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The antimicrobial and chemical properties of South African propolis

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302955



A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy

Johannesburg, 2015

Declaration

I, Tasneem Suleman, declare that this dissertation is my own work. It is being submitted in fulfilment of my degree of Master of Pharmacy at the University of the Witwatersrand, Johannesburg. I declare that it has not been submitted previously for any degree or examination at this university or any other.

_____

Tasneem Suleman

_____

Date

Dedication

I dedicate this dissertation to my family and friends, in particular my loving parents and in-laws, siblings and my adoring husband, Mohamed Moolla. Thank you all for your constant words of encouragement and utmost faith in me.

I could not have achieved all that I have without all of you.

Publications and conference presentations

Publications arising from this study

- D. Kasote, **T. Suleman**, W. Chen, M. Sandasi, A. M. Viljoen, S. van Vuuren. Chemical profiling and chemometric analysis of South African propolis. *Biochemical Systematics and Ecology*. 2014, 55, 156-163 (Refer to Appendix A for abstract).
- **T. Suleman**, S. van Vuuren, M. Sandasi and A. M. Viljoen. Antimicrobial activity and chemometric modelling of South African propolis. *Journal of Applied Microbiology*. 2015, 119, 981-990 (Refer to Appendix B for abstract).

Conferences

Oral presentations

- **T. Suleman,** D. M. Kasote, M. Sandasi, S. van Vuuren and A. M. Viljoen. Investigating the antimicrobial and chemical properties of South African propolis. Department of Botany and Plant Biotechnology Postgraduate Inter-university Symposium, University of Johannesburg, South Africa. October 29, 2013 (Refer to Appendix C for abstract).
- **T. Suleman,** D. M. Kasote, M. Sandasi, S. van Vuuren and A. M. Viljoen. South African propolis: Antimicrobial activity, chemical properties and interactive efficacy. Wits Research Day, University of the Witwatersrand, Gauteng, 17 September, 2014. [Podium presentation] (Refer to Appendix D for abstract).

Poster presentations

- D. Kasote, A. Viljoen, W. Chen, **T. Suliman**, S. van Vuuren – Chemical profiling of South African propolis. The 34th Annual Conference of the Academy of Pharmaceutical Sciences of South Africa- Cape Town, 4-6 October, 2013 [poster presentation] (Refer to Appendix E for poster).
- D. Kasote, **T. Suleman**, W. Chen, M. Sandasi, A. Viljoen and S. van Vuuren - HPTLC, UPLC-TOF-MS profiling and chemometric analysis of South African propolis. American Society of Pharmacognosy Annual meeting Oxford, Mississippi, USA. 2-6 August 2014 [poster presentation] (Refer to Appendix F for poster).

Abstract

Propolis is a sticky resin produced worldwide by honeybees (*Apis mellifera*). It is used to seal off holes in the hive from intruders, prevent putrefaction and thus prevent infections of the colony. The use of propolis as a natural or traditional product which dates as far back as 300 B.C. Globally, research has been extensively dedicated to studying the antimicrobial properties of propolis from various geographical and climatic regions. Brazilian propolis has, however, become a subject of increasing interest due to its characteristic favourable biological activities and is thus considered the "gold standard" of all propolis. This has resulted in the increased global demand for propolis. Despite this global outlook, research on the antimicrobial and chemical properties of propolis specifically from South Africa (SA) has been sorely neglected.

The aim of this study was to evaluate the antimicrobial activity of 39 SA ethanolic extracts of propolis (EEP) and three Brazilian EEP's(used as controls). The antimicrobial activities of EEP samples were evaluated using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays against two yeasts, two Gram-positive and two Gram-negative bacteria. Interactive efficacies of the ten most active propolis samples, combined with conventional antimicrobials and honey, were evaluated using the fractional inhibitory concentration (ΣFIC) assessment. The chemical profile and composition of propolis was determined using high performance thin layer chromatography (HPTLC) and ultra-performance liquid chromatography coupled to photodiode array detector-quadrupole/time-of-flight mass spectrometry (UPLC-PDA-qTOF-MS/MS) analysis.

All strains of bacteria and yeasts tested showed susceptibility to the 39South African EEP samples. Some noteworthy activities were observed with some samples (GP9, GP11 and WC8) displaying an MIC and MBC value as low as 6 µg/ml against *Staphylococcus aureus*. In this study it was found that the majority (56%) of South African EEP samples displayed average antimicrobial activity better than that of the Brazilian control samples; 71% of samples displayed noteworthy activity against *S. aureus* and 79% against *Cryptococcus neoformans*. Notable interactions were identified, such as the combination of EEP's with gentamicin where synergistic profiles were most often observed against *Pseudomonas aeruginosa* with ΣFIC ranging from 0.19 to 0.37. The Brazilian EEP sample was the only

sample found to display antagonism when combined with the antifungals; amphotericin B and nystatin.

Chemical analysis led to the identification six compounds namely; quercetin, galangin-5-methyl ether, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-butyrate or isobutyrate, pinobankin-3-*O*-pentanoate or 2-methylbutyrate and pinobanksin-3-*O*-hexanoatewhich were identified for the first time in SA propolis in this study. Chemometric analysis of LC-MS data revealed two distinct clusters and confirmed that the South African propolis is chemically distinct from Brazilian propolis. Furthermore, chemometric analysis was used to compare chemical data to antimicrobial activity. 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.

South African propolis displayed noteworthy antimicrobial activity, favourably comparable to that of the Brazilian control and global "gold standard". Interactive efficacy studies demonstrated notable synergistic profiles when combined with ciprofloxacin and gentamicin against Gram-negative bacteria. This could possibly have an impact on the future use of conventional antimicrobials with alternative therapies including propolis. Furthermore, SA propolis displayed not only superior activity in comparison to the Brazilian propolis but also exhibited superior antimicrobial activity in comparison to other extensively studied propolis from South America, Europe and Asia.

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List of acronyms and abbreviations

ADC- Automatic developing chamber

ATCC- American type culture collection

BR- Brazil

°C- Degrees Celsius

CAE- Chlorogenic acid equivalents

CAPE- Caffeic acid phenethyl ester

CFU- Colony forming units

EC- Eastern Cape province

EEP- Ethanolic extract of propolis

ESI- Electrospray ionisation

FIC- Fractional inhibitory concentration

FS- Free State province

FTIR- Fourier transform infrared spectroscopy

GC- Gas chromatography

GP- Gauteng province

HPLC- High performance liquid chromatography

HPTLC- High-performance thin layer chromatography

HSV- Herpes simplex virus

INT-*p*-Iodonitrotetrazolium chloride violet

KZN-KwaZulu-Natal province

LC-MS- Liquid chromatography in tandem with mass-spectrometry

LP- Limpopo province

m/z- Mass to charge ratio

MBC- Minimum bactericidal concentration

mg-Milligrams

mg/ml-Milligrams per millilitre

MIC- Minimum inhibitory concentration

min- Minutes

ml-Millilitres

mm-Millimetre

MRSA- Methicillin resistant *Staphylococcus aureus*

MS- Mass spectrometry

NC- Northern Cape province

NCCLS- National committee for clinical standards

NW- North-West province

OPLS- Orthogonal projections to latent structures

OPLS-DA- Orthogonal projections to latent structures-discriminant analysis

PCA- Principal component analysis

Rt-Retention time

SA- South Africa

SABIO- South African bee industry organisation

TIC- Total ion chromatogram

TLC- Thin layer chromatography

TSA- Tryptone Soya agar

TSB- Tryptone Soya broth

µg/ml- Micrograms per millilitre

µl-Microlitre

UPLC-ESI-MS- Ultra-performance liquid chromatography electrospray ionisation mass-spectrometry

UPLC-PDA-qTOF-MS/MS- Ultra-performance liquid chromatography coupled to photodiode array detector-quadrupole/time of flight mass spectrometry

w/v- Weight per volume

WC- Western Cape province

ZOI- Zone of inhibition

Chapter 1: Introduction

1.1. What is propolis?

Propolis is a resin produced worldwide by honeybees (*Apis mellifera*)(Figure 1.1).It varies in texture from sticky or mouldable to hard or friable and differs in colour, from light yellow to green and dark brown(Figure 1.2), according to the botanical source from which it is derived (Salatino *et al*., 2011; Maraschin *et al*., 2012). Once resin is collected from the buds of the various flora, it is masticated and mixed with the salivary enzymes. Propolis is hard and brittle at temperatures below 0°C and becomes sticky and soft once warm (Lofty, 2006). The word propolis simply put means "the defence system of the city", derived from the Greek words "pro" meaning "in front of" and polis meaning "the city".

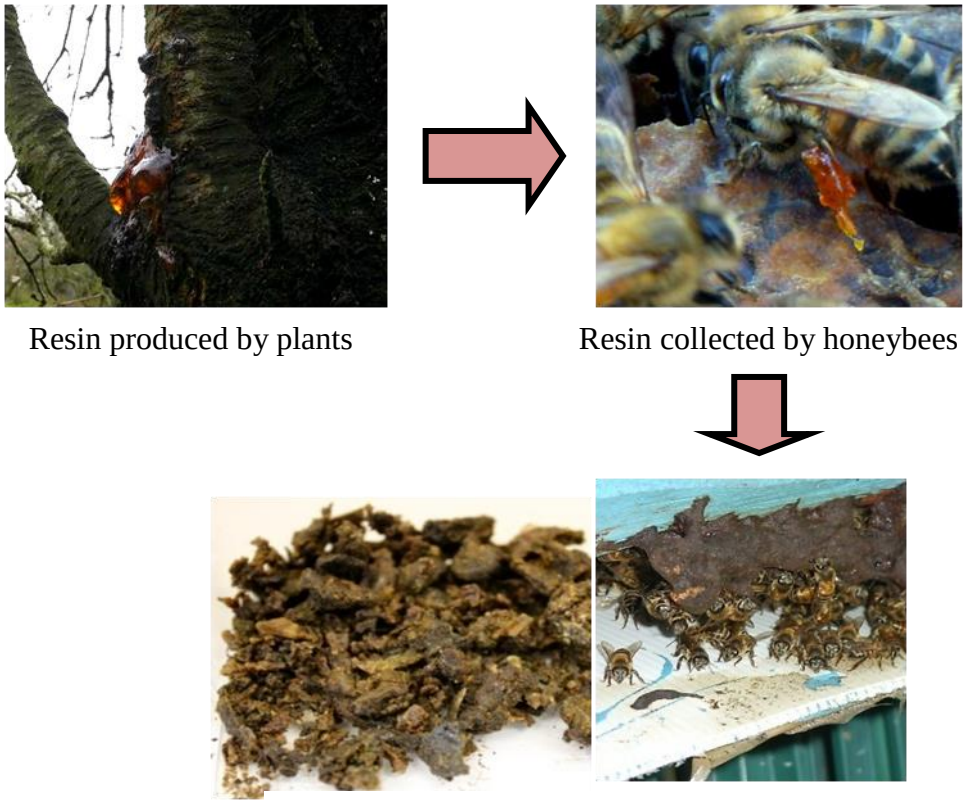


Figure 1.1: Production of propolis by honeybees *Apis mellifera*(www.biobees.com; www.foodwarriornetwork.com; mlmpropolis.com; www.fortunehoneyproducts.com)



Figure 1.2: Crude propolis illustrating different colours and textures.

Therefore, propolis maintains the homeostasis of the hive as well as provides protection to the bee colony (Fokt *et al*., 2010; Simone-Finstrom and Spivak, 2010; Miguel and Antunes, 2011). It is used as a disinfectant and antibiotic within the hive whereby infections within the colony are prevented (Castaldo and Capasso, 2002; Miguel and Antunes, 2011). Propolis extracts are known to be highly active against honeybee parasites such as *Paenibacillus larvae* and the wax moth *Galleria mellonella* (Bastos *et al.*, 2008).

1.2. Historical uses

The use of propolis, as a natural or traditional product for medicinal use dates back to 300 B.C (Burdock *et al.*, 1998; Melliou and Chinou, 2004; Ramos and Miranda, 2007; El Ashry and Ahmad, 2012). Egyptians used propolis for its antiputrefactive properties to embalm the deceased (Castaldo and Capasso, 2002; de Groot, 2013). Propolis has been used extensively since archaic times and holds many pharmacological uses. It was recognized by numerous physicians such as the Greek and Romans, Aristotle and Dioscorides for its vast amount of medicinal uses. During the Middle Ages and among the Arab physicians, propolis was used as an antiseptic, mouth disinfectant and cicatrizant in wound healing. The Incas used propolis as an anti-pyretic, and 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 (Castaldo and Capasso, 2002; de Groot, 2013; Wagh, 2013). Propolis is mentioned in Herst, Berlin and ancient Egyptian papyri as an effective treatment for sores and ulcers (El Ashry and Ahmad, 2012).More than 90 years ago, propolis was mixed with petroleum jelly and used during the Anglo-Boer war in South Africa (SA) for wound healing as well as tissue regeneration (Ghisalberti, 1979; Han *et al*., 2005; Ramos and Miranda, 2007; de Moura *et al*., 2011; El Ashry and Ahmad, 2012).

