperate zones, propolis is found to contain more flavonoids and phenolic acid esters as opposed to propolis found in Cuba and Venezuela. The main constituents found in Cuban red propolis are benzophenones. Furthermore, red Mexican propolis is found to contain a vast amount of flavones, isoflavans and pterocarpans (Hernandez *et al.* 2005; Lotti *et al.* 2010; Righi *et al.* 2011). Our earlier study (Kasote *et al.* 2014a,b) was the first comprehensive report to be published on the chemical profiling of propolis samples from various regions in SA, which included an extensive sample bank consisting of propolis samples from the various provinces of SA. Results of this study supported the findings of earlier studies confirming that SA propolis is rich in flavonoids with the main constituents being, pinocembrin, galangin, chrysin, myricetin and pinobanksin and is chemically distinct from Brazilian propolis which was found to contain high concentrations of only one flavonoid, artepillin C.

The presence of flavonoids and derivatives of caffeic acid are known to be associated with the bactericidal activities of propolis (Bosio *et al.* 2000). The antimicrobial properties of propolis are also known to be attributed to high flavonoid content, especially galangin, chrysin and pinocembrin (Bosio *et al.* 2000; Hegazi and Abd El Hady 2001; Cushnie and Lamb 2005). Due to the high flavonoid content, it is noted to have good activity against dermatophytes, and *Candida* spp. (Cafarchia *et al.* 1999; Cushnie and Lamb 2005). Chrysin, a flavo-

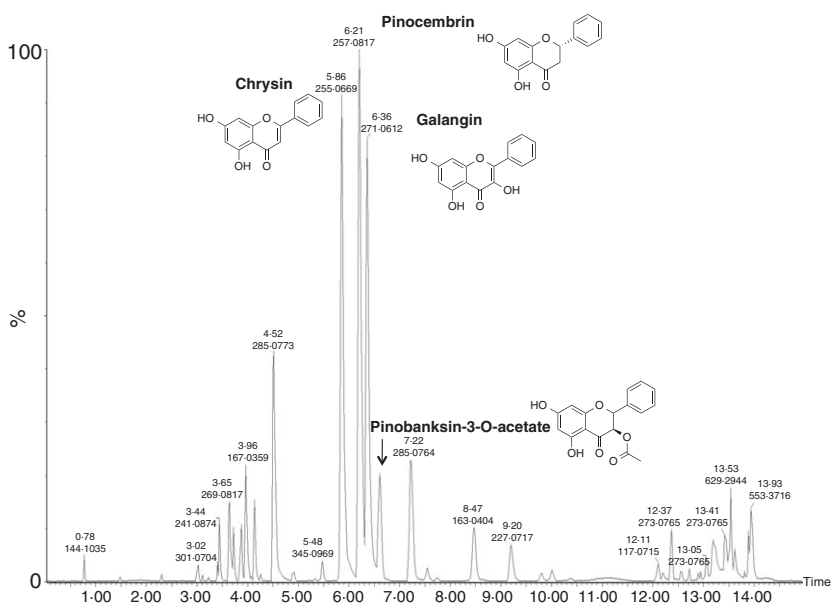

Figure 3 UPLC-ESI+-MS chromatograph of propolis methanol extract showing the biomarkers contributing significantly to the antimicrobial activity as identified in the S-plot.

noid also found in propolis has been proven to inhibit the viral replication of the herpes simplex virus (HSV) and rotavirus and has been reported to have good antibacterial activity (Melliou and Chinou 2004; Cushnie and Lamb 2005). Galangin has also been found to display good antimicrobial activity against *Aspergillus* and *Penicillium* spp. as well as antiviral activity against HSV (Cushnie and Lamb 2005). In a recent study, pinocembrin was found to be the compound responsible for observed antifungal activity of propolis, whilst pinobanksin was reported to be responsible for observed antibacterial activity (Kasote *et al.* 2014a,b). In our previous study, the chemical profiling techniques demonstrated samples that were chemically distinct from the Brazilian comparator samples (Kasote *et al.* 2014a,b). The LC-MS analysis confirmed that pinocembrin, chrysin, galangin and pinobanksin are the major constituents of SA propolis, whilst the major constituent of the Brazilian propolis samples was Artepillin C. Pinocembrin and chrysin are flavanones, while galangin and pinobanksin-3-*O*-acetate are flavonols. Flavonones and flavonols are types of flavonoids found in propolis and other natural products such as honey and medicinal plants (Melliou and Chinou 2004). Melliou and Chinou (2004) reported on the antimicrobial activity of flavonoids found in propolis, the study reported that pinocembrin and chrysin displayed respective MIC values of 0·25 mg ml^{-1} (250 μg ml^{-1}) and 3·4 mg ml^{-1} (3400 μg ml^{-1}) against *Staph. aureus*, and 0·10 mg ml^{-1} (100 μg ml^{-1}) and 0·05 mg ml^{-1} (500 μg ml^{-1}) against *C. albicans*. The activity of 23 EEP samples tested in this study against *Staph. aureus* (6–49 μg ml^{-1}) and 15 EEP samples tested against *C. neoformans* (12–49 μg ml^{-1}) displayed antimicrobial activities lower than the activities displayed by the single bioactive compound pinocembrin reported by Melliou and Chinou (2004). Interactive efficacy studies of these compounds in combination are recommended for future studies.

In conclusion, this study demonstrated that a wide range of SA propolis displayed noteworthy bactericidal activity. *Staphylococcus aureus* was found to be inhibited and killed by very low concentrations of propolis (6 μg ml^{-1}), thus possibly making SA propolis a valuable future alternative in anti-infective therapy.

Acknowledgements

We express our greatest appreciation to the bee keepers and the South African Bee Industry Organisation (SABIO) for their contribution of propolis samples. National Research Foundation (South Africa) and the Faculty Research Committee (WITS) are acknowledged for funding. We thank Dr Weiyang Chen for performing the LC-MS analyses.

Conflict of Interest

No conflict of interest declared.

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