1.3. Pharmacological uses

Propolis has been shown to exhibit immunomodulatory and immunostimulatory effects (Castaldo and Capasso, 2002). Also reported are its antioxidant, anti-tumor, anti-inflammatory, antimicrobial, antiviral, antiparasitic, anti-carcinogenic, anti-diabetic properties, cholesterol lowering effects, as well the treatment of duodenal and gastric ulcers; it is for these purposes that modern day herbalists have been prescribing propolis (Banskota *et al.*, 2001;Borrelli *et al.*, 2002; Ramos and Miranda, 2007; Viuda-Martos *et al.*, 2008; du Toit *et al*., 2009; Abu-Mellal *et al.*, 2012; El Ashry and Ahmad, 2012; da Silva Frozza *et al*., 2013; Król *et al*., 2013; Kuropatnicki *et al.*, 2013; Wagh, 2013). Other studies have reported on its strong hepatoprotective and antioxidant properties (Banskota *et al*., 2001; Sforcin, 2007; Viuda-Martos *et al*., 2008). Propolis has been reportedly used for the treatment of various ailments such as influenza and upper respiratory tract infections (Rahman *et al*., 2010). Numerous studies have been conducted on propolis worldwide, both *in vitro* and *in vivo*, through clinical studies, either alone or in combinations with calcium hydroxide, detailing the beneficial effects of propolis in dental health and in the reduction of dental caries (de Rezende *et al*., 2008; Awawdeh *et al*., 2009; Bertolini *et al*., 2010; Arslan *et al*., 2011; Madhubala *et al*., 2011; Kousedghi *et al*., 2012; Mattigatti *et al*., 2012;Bezerra *et al*., 2015; Pimenta *et al*., 2015). Lu *et al*. (2005) documented the use of propolis in the prevention of gingivitis among other oral infections and also confirmed the usefulness of propolis in the treatment of allergies and different pathological conditions such as tumours and diabetes.

In Japan, propolis is believed to be beneficial to health and is used in numerous health products found on the market (Nagai *et al*., 2003).Propolis has also been used increasingly as a preservative in various food substances (Tosi *et al*., 2007), as well as in veterinary medicine for the treatment of various ailments such as mastitis in cows, canine otitis and *Salmonella* infections (Cardoso *et al*., 2010;Al-safi, 2013; Aamer *et al*., 2015).The properties of propolis are now being scrutinized in greater detail with various studies reporting on the broad-spectrum antimicrobial activity, anti-inflammatory and anticarcinogenic and immunomodulatory effects (Banskota *et al*., 2002; Simone-Finstrom and Spivak, 2010).Miguel and Antunes (2011) reported that propolis has become a substance of increased importance used therapeutically either alone or in combination therapy with a variety of medicines and homeopathic products. Salatino *et al*. (2011) reported that propolis is gaining a wide acceptance in popular medicine in various parts of the globe. Brazilian propolis in particular has become a subject of increasing interest due to its characteristic favourable biological activities. The main Brazilian plant sources are *Araucaria* spp., *Baccharis* spp. and

Eucalyptus spp. (Bankova *et al*., 2000; Kumazawa *et al*., 2003). This has resulted in the increased global demand for Brazilian propolis, with the State of Paraná producing over 36 tons of propolis annually (de Castro, 2001).

1.4. Antimicrobial studies

Globally, research has been dedicated to studying the antimicrobial properties of propolis from various geographical and climatic regions especially Brazil, Turkey, Argentina and Italy, as well as Colombia, Ethiopia, Russia, Bulgaria, Greece, 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.

Appendix G provides an overview of the antimicrobial studies undertaken on propolis worldwide. An in-depth review of these studies conducted on the antimicrobial properties of propolis clearly showed that propolis; irrespective of the country of origin has remarkable antimicrobial properties when compared to other natural products. Furthermore, it was noted that in these studies, propolis was most frequently tested against micro-organisms such as; *S. aureus, P. aeruginosa, C. albicans, E.coli, E. faecalis,* various *Streptococcus* spp. as well as some resistant microbial strains such as methicillin-resistant *S. aureus.* It is, however, important to note that whilst propolis worldwide has been rather extensively studied, the antimicrobial efficacies of African, specifically South African propolis in particular have been poorly investigated. Figure 1.3 further supports this observation in the lack of investigations, as it illustrates the scarcity of studies conducted within the African continent in comparison to South America (i.e. Brazil and Argentina), Europe (i.e. Turkey and Italy) and Asia (i.e. India and Iran).

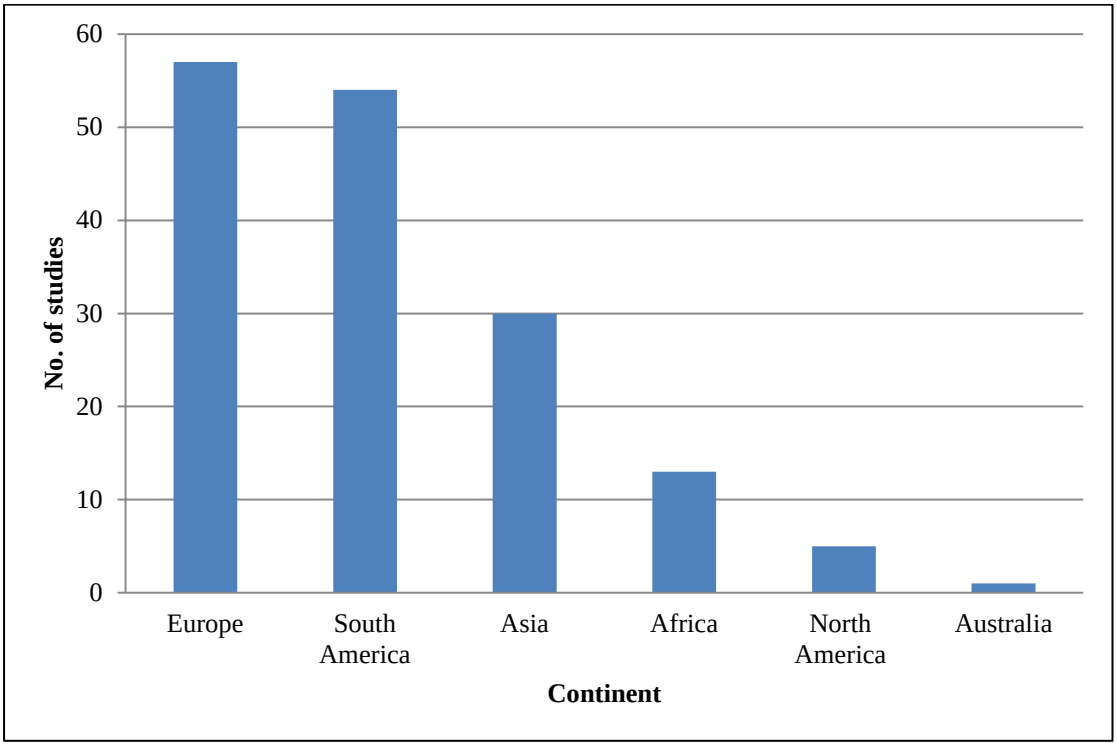


Figure 1.3: Studies conducted worldwide on the antimicrobial properties of propolis in comparison to Africa.

Although research may be lacking in other continents, such as Australia and North America, Africa is still poorly studied in comparison to South America, Europe and Asia (Figure 1.3), with less than 15 studies being conducted on African propolis. From a SA perspective, the antimicrobial investigation of propolis has been somewhat neglected, with only three studies by other authors investigating the properties of SA propolis and only one of these on its antimicrobial property. A study conducted by du Toit *et al.* (2009), investigated the antimicrobial properties of SA propolis, however, the study was more focused 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. Seidal *et al.* (2008) reported on the antimicrobial activity of propolis from different geographical and climatic zones, which included only one sample from SA. Considering that the antimicrobial efficacy of SA plants has demonstrated a number of promising anti-infective properties (van Vuuren, 2008), the probability that propolis derived from the unique 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.

1.5. Chemical composition

The chemical composition of propolis is extremely complex with more than 300 constituents having already 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). Composition is largely dependent on the climate, bee species and local flora available (Markham *et al*., 1996; Righi *et al*., 2011; Salatino *et al*., 2011). Amongst the constituents contained in propolis are wax, resins, balsams, essential oils, amino acids, sugars, polyphenols, esters of phenolic acids, flavonoids, sesquiterpenes, diterpenes, triterpenes, lignans, prenylated benzophenones, aldehydes, steroids and coumarins (de Castro, 2001; Mohammadzadeh *et al*., 2007; Hernández *et al*., 2005; Farooqui and Farooqui, 2012; Mavri *et al*., 2012). Phenolics (mostly flavonoids) are reported as constituting over 50% of the total weight of a given propolis sample (Bankova *et al*., 1996).

Different chemotypes of propolis have been reported. The most common types of propolis reported are Temperate -produced from the bud exudates of Populus trees, Birch, Tropical, Mediterranean and Pacific (Popova *et al*., 2004; Bankova, 2005b). In Europe and 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 prenylated benzophenones. Furthermore, red Mexican propolis is found to contain a vast amount of flavones, isoflavans and pterocarpans (Hernández *et al*., 2005; Lotti *et al*., 2010; Righi *et al*., 2011). Poplar-derived propolis has been found to be rich in flavonoids and aromatic acids (Sawaya *et al*., 2010). Birch propolis is found more specifically in Russia and is chemically distinct from Poplar propolis (Christov *et al*., 1999). Pacific propolis is characteristically rich in prenylated flavanones (Popova *et al*., 2010). Propolis obtained from the tropical regions mainly contains prenylated *p*-coumaric acid derivatives, flavonoids, benzophenones, lignans and terpenes (Popova *et al*., 2009). Brazilian propolis is a highly valued propolis and has gained tremendous commercial importance due to its wide range of health benefits (Salatino *et al*., 2011). The green and brown Brazilian propolis samples 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). Propolis has repeatedly 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).

Figure 1.4 provides a summary of the most frequently identified compounds present in propolis (i.e. pinocembrin, galangin and chrysin), based on studies conducted globally (Appendix H). In addition to these aforementioned compounds it can be seen that caffeic acid, kaempferol, quercetin, *p*-coumaric acid, ferulic acid, pinobanksin and apigenin are also compounds often reported by other studies as major constituents of propolis. From Figure 1.4 it can further be seen that pinocembrin and chrysin are the compounds most commonly found and reported in propolis. The only factor that may vary is the concentration in which these compounds are found.

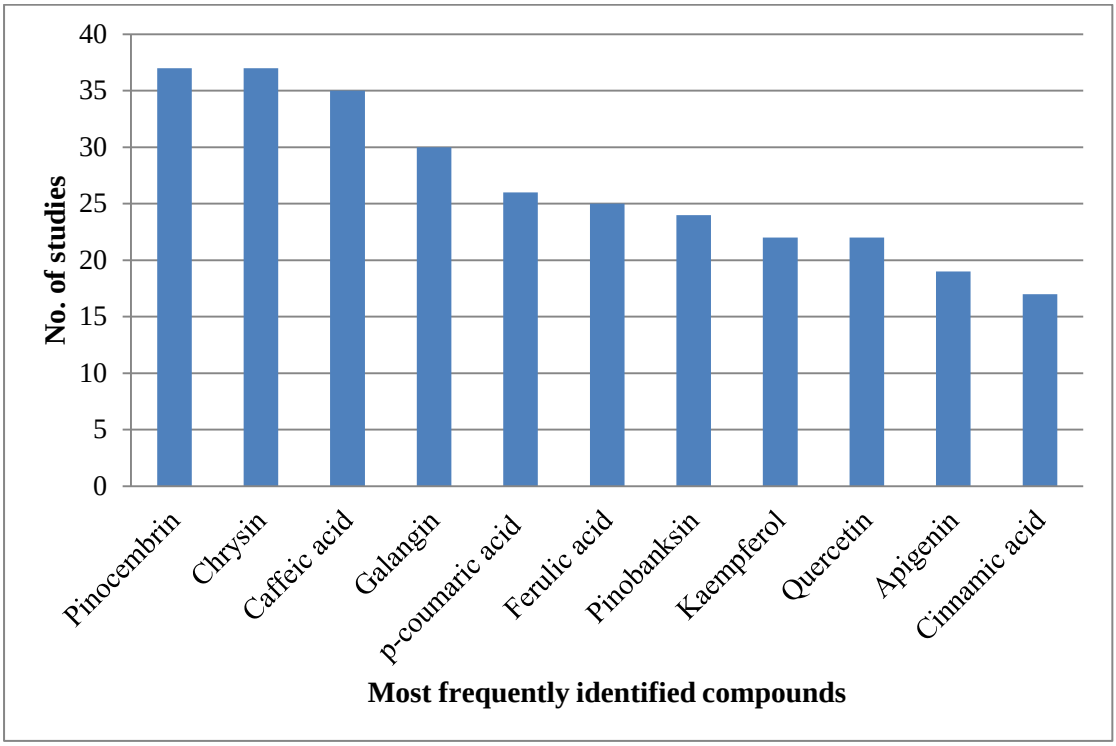


Figure 1.4: Compounds most commonly identified in propolis, globally.

A study conducted by Maraschin *et al*. (2012) utilized Fourier transform infrared spectroscopy (FTIR) and UV-visible spectroscopies coupled with chemometrics to differentiate between samples produced in southern Brazil, a region known to be rich and diverse in botany. Poplar propolis has been identified using UV-visible spectrophotometry by Popova *et al*. (2004) and other chromatographic methods such as high-performance liquid chromatography (HPLC), thin layer chromatography (TLC) and gas chromatography (GC) (Sârbu and Mot, 2011). Bankova *et al*. (2000), argues that there are some constraints with such an analytical approach. Therefore, a need arises for a faster screening method that can

characterize propolis by chemical composition from different geographical regions. This led scientists to the discovery of a novel and faster method of analysis known as direct insertion mass spectrometric fingerprinting technique. This technique has proven to be a robust propolis characterization method (Sawaya *et al*., 2011). Chemometric methods are currently being considered in order to analyse the huge data sets resulting from non-selective analytical techniques (Maraschin *et al*., 2012). It is due to this information that this study resolved to conduct chemical analysis on SA propolis using HPTLC, UPLC-PDA-qTOF-MS/MS and chemometric analysis.

Propolis samples from SA have previously been documented to contain different types of phenolic compounds and reported as possibly displaying some similarities with poplar propolis found in temperate regions (Kumazawa *et al*., 2004). However, limited research has been conducted on the chemical properties of SA propolis. Studies conducted by du Toit *et al.* (2009), reported on the flavonoids as well as a non-flavonoid caffeic acid phenethyl esters (CAPE). Zhang *et al*. (2014) reported on the chemical profiles of a limited number of samples (i.e. five samples) obtained from the KwaZulu-Natal province of SA. Of the five samples tested by the study, three samples displayed chemical profiles similar to that of temperate region poplar propolis whilst the other two samples were found to be rich in diterpene acids, characteristic of propolis originating from the eastern Mediterranean regions. Although SA has a very active bee-keeping community, there is a lack of scientific research on both the biological properties (i.e. antimicrobial) and chemical composition of locally produced propolis from all provinces.

1.6. Combination studies

Resistance to antimicrobials is a growing concern in the industry of medical health and scientists are constantly looking for an alternative means of inhibiting microbial growth as well as enhancing the potencies of the current available antimicrobials. The use of propolis in combination with other natural products has shown a dramatic increase in popularity (Miguel and Antunes, 2011). Studies have been conducted in specific countries such as China, Brazil, Cuba, Bulgaria and Russia introducing propolis as a vaccine adjuvant, demonstrating the positive effects of the combined use of propolis with other therapeutic regimens (El Ashry and Ahmad, 2012). Propolis has also been reported to display synergism with conventional antimicrobials (Fernandes *et al*., 2005; Orsi *et al*., 2006; Rahman *et al*., 2010; Helaly *et al*., 2011; Naher *et al*., 2011; Orsi *et al*., 2012). Some examples include the combination with clarithromycin for the treatment of *Helicobacter pylori* infections, implicated in the

development of peptic ulcers (Nostro *et al*., 2006) and with gels used for wound healing (Berretta *et al.*, 2012) as well as muco-adhesive gels in the treatment of vulvovaginal candidiasis (Berretta *et al*., 2013). Propolis has been proven to enhance the activity of some antibiotics against the *Staphylococcus* species (Rahman *et al*., 2010).

Propolis has been tested against *Salmonella typhimurium* in combination with gentamicin and ampicillin. The study reported synergism when EEP was combined with ampicillin, whilst combinations with gentamicin produced only additive interactions (Al-Safi, 2013). Synergistic interactions were also found with gentamicin and amoxicillin against *S. aureus* (de Lima Silva *et al*., 2015). Wojtyczka *et al*. (2013a) tested the interactive efficacy of Polish EEP samples against a multitude of antibiotics; cefoxitin, clindamycin, tetracycline, tobramycin, penicillin, ciprofloxacin and more. Synergistic activity was reported with chloramphenicol against *S. aureus* (Araújo *et al*., 2015). Speciale *et al*. (2006), tested propolis in combination with beta-lactams, macrolides and fluoroquinolones, however, no synergism was reported.

Mantovani *et al*. (2008) reported synergism between EEP from the south of Brazil and six out of the seven antibiotics tested in the study against coagulase-negative *Staphylococcus* strains. These included; cephalotin, netilmicin, clindamycin, vancomycin, oxacillin and tetracycline. In a study conducted by Scheller *et al*. (1999), synergistic interactions were reported between EEP and anti-tuberculosis drugs; rifampicin, streptomycin, ethambutol and isoniazid. The study argues one antagonistic interaction between EEP and ethambutol, the study reasoned that this antagonism may be due to the development of a chemical bond between ethambutol and one of the active constituents in propolis.

Studies conducted on Serbian propolis by Stepanović *et al*. (2003) reported synergism between ethanolic extracts of propolis (EEP) and ampicillin, ceftriaxone, doxycycline, amikacin, nalidixic acid, trimethoprim/sulfamethoxazole and nystatin. Helaly *et al*. (2011) investigated the interactive efficacies of propolis when combined with polymyxin B, colistin sulphate, vancomycin, sulphamethoxazole-trimethoprim, erythromycin, tetracycline, chloramphenicol, bacitracin, fusidic acid, ampicillin-sulbactam, amoxicillin-clavulanic acid, methicillin, cefaclor, cefoperazone, cefipime, imipenem, ciprofloxacin, ofloxacin, levofloxacin, gentamicin, neomycin and tobramycin. The study reported synergistic effects for most of the antibiotics tested against *S. aureus* and the organism which was resistant to fluoroquinolones, cephalosporins and imipenem became sensitive to these antibiotics in the

presence of EEP compared to when these antibiotics were tested alone. Naher *et al*. (2011) tested the interactive efficacy of propolis originating from Iraq and established that the following combinations displayed synergy; propolis with amoxicillin displayed synergy against *P. aeruginosa*, *H. pylori*, *E. coli* and *Klebsiella pneumoniae*; clindamycin with propolis displayed synergy against *P. aeruginosa*, *H. pylori*, *K. pneumoniae,* and *Enterococcus aerogenes*; rifampicin with propolis displayed synergy against *P. aeruginosa*, *E. coli*, *K. pneumoniae, S. pyogene*s, and *E. aerogenes*; and nystatin with propolis was reported to display additive activity against *C. albicans*. Stepanović *et al*. (2003) investigated the synergism of ethanolic extracts of propolis (EEP) with the antibiotics ampicillin, ceftriaxone, doxycycline, amikacin, nalidixic acid, trimethoprim/sulfamethoxazole and nystatin against *S. aureus*, *K. pneumoniae* and *C. albicans.*

In vivo studies conducted by Onlen *et al*. (2007), evaluated the effects of antibacterial combinations of propolis and ciprofloxacin tested in rabbits for the treatment of *P. aeruginosa* keratitis. Using 3% propolis drops, instilled into both eyes of the infected rabbits, did not cause a noteworthy or desirable effect, however, when combined with various concentrations of ciprofloxacin the study yielded synergistic results. A similar study conducted by Oksuz *et al*. (2005) also tested the interactive efficacy of ciprofloxacin and EEP on rabbits infected with *S. aureus* keratitis. A bacterial count was conducted on the corneas of treated rabbits. The study reported lower levels of bacteria in the eyes of rabbits treated with ciprofloxacin and propolis than those treated with ciprofloxacin or propolis independently, thereby suggesting synergism between ciprofloxacin and propolis. Sforcin and Bankova (2011) postulated that propolis may hold the potential for novel drug developments, as *in vitro* studies pave the way to the understanding of the mechanism of action of propolis.

A clinical study conducted by Adewumi and Ogunjinmi (2011) in Nigeria, combined propolis with another natural product of the hive, honey. The honey and propolis were combined in a lotion and applied to the septic wounds of 50 patients, three times a day. The study reported that 60% of wounds were completely healed by the end on the 10^{th} day and the remaining 40% completely healed by the end of the 15^{th} day, all wounds were reported to have healed without any remnants of scars or blisters. Propolis has been reported to be combined in other formulations such as toothpastes (Groppo *et al*., 2008; Palombo, 2011; Skaba *et al*., 2013) and in collagen-based dressings used in the treatment of burns (de Almeida *et al*., 2013).
The activity of 10% w/v propolis in combination with fennel honey was tested against various strains of *Staphylococcus* sp. implicated in bovine mastitis. The study reported that

although all pathogens displayed antimicrobial sensitivity to the samples of honey tested individually, the combination of 10% propolis and fennel honey displayed the best synergistic interactive efficacy with MICs and MBCs of 13.96% and 28.26% v/v, respectively. The study further concluded that the incorporation of propolis into the tested honey patch would potentiate the antimicrobial activity of the honey (Aamer *et al*., 2015). Furthermore, in dentistry, Pimenta *et al*. (2015) tested the interactive efficacy of brown Brazilian propolis at a concentration of 20% with calcium hydroxide in the treatment of oral *E. faecalis* infections. The study reported good interactive efficacy with a percentage inhibition of 41% compared to 21% inhibition when calcium hydroxide was used alone. Al-Waili *et al*. (2012) reported a synergistic interaction between honey and propolis against multi-drug resistant *S. aureus, E. coli* and *C. albicans.* In spite of the number of studies conducted on propolis in various combinations, studies on SA propolis, in particular, in combination with antimicrobials and other natural products such as honey have been neglected.

1.7. Aim and objectives

The overall aim of this study was to investigate the antimicrobial efficacy and chemical composition of SA propolis by undertaking the following objectives;

- Source and prepare propolis extracts using absolute ethanol as the solvent of choice.
- Determine the antimicrobial activity of the varied propolis samples (n=42) using the micro-titre plate serial dilution minimum inhibitory concentration (MIC) method against relevant pathogens.
- Determine the minimum bactericidal concentration (MBC) using Tryptone Soya agar (TSA).
- Determine the chemical composition and structure activity relationship of propolis using high performance thin layer chromatography (HPTLC) and Ultra-performance liquid chromatography coupled to photodiode array detector-quadrupole/time-of flight mass spectrometry (UPLC-PDA-qTOF-MS/MS).
- Investigate antimicrobial interactions of propolis with ciprofloxacin, gentamicin, nystatin, amphotericin B and honey by determination of the fractional inhibitory concentration index (ΣFIC) of 1:1 combinations.
- Correlate chemical composition with geographical variance and the observed antimicrobial activity of propolis using chemometric analysis.

Chapter 2: South African propolis: Chemical profiles and chemometric analysis

2.1. Introduction

The progress of chemical studies conducted on propolis samples from SA thus far, has been outlined in Chapter 1, Section 1.5.Only three studies were found to discuss the composition of SA propolis. Two focused on chemical composition, however, the number of samples tested from such a diverse country with diverse flora was limited, with the majority number of samples being five; of which all five were collected from the same province in SA (Kumazawa *et al*., 2004; Zhang *et al*., 2014). The third study conducted by du Toit *et al*., (2009) focused mainly on the anti-inflammatory properties of SA propolis rather than its chemical composition.

The objective of this Chapter was to develop fingerprinting and metabolite profiles of 39 SA propolis samples obtained from the different provinces of SA using high performance thin layer chromatography (HPTLC), ultra-performance liquid chromatography-photodiode array detector-quadrupole-time-of-flight mass spectrometry (UPLC-PDA-qTOF-MS). Chemometric modelling was also used in order to observe possible chemo-geographical patterns.

2.2. Materials and methods

2.2.1. Sourcing of propolis samples

Propolis samples (n=42) were sourced from different provinces of SA in collaboration with the SA Bee Industry Organisation (SABIO). Of these samples, 39 (i.e. samples 4-42) were from SA and three control samples (i.e. samples 1-3) from Brazil. Brazilian samples were included for comparative purposes as they are generally considered superior in activity 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. Table 2.1 lists samples used for chemical characterization, with their corresponding locations and extract yields. Figure 2.1 serves as an illustration of the specific sample locations across the various provinces of SA.

Table 2.1: Propolis samples: extract yields and localities.

No.	Location	CODE	Weight of sample used (g)	Weight of extract (g)	% yield
1	Orange River	NC1	11	1.84	16.76
2	Northern Pretoria	GP1	17.3	2.72	15.75
3	North West	NW1	15.7	2.49	15.87
4	Christiana- North West	NW2	16.7	5.85	35.01
5	Christiana- North West	NW3	17.4	5.66	32.56
6	Outeniqua Mountains, Oudtshoorn	WC1	5.7	2.57	45.12
7	Brazil	BR1	21.2	5.99	28.24
8	Brazil	BR2	17.8	7.19	40.39
9	Walkerville (Vereeniging)	GP2	9.2	3.76	40.87
10	Springs	GP3	10	3.70	37.00
11	Johannesburg	GP5	10	5.95	59.50
12	Johannesburg	GP6	9	4.45	49.44
13	Johannesburg	GP7	9	5.26	58.44
14	Lakeside/Westlake	GP8	21	5.68	27.05
15	Northern Cape	NC2	10	3.70	37.00
16	Western Cape	WC2	7	5.21	74.43
17	Somerset West	WC3	13	5.12	39.38
18	Botrivier	WC4	12	6.19	51.58
19	Graafwater	WC5	7	2.02	28.86
20	Brazil	BR3	10	4.65	46.50
21	Douglas- Northern Cape	NC3	6	2.35	39.17
22	Bloemfontein	FS1	18	10.02	55.67
23	Honeydew	GP9	12	6.13	51.08
24	Edenvale	GP10	9.3	5.25	56.45
25	Edenvale	GP11	4.8	3.42	71.25
26	Baviaanskloof – PE	EC1	6.7	1.12	16.72
27	Pretoria	GP12	7.3	2.71	37.12
28	Pretoria	GP13	7.3	3.42	46.85
29	Beaufort West	WC6	14.1	2.5	17.73
30	Lydiana Gardens- Pretoria	GP14	8.8	2.92	33.18
31	Lydiana Gardens- Pretoria	GP15	14.4	4.37	30.35
32	Northern Cape	NC4	5.5	1.08	19.64
33	Western Cape	WC7	3.3	0.24	7.27
34	KwaZulu-Natal	KZN1	3.8	1.07	28.16
35	Southern Suburbs - Cape Town	WC8	7.3	2.22	30.41
36	Southern Suburbs - Cape Town	WC9	4.1	1.97	48.05
37	Southern Suburbs - Cape Town	WC10	7.9	3.38	42.78
38	Wilgerivier – Bronkhorstspruit	GP16	7.6	2.05	27.01

No.	Location	CODE	Weight of sample used (g)	Weight of extract (g)	% yield
39	Beaulieu – Midrand	GP17	12.1	4.02	33.31
40	President Park – Midrand	GP18	12.0	5.94	49.67
41	Devon - Sedibeng area (Gauteng)	GP19	4.2	1.47	35.08
42	Mooinooi - North West	NW4	7.3	3.05	41.67

GP=Gauteng; NW=North West; WC=Western Cape; NC=Northern Cape; EC=Eastern Cape; KZN=KwaZulu-Natal; FS= Free State; BR = Brazil

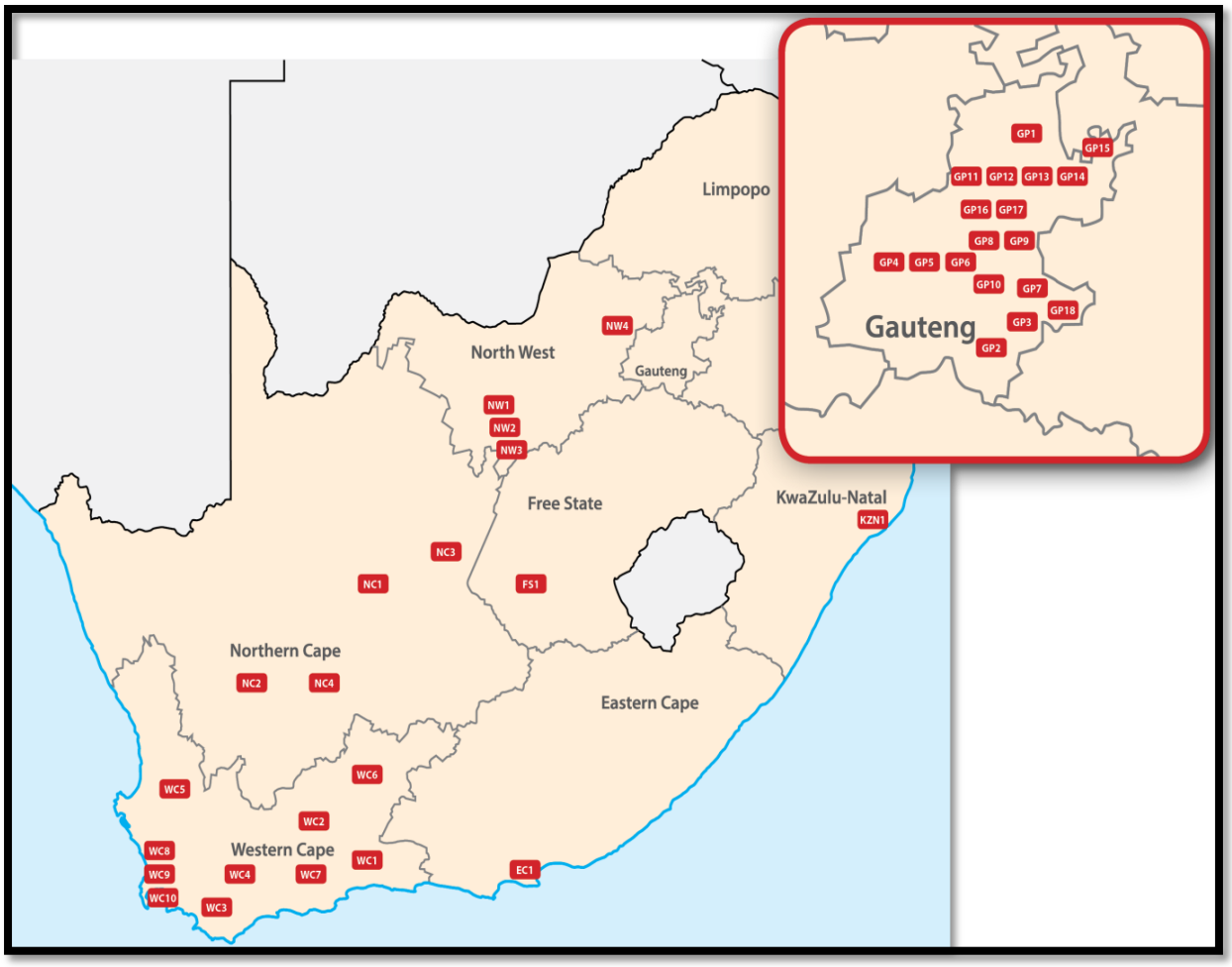


Figure 2.1: Geographical locations of propolis samples used in this study.

2.2.2. Preparation of extracts for chemical studies

Preliminary studies were conducted using various solvents in order to investigate which solvent system extracted the most compounds out of samples. It was proven that 80% aqueous ethanol effectively extracted compounds and produced the best HPTLC plate with clear separation of compounds, unlike other solvents tested that led to the "bleeding" or rather "merging" of compounds. Therefore, a 100 mg of each sample was submerged in 10

ml of 80% aqueous ethanol and placed in an orbital shaker incubator for a period of 24 hours at 27°C. After 24 hours the final suspension was filtered through a 0.22-µm Clarinert™ syringe filter for chemical profiling. The standards were prepared in ethanol.

2.2.3. HPTLC fingerprinting and profiling

HPTLC analysis of SA propolis samples and Brazilian control samples was carried out using the CAMAG semi-automated HPTLC instrument, which consisted of an automatic sampler 4 (connected to a nitrogen tank), an automatic developing chamber (ADC2), a chromatogram immersion device and a documentation device Reprostar 3 all of which utilize winCATS version 1.4.4.6337 planar chromatography manager software (Figure 2.2). HPTLC pre-coated silica gel $60F_{254}$ (MERCK, Germany) glass plates were used for the HPTLC analysis. By means of a 25 µl Hamilton micro-syringe in the automatic TLC sampler 4.A volume of 5 µl (0.05 mg/µl) of each propolis extract was applied onto the plates as an 8 mm band, 5 mm from the bottom edge of the plate.

The separation was then carried out by using a solvent system of chloroform: methanol: formic acid (38: 2.8: 2; v/v). The chromatogram was developed in a glass twin-trough chamber in ADC2 which was automatically saturated with the prepared mobile phase vapour for 20 minutes. The ascending development was carried up to a distance of 70 mm. Once developed, the plate was dried using a dryer and resulting images were captured under ultraviolet (UV) light at 254 and 365 nm. Hereafter derivatization was carried out with natural product reagent (1% methanolic diphenylboric acid-β-ethyl amino ester: 5% ethanolic polyethylene glycol - 9000) and 85 ml methanol, 10ml glacial acetic acid, 5ml of concentrated sulphuric acid added very slowly and 0.5 ml of anisaldehyde. The HPTLC plate was placed in a dipping tank in the aforementioned solution and ascending development was carried up to a distance of 70 mm as done previously. This was done in order to permanently stain the plate, capture chromatographic fingerprinting profiles and record their profiles.

The retention factor (R_f) was calculated as follows:

$$R_f = \frac{\text{distance travelled by sample}}{\text{distance to solvent front}}$$

Hereafter, plates were further analyzed, dominant bands were identified and new HPTLC plates were prepared containing each of the chosen SA propolis samples and Brazilian control samples. All SA samples displayed the same dominant band and all Brazilian controls displayed the same dominant band (Figure 2.3).



Figure 2.2: HPTLC process using **A:** automatic TLC sampler 4; **B:** Automatic developing chamber 2 and; **C:** Reprostar 3 documentation system (Camag).

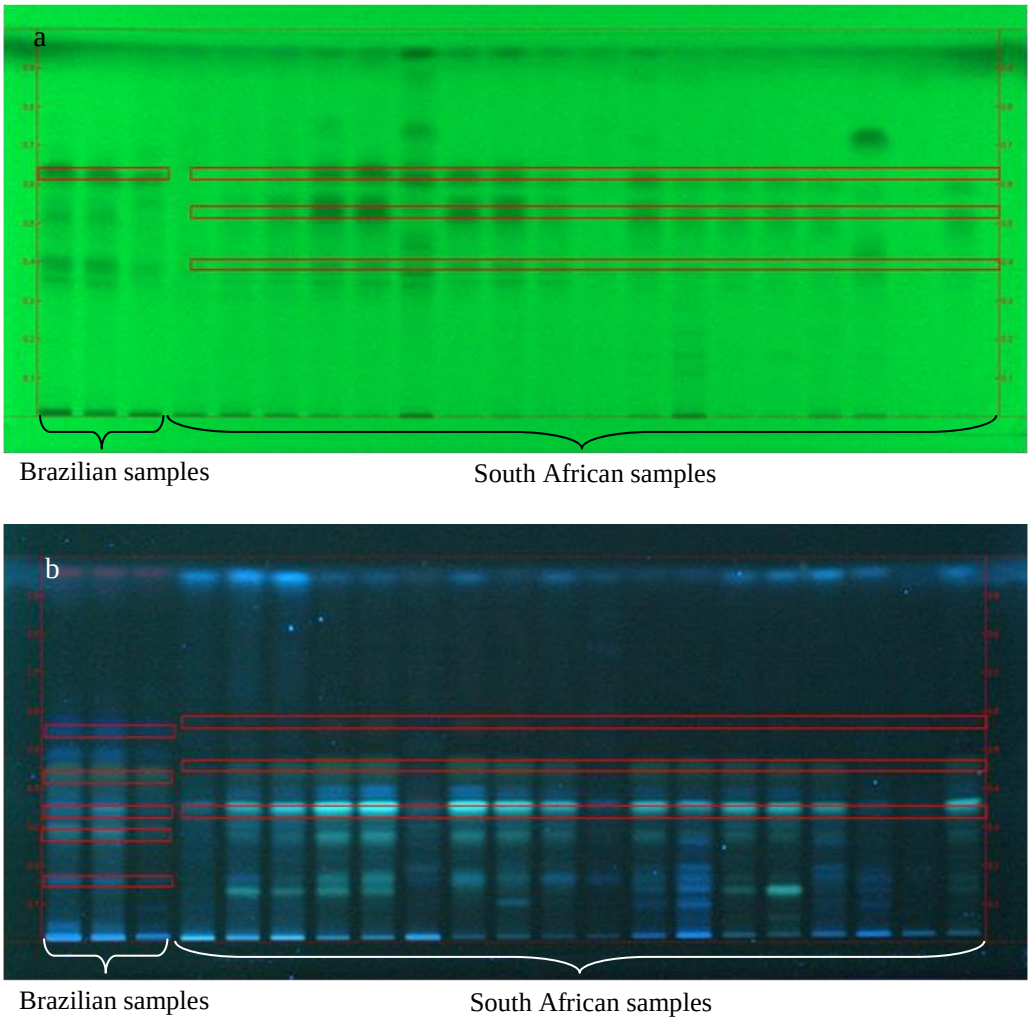


Figure 2.3: a- HPTLC plate at 254 nm indicating dominant bands in all SA samples and Brazilian control samples; **b-** HPTLC plates at 366 nm indicating dominant bands in all SA samples and Brazilian control samples.

A volume of 3 µl (0.03 mg/µl) of the chosen propolis extract was applied onto the plates in the same manner as elaborated previously. Separation was carried out using the same solvent system of chloroform: methanol: formic acid (38: 2.8: 2; v/v). However, no derivatization of plates was done. Four pre-coated silica gel $60F_{254}$ (MERCK, Germany) glass plates were prepared for each of the samples, dominant bands were then carefully scratched off, placed into sample bottles and 3 ml of HPLC grade methanol was added. These samples were then used for UPLC-PDA-qTOF-MS analysis in order to identify these prominent compounds.

2.2.4. UPLC-PDA-qTOF-MS chemical profiling

Ultra-performance liquid chromatography-photodiode array detector-quadrupole-time-of-flight mass spectrometry (UPLC-PDA-qTOF-MS) for chemical profiling analysis of SA and Brazilian propolis samples was performed using a Waters ACQUITY™ UPLC™ system obtained from Waters Corp., Milford, MA, USA. The UPLC system was equipped with a PDA detector and quadrupole-TOF mass spectrometer. A volume of 1 µl (0.01 mg/µl) of each sample was injected into the system, and separation was then achieved on a BEH C_{18} (2.1 x 150 mm, 1.7 µm) column. The binary mobile phase contained 0.1% formic acid in water (phase A) and acetonitrile (phase B) (phase A and B were obtained from ROMIL-SpS™, Cambridge, United Kingdom).

The following gradient programme was used;

- 10 to 40% of phase B within 2.5 minutes
- 40 to 45% of phase B for the next 8.5 min
- 45 to 80% of phase B over a further 0.5 min, then returned to the initial conditions (10 to 40% of phase B) and conditioning at 10% B over 1 min, finished at 14 min.

The flow rate was set at 0.45 ml/min and the temperature of the column was kept constant, at 35°C. The UV spectrum of each sample was recorded within range of 200 to 400 nm using the PDA detector. The acquisition of mass spectra was performed at negative mode by electrospray ionisation (ESI). The conditions for the ESI source were; a capillary voltage of 34.0 kV; sampling cone at 40 V; source temperature set at 100°C; and desolvation temperature set at 500°C. Nitrogen was used as a desolvation gas at a flow rate of 600 L/h. The prominent ions were further selected for MS/MS fragmentation analysis, in which the collision energy was ramped from 6 to 40 V. MassLynx software version 4.0 (Waters) was used for instrument control and data acquisition (i.e. chemical profiling).

2.2.5. Chemometric analysis

The UPLC-ESI-MS data of all samples was analysed using chemometric methods. The chromatograms were first pre-processed using the MarkerLynx 4.1 system in which Apex Track^{TM} detected the chromatographic peaks and assigned retention times accordingly across the entire data set attained. The ions which were associated with these peaks were identified, and their corresponding ion intensities, retention times and masses (m/z) were then captured and aligned accordingly (Stumpf and Goshawk, 2004). This aligned data was then further assembled in a single matrix, imported into SIMCA-P+ 12.0 and analysed firstly using principal component analysis (PCA). This is a mathematical algorithm that reduces multidimensional data, to a co-ordinate system which then allows for a graphical interpretation of the data. The spatial distribution of the samples can also be observed on a score plot where samples with a similar chemical nature will then cluster close together and dissimilar samples will fall further apart. Secondly, classification and discriminant analysis of these propolis samples was performed using orthogonal projections to latent structure-discriminant analysis (OPLS-DA). A loadings plot was used in order to describe the relationships amongst the measured variables. Those samples furthest from the origin of the plot were then regarded as the highest contributors to variance within the chemical space.

2.3. Results

2.3.1. HPTLC fingerprinting of South African propolis

In this study, a novel HPTLC chromatographic fingerprinting method was developed for the characterization and authenticity of SA propolis samples in comparison to the control Brazilian propolis samples, this study (Appendix A) is the first to report on this fingerprinting technique unused by other studies (Kasote *et al*., 2014a). Figures 2.4a and 2.4b show the HPTLC fingerprinting images of 39 SA propolis samples under the wavelengths 254 and 366 nm before derivatization with a natural product reagent in comparison to the three control Brazilian propolis samples. These HPTLC fingerprinting profiles of SA propolis samples were notably different from the three control Brazilian propolis samples. Most of the SA samples displayed distinctive greenish yellow florescent bands under a wavelength of 366 nm (Figure 2.4b).This may be attributed to the presence of flavonoids and phenolic acids.

Figures 2.4c and 2.4d show images of SA samples in comparison tithe control Brazilian propolis samples under wavelengths 254 and 366 nm after derivatization with a natural product reagent. Primarily, all propolis samples look alike and no distinct chemical difference may be seen between Brazilian and SA propolis. However, with further investigation and

after derivatization of HPTLC plates with a natural product reagent some clear distinctions may be seen at this point. Moreover, some chemical variations were apparent within SA samples themselves (Figure 2.4a, b, c and d.). Most SA propolis samples tested displayed distinctive greenish-yellow florescent bands under wavelength 366 nm before as well as after derivatization with the natural product reagent (Figures 2.4c and 2.4d).This may possibly confirm the presence of chemical compounds such as flavonoids and phenolic acids as observed in other studies (Appendix H).

2.3.2. UPLC-PDA-qTOF-MS chemical profiling

Preparative UPLC-PDA-qTOF-MS/MS analysis in negative mode was used to identify and confirm the phenolic and flavonoid compounds from SA propolis samples seen in HPTLC. UPLC-ESI-MS Total ion chromatogram (TIC) of representative SA propolis samples in comparison with Brazilian propolis is shown in Figure 2.5. From this chromatographic fingerprint it can be seen that the SA propolis sample was different in comparison to the control Brazilian propolis samples. Both the HPTLC and UPLC-ESI-MS chromatographic fingerprints therefore confirmed that SA propolis is different from Brazilian propolis.

The identification of these compounds was done by matching their retention times (Rt), maximum UV absorptions, pseudomolecular ion mass values and MS/MS fragmentation patterns with authentic compounds, confirmed from a standard when available or confirmed with data previously reported in literature. Fifteen phenolic and flavonoid compounds, reported in Table 2.2, were identified in the SA propolis samples. Figure 2.6 is a UPLC-ESI-MS total ion chromatogram (TIC) of SA propolis displaying the peak numbers of compounds as reported Table 2.2. Peak 8 had a UV_{max} of 288 nm and was found to be the compound most prominent in SA propolis and was identified by the study as being the marker compound for SA propolis samples tested. Peak 8 was identified as pinocembrin (Pellati et al., 2011).

Furthermore, this analysis of SA propolis samples resulted in the identification of six new additional compounds unidentified in SA propolis. These included quercetin, galangin-5-methyl ether, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-butyrate or isobutyrate, pinobankin-3-*O*-pentanoate or 2-methylbutyrate and pinobanksin-3-*O*-hexanoate (Figure 2.7). Among the compounds identified, pinocembrin was found to be the major constituent in the SA propolis samples. In addition, artepillin C and cinnamyl acetate were identified as the marker compounds for the Brazilian propolis samples.

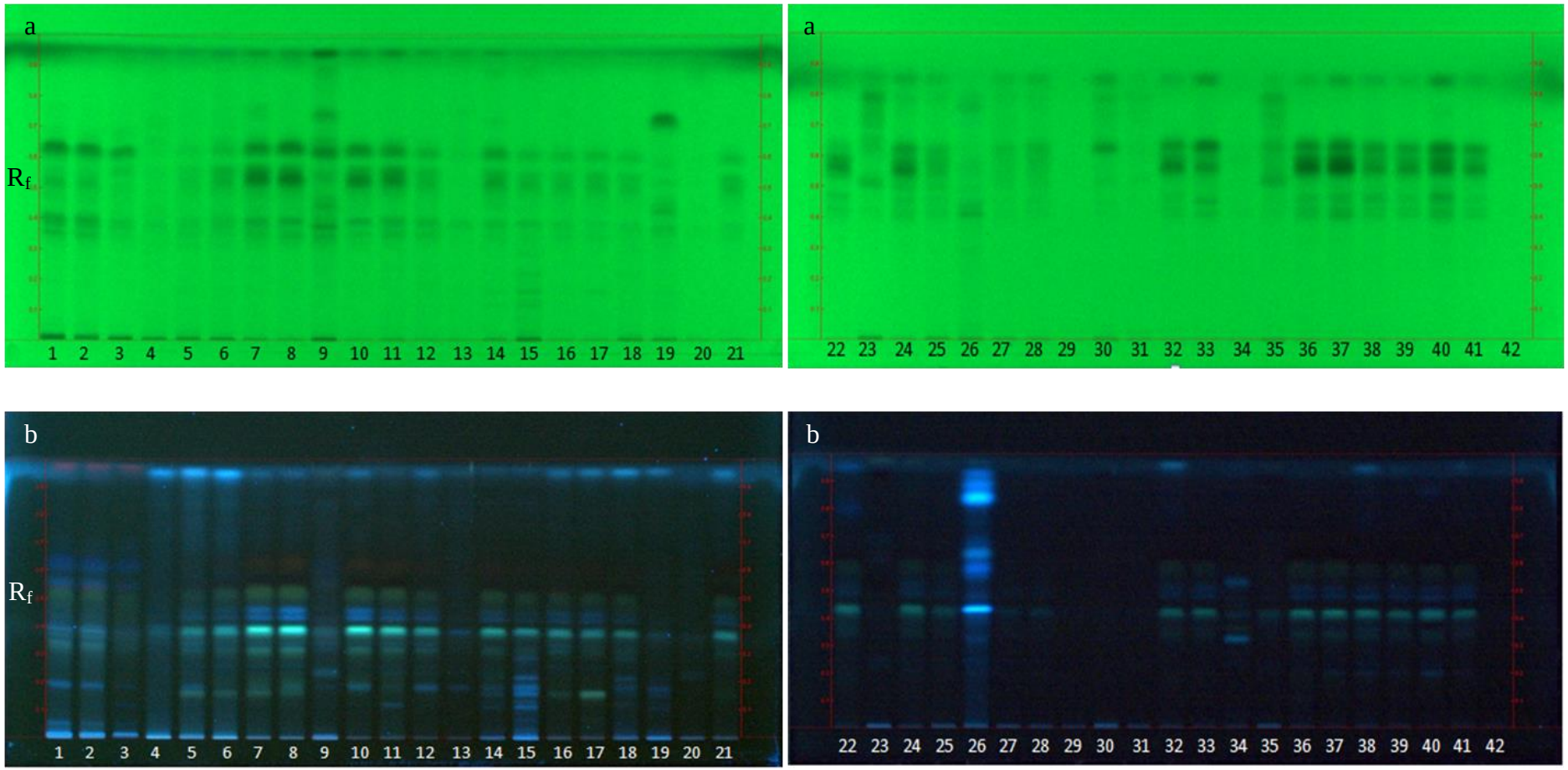


Figure 2.4: a-HPTLC plates at 254 nm before derivatization (samples 1-3 = Brazilian; samples 4-42 = SA); **b**- HPTLC plates at 366 nm before derivatization (samples 1-3 = Brazilian; samples 4-42 = SA).

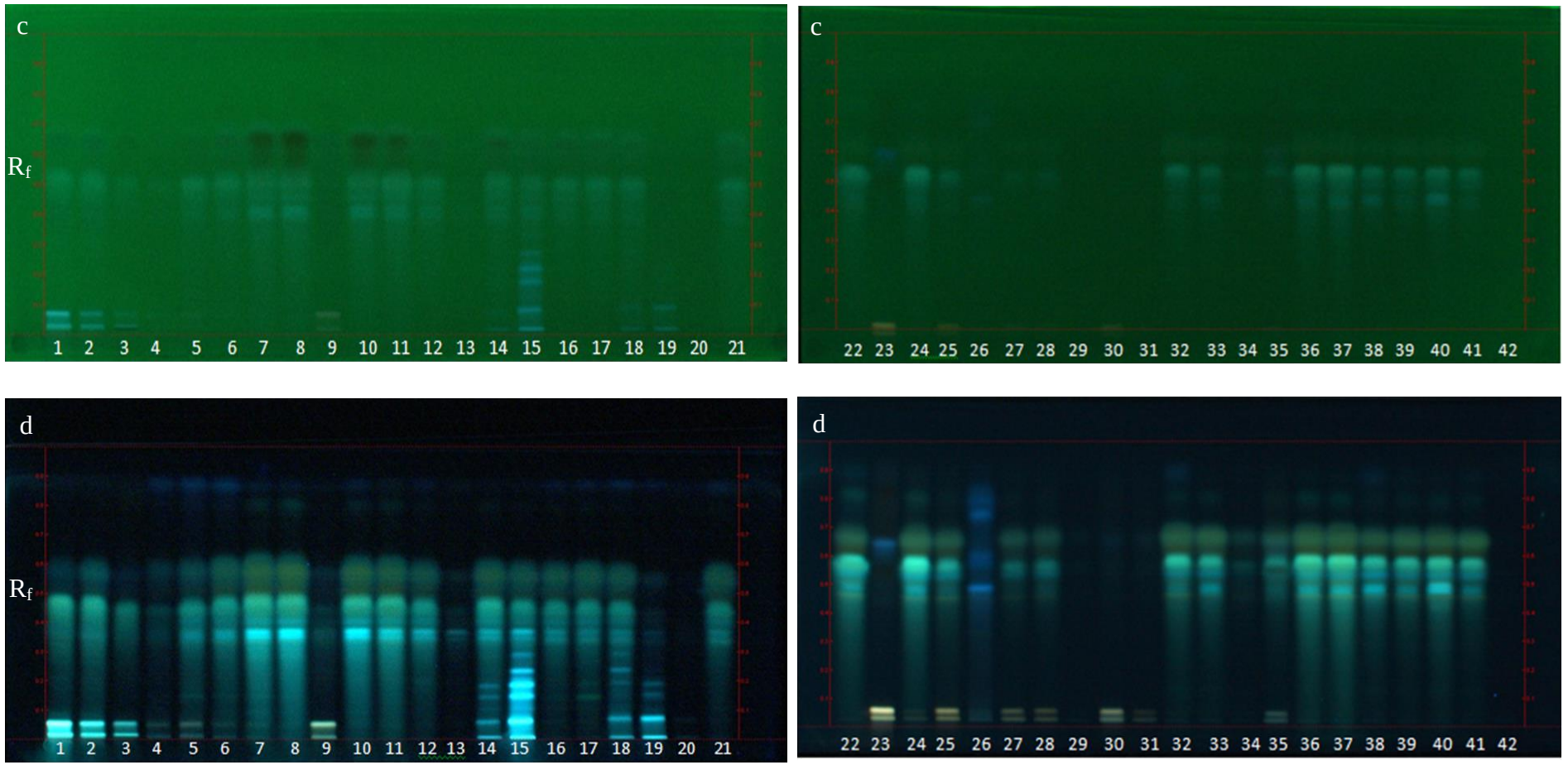


Figure 2.4: **c-** HPTLC plates of samples 1-21 after derivatization (samples 1-3 = Brazilian; samples 4-42 = SA) with natural product reagent at 254; **d-** HPTLC plates of samples 22-42 after derivatization (samples 1-3 = Brazilian; samples 4-42 = SA) with natural product reagent at 366 nm.

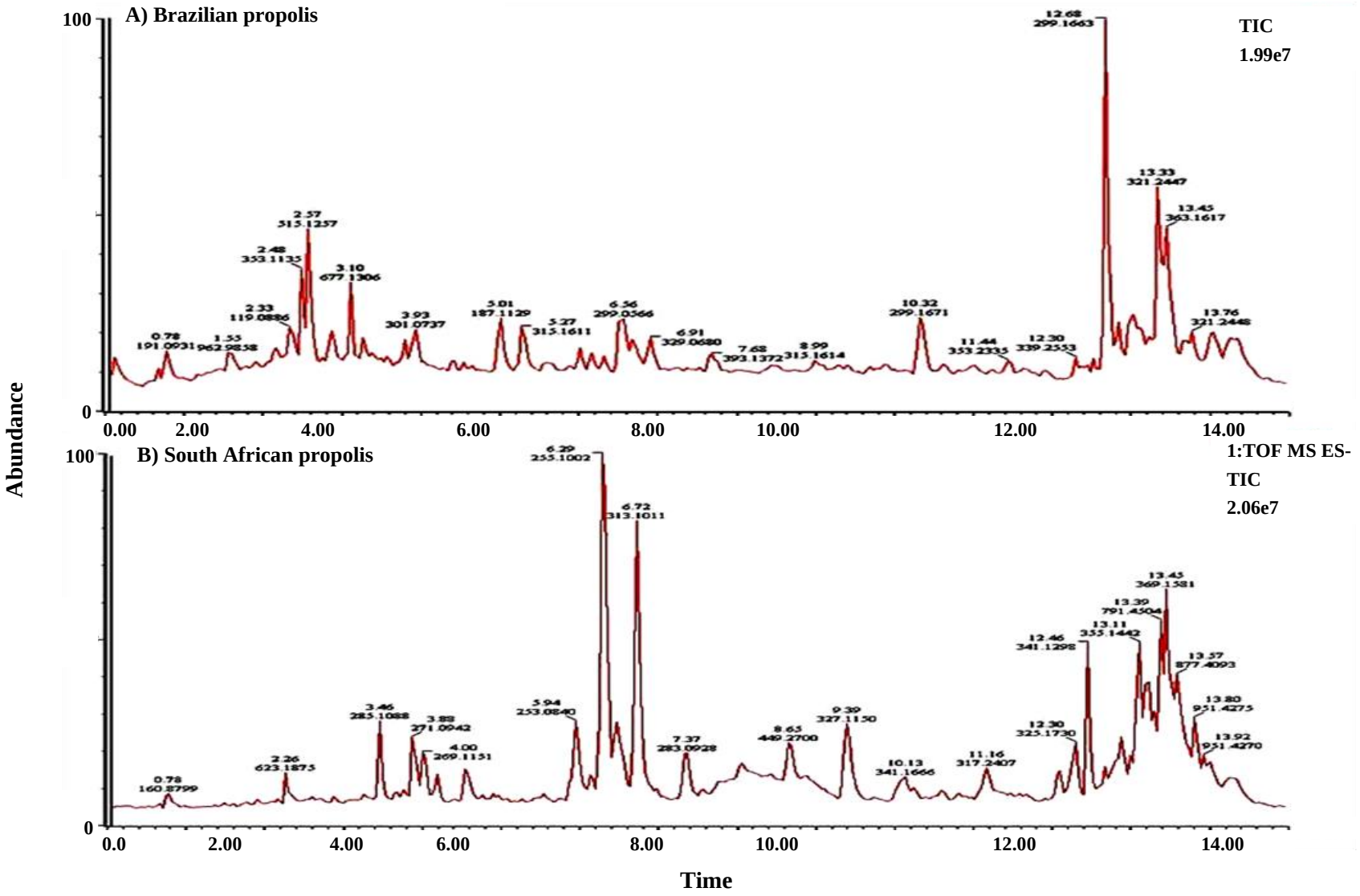


Figure 2.5: UPLC-ESI-MS total ion chromatogram (TIC) of one SA propolis sample in comparison with one control Brazilian propolis sample.

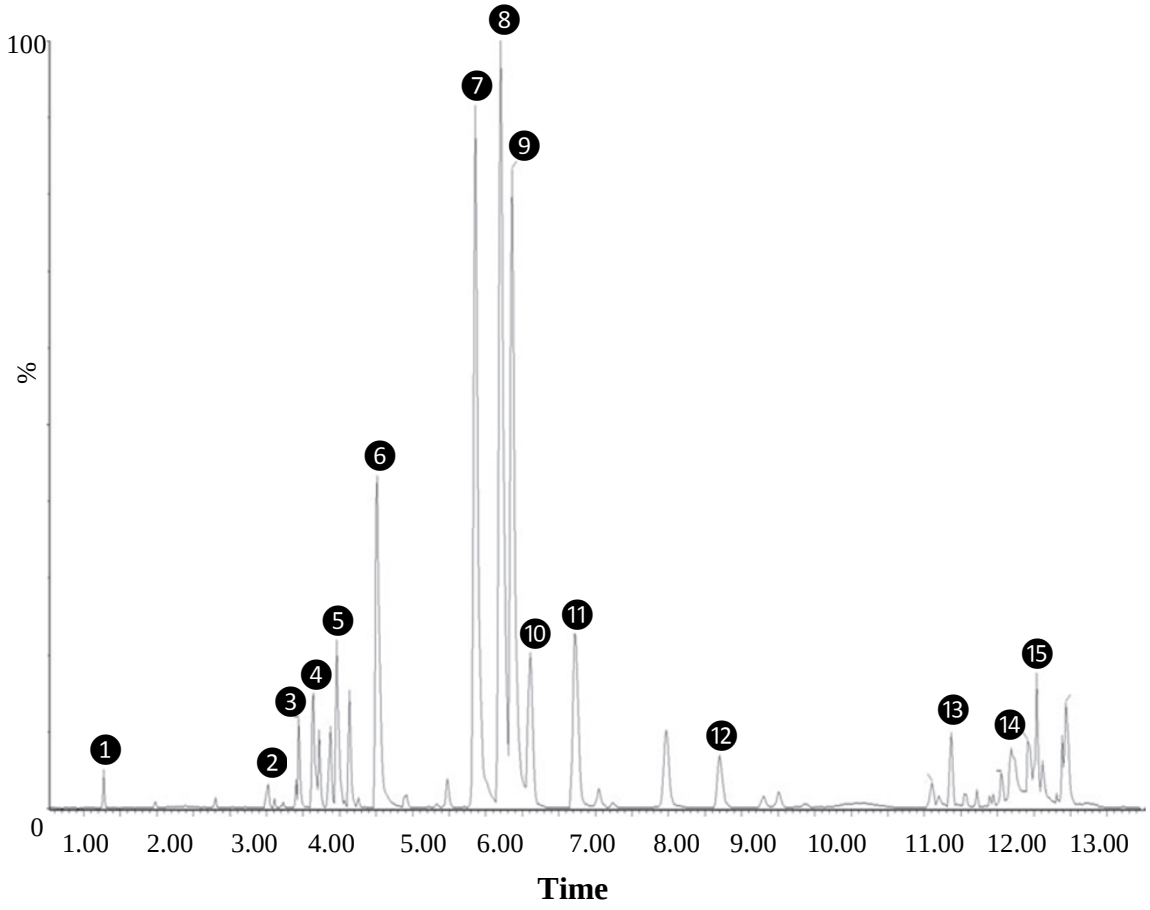


Figure 2.6: UPLC-ESI-MS total ion chromatogram (TIC) of SA propolis displaying the peak numbers of compounds.

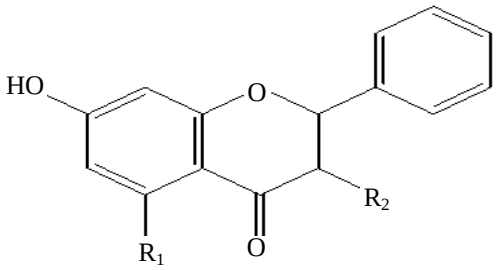
Table 2.2: Identification of phenolic and flavonoid compounds from SA propolis by UPLC-PDA-qTOF-MS/MS.

Peak No.	Rt (min)	UV_{max}	Pseudomolecular ion $[M-H]^-$ (m/z)	MS/MS fragmentation of [M-H]-	Fragment Identification	Compound
1	1.91	238,285, 291,308	179	177, (133)	$[M-H-2H]^-$,$[M-H-2H-CO_2]^-$	Caffeic acid[a]
2	2.29	229,262, 277,306	163	163, (117)	$[M-H]^-$,$[M-H-H_2O-CO]^-$	*p*-Coumaric acid[a]
3	3.26	291	301	-301	$[M-H]^-$	Quercitin[a]
4	3.43	284	285	283,265,(250), 237	$[M-H-2H]^-$,$[M-H-2H-H_2O]^-$, $[M-H-2H-H_2O-CH_3]^-$, $[M-H-2H-H_2O-CO]^-$	Pinobanksin-5-methyl ether[b]
5	3.86	289	271	(269),251,223,	$[M-H-2H]^-$,[M-H-	Pinobanksin[b]

Peak No.	Rt (min)	UV_{max}	Pseudomolecular ion $[M-H]^-$ (m/z)	MS/MS fragmentation of [M-H]-	Fragment Identification	Compound
				195,149, 105	2H-$H_2O]^-$, [M-H-2H-H_2O-$CO]^-$,[M-H-2H-H_2O-2$CO]^-$, [^{1,3}A-2H], [^{1,3}A-2H-CO_2]	
6	4.53	259,350	283	281,266,(237), 209	[M-H-2H]$^-$,[M-H-2H-$CH_3]^-$, [M-H-2H-$CO_2]^-$, [M-H-2H-CO_2-$CO]^-$	Galangin-5-methyl ether[b]
7	5.92	266, 313	253	(251),207,179, 149,105	[M-H-2H]$^-$,[M-H-2H-$CO_2]^-$, [M-H-2H-CO_2-$CO]^-$, [^{1,3}A-2H], [^{1,3}A-2H-CO_2]	Chrysin[b]
8	6.27	288	255	(253),211,185, 169,149,105	[M-H-2H]$^-$,[M-H-2H-$C_2H_2O]^-$, [M-H-2H-$C_3O_3]^-$, [M-H-2H-2$C_2H_2O]^-$, [^{1,3}A-2H], [^{1,3}A-2H-CO_2]	Pinocembrin[b]
9	6.44	264,357	269	(267),211,195, 167	[M-H-2H]$^-$,[M-H-2H-2$CO]^-$, [M-H-2H-CO-$CO_2]^-$, [M-H-2H-2CO-$CO_2]^-$	Galangin[b]
10	6.72	291	313	313,269,(251)	[M-H-2H]$^-$,[M-H-2H- acetate]$^-$, [M-H-2H-acetate-$H_2O]^-$	Pinobanksin-3-*O*-acetate[b]
11	7.32	264	283	281,266,(237), 209	[M-H-2H]$^-$,[M-H-2H-$CH_3]^-$, [M-H-2H-$CO_2]^-$, [M-H-2H-CO_2-$CO]^-$	Tectochrysin[b]
12	9.37	291	327	325,269,(251)	[M-H-2H]$^-$,[M-H-2H- propionate]$^-$, [M-H-2H-propionate -$H_2O]^-$	Pinobanksin-3-*O*-propionate[b]
13	12.42	291	341	339,269,(251)	[M-H-2H]$^-$,[M-H-2H-butyrate/isobutyrate]$^-$, [M-H-2H-butyrate/isobutyrate -$H_2O]^-$	Pinobanksin-3-*O*-butyrate or isobutyrate[b]
14	13.07	290	355	251	[M-H-2H-pentanoate/2-methylbutyrate-	Pinobankin-3-*O*-pentanoate or 2-methylbutyrate[b]

Peak No.	Rt (min)	UV_{max}	Pseudomolecular ion $[M-H]^-$ (m/z)	MS/MS fragmentation of [M-H]-	Fragment Identification	Compound
					$H_2O]^-$	
15	13.42	290	369	269,(251)	[M-H-2H-hexanate]$^-$, [M-H-2H- hexanoate - $H_2O]^-$	Pinobanksin-3-*O*-hexanoate[b]

[a] Confirmed from standard.[b] Confirmed from literature (Pellati *et al*., 2011; Falcão *et al*., 2013)



Pinocembrin - R_1 = OH, R_2 = H

Pinobanksin - R_1 = OH, R_2 = OH

Pinobanksin-5-methyl-ether - R_1 = OCH_3, R_2 = OH

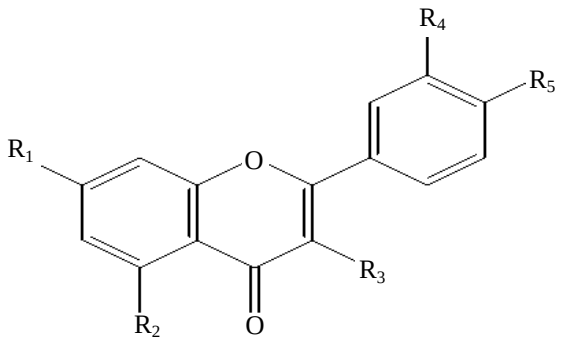
Pinobanksin-3-*O*-acetate - R_1 = OH, R_2 = $OCOCH_3$

Pinobanksin-3-*O*-propionate - R_1 = OH, R_2 = $OCOC_2H_5$

Pinobanksin-3-*O*-butyrate - R_1 = OH, R_2 = $OCOC_3H_7$

Pinobanksin-3-*O*-pentanoate - R_1 = OH, R_2 = $OCOC_4H_9$

Pinobanksin-3-*O*-hexanoate - R_1 = OH, R_2 = $OCOC_5H_{11}$



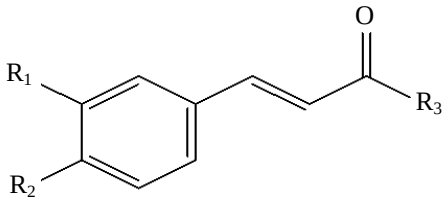
Quercetin - R_1 = OH, R_2 = OH, R_3 = OH, R_4 = OH, R_5 = OH

Chrysin - R_1 = OH, R_2 = OH, R_3 = H, R_4 = H, R_5 = H

Tectochrysin - R_1 = OCH_3, R_2 = OH, R_3 = H, R_4 = H, R_5 = H

Galangin - R_1 = OH, R_2 = OH, R_3 = OH, R_4 = H, R_5 = H

Galangin-5-methyl-ether - R_1 = OH, R_2 = OCH_3, R_3 = OH, R_4 = H, R_5 = H



Caffeic acid - R_1 = OH, R_2 = OH, R_3 = OH

p-Coumaric acid - R_1 = H, R_2 = OH, R_3 = OH

Figure 2.7: Structures of identified phenolic and flavonoid compounds from SA propolis.

2.3.3. Chemometric analysis of UPLC-ESI-MS data of propolis samples

Chemometric studies were undertaken in order to investigate the possibilities of regional variations among SA propolis samples. Figure 2.8a shows a scatter plot of PCA scores for the 42 (39 SA and three Brazilian controls) propolis samples. These plots were constructed using UPLC-ESI-MS data which was pre-treated with MarkerLynx. The PCA plot displayed three distinct clusters with the Brazilian samples (red cluster) falling outside the Hotelling's T^2 (95% CI) in the 2nd quadrant. Few samples from both the Gauteng (GP) and Western Cape (WC) provinces of SA occupy a separate cluster in the first quadrant (green cluster).These are separated from the majority of SA samples which lie within the blue cluster. Samples present in the green cluster demonstrated a chemical variation which resulted in the spatial distribution of these samples within the first quadrant. The majority of SA propolis samples (i.e. blue cluster) exhibit similar chemical profiles, and they are therefore seen to be tightly clustered. The chemical variation within the dataset which was responsible for the formation of these three clusters along PC1 and PC2 was estimated to be 49% ($R2X_{cum}$ (PC1+2)). Figure 2.8b, a corresponding dendrogram shows the three distinct clusters identified with the green cluster showing more variation as compared to the blue cluster.

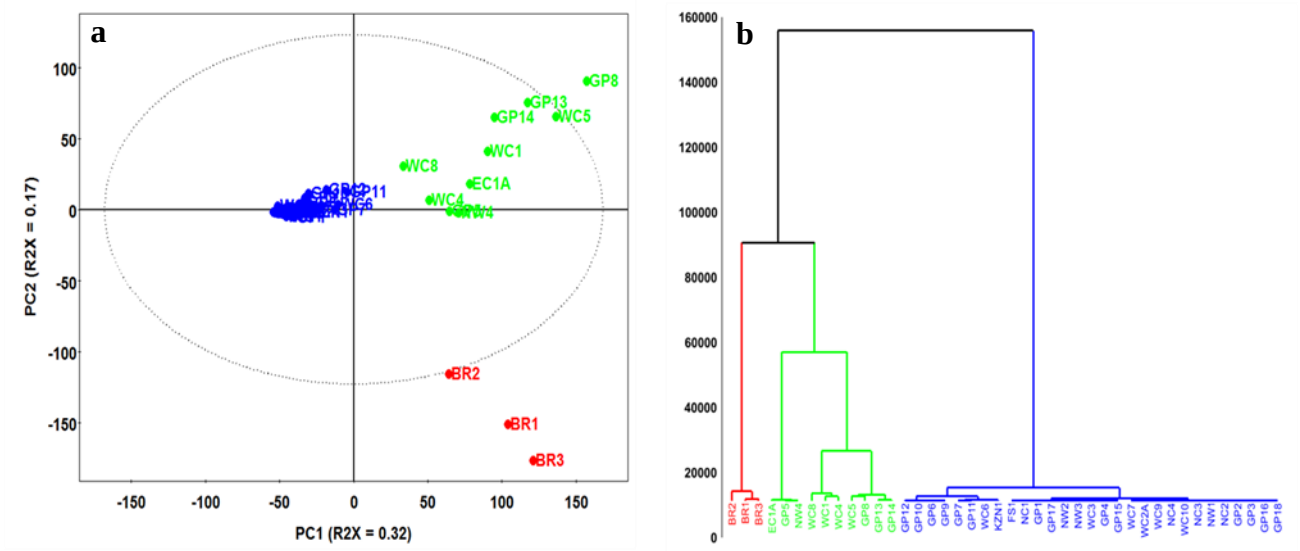


Figure 2.8: A PCA score scatter plot **(a)** showing separation of propolis extracts into three clusters and **(b)** the corresponding dendrogram showing the linkages of the three clusters.

Assigning of classes to the three clusters in Figure 2.8a, allowed for the construction of an OPLS-DA model. Figure 2.9a is an OPLS-DA scores plot, this plot demonstrates a similar pattern of chemical variability as seen in the PCA plot however, tighter clustering of the propolis samples can be seen. The chemical variation observed within the propolis samples related to the differentiation of the clusters (predictive) was estimated at 31% (R2X = 0.31). An orthogonal variation of 16% is what separated the Brazilian propolis from both SA propolis clusters along PC2, this resulted in the red cluster falling outside of the Hotelling's T^2 in the 4^{th} quadrant. This observation demonstrated and confirmed what was seen during HPTLC analysis, UPLC-PDA-qTOF-MS analysis as well as on the UPLC-ESI-MS TIC (Figure 2.5), that is that SA propolis is chemically distinct from Brazilian propolis. The SA propolis samples tested also displayed variable chemical profiles, which resulted in two chemotypes being identified in OPLS-DA. The possibility of the presence of more than two chemotypes in the SA propolis cannot be ignored, as PCA demonstrated in this study that some chemical differences between propolis samples in the green cluster is apparent.
In order to further investigate the chemical differences between the three clusters, a loadings scatter plot (Figure 2.9b) of the X-variables was constructed. This loadings plot displays the relationship between variables and aims to correlate X-variables to patterns that were observed in the scores plot. Based on the position of the observation in question on the scores plot, the X-variables, in a similar position to these, in the loadings plot can be reported to be noteworthyly influential in distinguishing or identifying these observations. Figure 2.9 shows the variables that may be used as markers to differentiate the propolis chemotypes.

Figure 2.10 is an illustration of the LC-MS chromatographs representing each of the three clusters (chemotypes) seen in the PCA and OPLS-DA graphs. In Figure 2.10 each major peak represents a compound that is eluted at that specific retention time and their corresponding abundance; these compounds are reported in Table 2.3. Figure 2.10a shows specifically that the compound displaying a retention time of 12.64 minutes occurs more abundantly in Brazilian propolis than in the SA propolis samples. The green cluster in Figure 2.10a is characterised by high levels of compounds found at 10.96, 12.18 and 13.55 minutes respectively (Figure 2.10b). However, the majority of the SA samples found in the blue cluster, hereby referred to as common SA propolis, showed a more consistent profile which was characterised by compounds eluting at 3.89, 5.86, 6.19, 6.38, 12.37 and 13.41 minutes (Figure 2.10c).

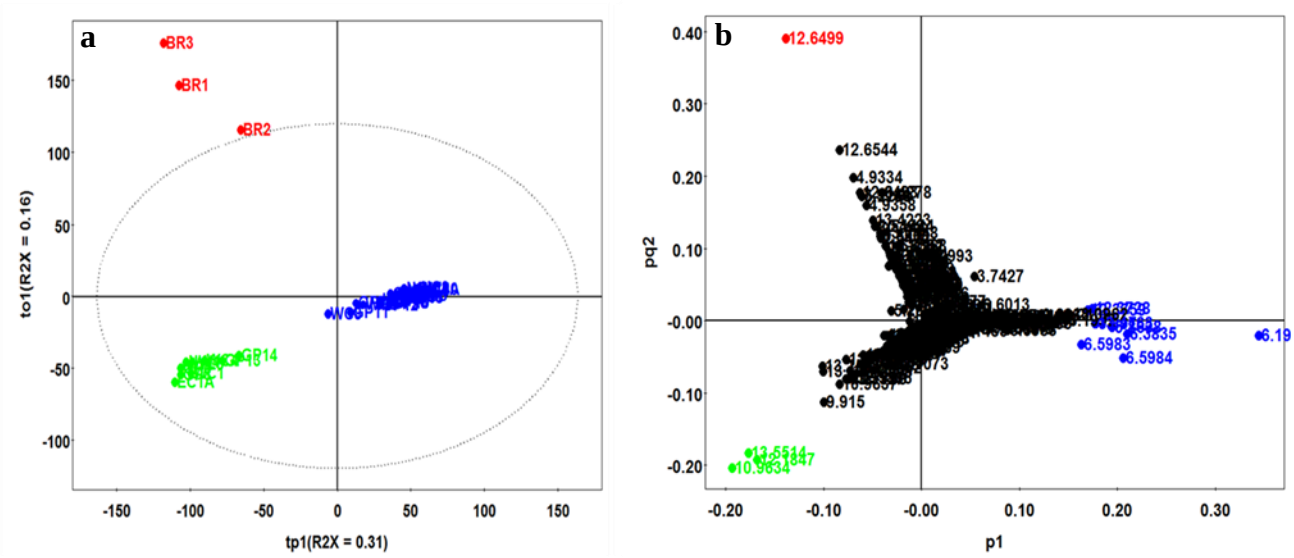


Figure 2.9: An OPLS-DA score scatter plot **(a)** showing separation of propolis extracts into three clusters and the corresponding loadings scatter plot showing the X-variables that are correlated to the three clusters **(b)** are shown.

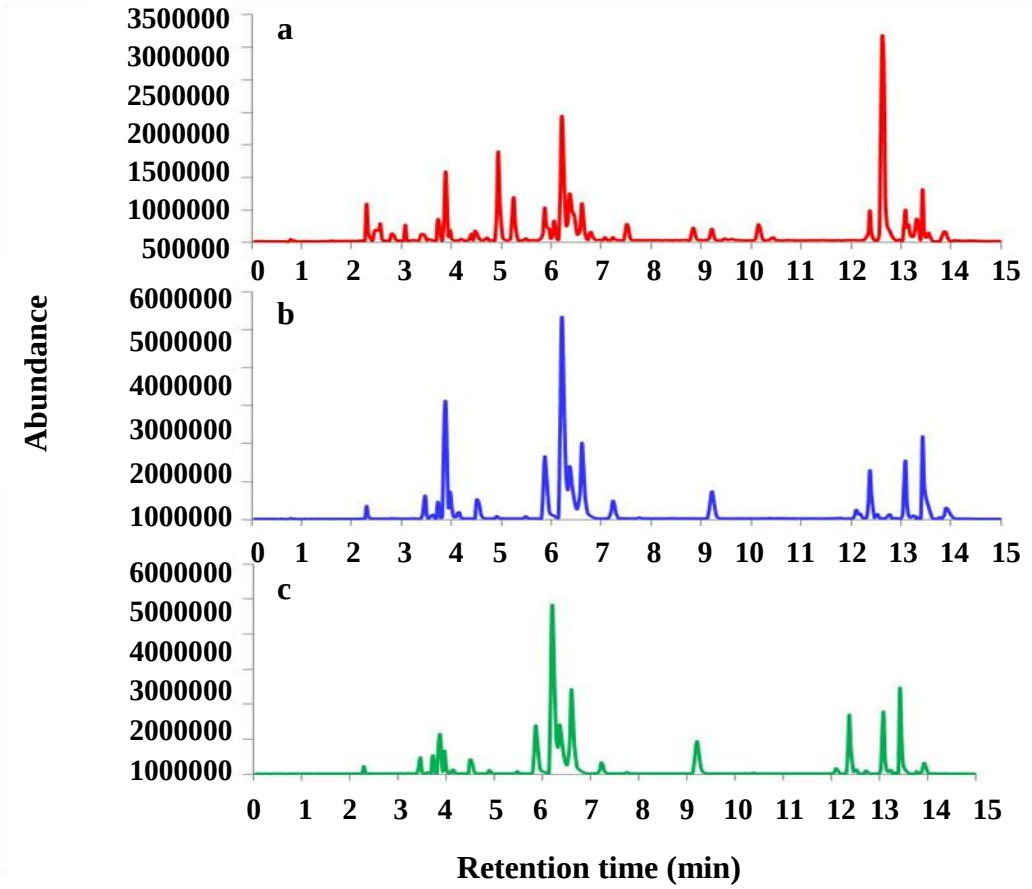


Figure 2.10: LC-MS fingerprint chromatograms representing each of the three chemotypes. **a)**red cluster, **b)** blue cluster and **c)** green clusters.

Table 2.3 shows the marker compounds identified within the three identified clusters, these compounds were identified using chemometric methods and available literature in order to confirm the identities of the molecules. On inspection of the UPLC-ESI-MS chromatograms of three representative samples from the three clusters clear differences in the chemical profiles were observed (Figure 2.10). Ultimately, the chemical profiling of propolis samples has demonstrated clear chemical differences between Brazilian propolis and SA propolis.

Table 2.3: List of marker compounds identified for the three propolis clusters.

Cluster	Retention time(R_t)	m/z ratio	Compound
Red cluster	12.64	299	Artepillin C[b]
Green cluster	10.96	317	Myricetin[b]
	12.18	325	n.i.
	13.55	371	Pentamethoxyflavone[b]
Blue cluster	3.89	271	Pinobanksin[b]
	5.86	253	Chrysin[b]
	6.19	255	Pinocembrin[b]
	6.38	269	Galangin[b]
	12.37	341	Pinobanksin-3-*O*-butyrate or isobutyrate[b]
	13.41	369	Pinobanksin-3-*O*-hexanoate[b]

n.i.: not identified.[b] Confirmed from literature (Hegazi and Abd El Hady, 2002; Gardana *et al*., 2007; Pellati *et al*., 2011; Falcão *et al*., 2013).

2.4. Discussion

Chromatographic fingerprinting techniques such as HPTLC have been established as very promising tools for the standardization of crude drugs, particularly when authentic standards or marker compounds are not available for comparison. HPTLC is a rapid, sensitive, reproducible and low cost fingerprinting technique (Chen *et al*., 2006). UPLC-PDA-qTOF-MS is a powerful tool that has increasingly been used in the fingerprinting and chemical profiling of herbal drugs due to its sensitivity, resolution and throughput capacity (Grata *et al*., 2008; Li *et al*., 2010). This technique provides accurate MS data and UV spectra for the identification of unknown compounds (Li *et al*., 2010). Mass spectroscopic analysis in negative mode was found to be the most sensitive in the determination of flavonoid structures

(Cuyckens and Claeys, 2004). Hence, UPLC-PDA-qTOF-MS/MS in negative mode was used to identify phenolic and flavonoid compounds from SA propolis samples.

The chemical composition of propolis is known to be extremely complex with a vast number of constituents having already been identified (Appendix H). Amongst the constituents found in propolis phenolic compounds (mostly flavonoids) are reported as constituting over 50% of the total weight of any propolis sample (Bankova *et al*., 1996). An earlier study conducted by Kumazawa *et al*. (2004) also reported the presence of pinobanksin, pinobanksin 5-methyl ether, chrysin, pinocembrin, galangin and pinobanksin-3-*O*-acetate in a SA propolis sample tested by the study, however, the presence of quercetin was not reported by Kumazawa *et al*. (2004). As mentioned in Chapter 1, Section 1.5, Zhang *et al*. (2014) reported on a limited number of SA propolis samples obtained from one region of SA, namely KwaZulu-Natal. The study concluded that a majority of SA samples tested displayed chemical profiles similar to temperate region poplar propolis. The overall chemical pattern observed in this study was in alignment with reports by Zhang *et al*. (2014) and led to the conclusion that the majority of SA propolis samples tested in this study displayed a similar profile to that of the common temperate poplar (i.e. European) propolis samples.

Chemometric analysis was conducted in order to further analyse and confirm the distinct chemical difference between SA propolis and the Brazilian controls. Chemometric modelling led to the construction of a PCA plot that displayed 74% of SA samples as having similar chemical profiles and hence were seen as a tight blue cluster, whilst the Brazilian controls were observed as completely separated as a red cluster in Figure 2.8a. The construction of an OPLS-DA model led to a similar pattern as seen in the PCA graph in Figure 2.6a. The advantage of an OPLS-DA plot is that it allows for the variation which is identified in a PCA to be analysed separately as these correlate to the classes (predictive) and orthogonal variation which could be due to other environmental factors. The LC-MS chromatogram (Figure 2.10) provided for another chemical distinction to be made between SA propolis and the Brazilian control samples, thereby confirming patterns seen in both HPTLC and chemometric analysis. From Figure 2.10, SA propolis (Figure 2.10b and c) is once again seen as chemically distinct from Brazilian propolis (Figure 2.10a) which contained an abundant amount of artepillin C (higher peak seen at retention time 12.64 with an abundance of > 30 000), whilst SA propolis was found to contain greater amounts of two compounds in addition to other compounds

reported in Table 2.3, namely pinocembrin and galangin (higher peaks seen at retention times of 6.19 and 6.38, respectively with an abundance of >50 000).

In Chapter 1, Figure 1.4 provided a brief summary of the data tabulated in Appendix H, by highlighting the specific compounds that have been frequently reported in literature reviewed in this study. In alignment with these previous studies and Figure 1.4, this study established findings of the presence of pinobanksin, quercetin, chrysin, galangin, caffeic acid, *p*-coumaric acid and pinocembrin. Furthermore, this study found that these compounds with the exception of *p*-coumaric acid, caffeic acid and quercetin have not been reported in Brazilian propolis to date. In this study the major compound found in the Brazilian comparator samples was artepillin C, also found by other studies (de Paula *et al*., 2009; de Aguiar *et al*., 2013).

2.5. Conclusion

This study is the first comprehensive report on the chemical profiling of SA propolis from various regions using HPTLC, UPLC-PDA-qTOF-MS/MS and chemometric analysis. This approach confirmed that the majority of SA propolis samples are characteristically rich in phenolic acids and flavonoids, and these chemical profiles are highly consistent with propolis produced in the temperate regions of the world. UPLC-PDA-qTOF-MS/MS analysis studies of the 39 SA propolis samples led to the identification of six novel compounds never previously identified in SA propolis. The use of chemometric algorithms has revealed a clear distinction between SA propolis and Brazilian propolis. Variations in the chemical profiles of SA propolis were also observed resulting in two clear clusters each representing a different chemotype. An uncommon type of propolis observed in Gauteng and the Western Cape provinces, which exhibited distinct chemical profiles from propolis produced in the temperate and tropical regions, could be a characteristic and unique chemotype from SA. Findings of this study will contribute to the future chemical characterisation of SA propolis and will assist in the development of quality control standards for raw and finished products containing propolis.

Chapter 3: Antimicrobial efficacy and chemometric modelling of SA propolis.

3.1. Introduction

Globally, research has been dedicated to studying the antimicrobial properties of propolis from various geographical and climatic regions (Appendix G). However, as mentioned in Chapter 1, Section 1.4; from a SA perspective, the antimicrobial investigation of propolis has been neglected. Considering that SA plants has previously been reported as demonstrating promising anti-infective properties (van Vuuren, 2008), the probability that propolis derived from SA flora could potentially be an effective antimicrobial is high. Therefore, an in-depth antimicrobial investigation of propolis from all geographical areas within SA warranted attention.

The objective of this chapter was to investigate the antimicrobial properties of 39 SA propolis samples obtained from different provinces of SA using the minimum inhibitory concentration assay and the minimum bactericidal concentration assay and compare activity to Brazilian control samples. Hereafter, the interactive efficacy of the ten most active SA propolis samples with conventional antimicrobials and an antimicrobially active SA honey sample was determined and the fractional inhibitory concentration (ΣFIC) calculated. The correlation between liquid chromatography-mass spectrometry (LC-MS) chemical data and the antimicrobial activity of propolis extracts was investigated using chemometric modelling.

3.2. Materials and methods

3.2.1. Preparation of extracts

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(Figure 3.1) (Sawaya *et al*., 2004).Extraction took place in an orbital shaker incubator (Labcon) at 37°C for 7 days. Thereafter, ethanol was siphoned off and the extract was allowed to dry at ambient temperature, dry ethanolic extracts of propolis (EEP) were stored at -4°C (Bosio *et al*., 2000; Sawaya *et al*., 2004; Scazzocchio *et al*., 2006;Sawaya *et al*., 2010).

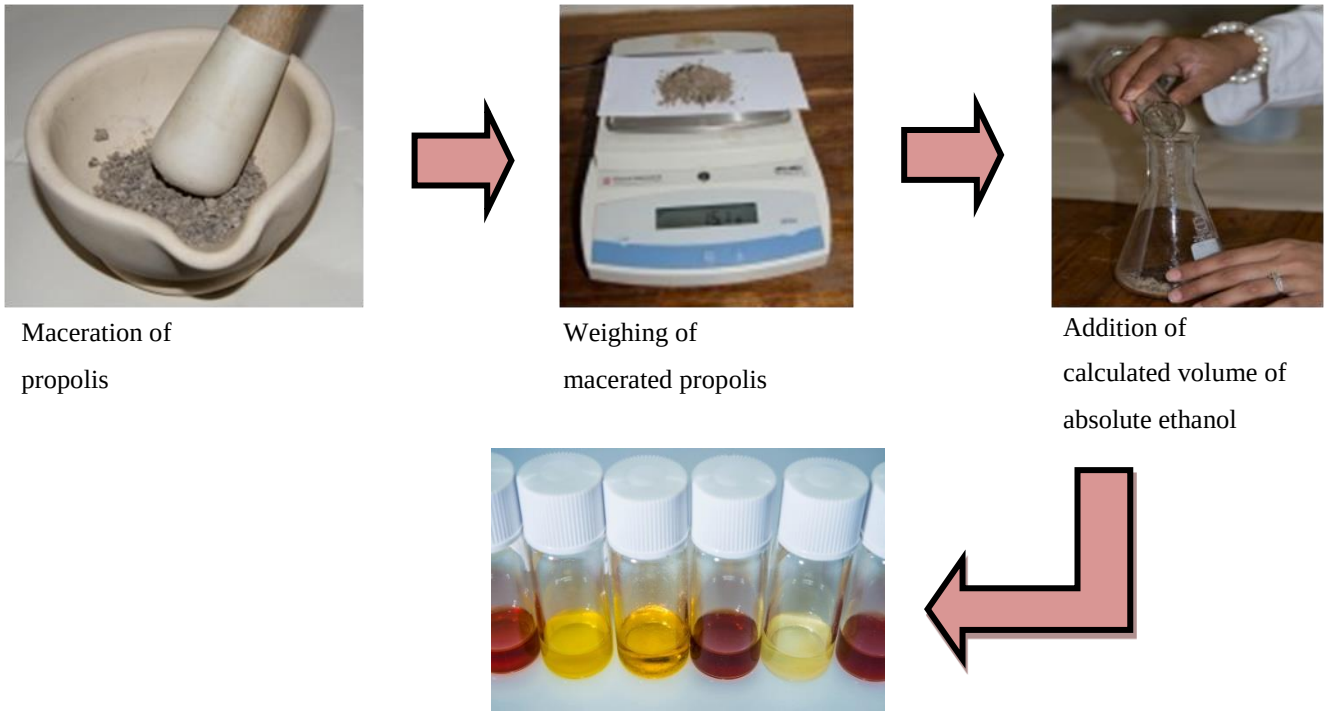


Figure 3.1: Preparation of extracts for antimicrobial studies.

3.2.2. Screening of propolis for antimicrobial activity

3.2.3.1. Preparation of extracts for antimicrobial testing

Samples used for the minimum inhibitory concentration (MIC) assays were prepared by dissolving a known amount of dry crude extract in acetone to a final standard concentration of 25 mg/ml. Acetone was used as the solvent as it is reported to have negligible antimicrobial effects (van Vuuren *et al.*, 2010).

3.2.3.2. Preparation of cultures for antimicrobial assays

Cultures were prepared as stated in the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (2009). Six American Type Culture Collection (ATCC) strains were selected for this study. These included two Gram-positive micro-organisms; *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, two Gram-negative micro-organisms; *Pseudomonas aeruginosa* ATCC 27858, and *Escherichia coli* ATCC 25922; and also included were two yeast strains; *Candida albicans* ATCC 10231 and *Cryptococcus neoformans* ATCC 14116. In order to correlate this study to previous studies conducted worldwide, pathogens were selected based on the most common pathogens tested against by these previous studies (Appendix G). 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; Appendix I).

Bacterial cultures were grown in Tryptone Soya broth (TSB) (Oxoid) at 37°C for 24 hours for bacterial strains and 37°C for 48 hours for yeast strains. All micro-organisms were kept viable by sub-culturing every two weeks. Cultures were streaked out onto Tryptone Soya agar (TSA) plates and incubated under optimal conditions in order to confirm purity of cultures.

3.2.4. Antimicrobial assay

3.2.4.1. Minimum inhibitory concentration assay (MIC)

Serial micro-dilution assays were conducted in order to quantify the MIC values for the ethanolic extracts of propolis (EEP), using *p*-Iodonitrotetrazolium violet solution as an indicator of bacterial/yeast growth (van Vuuren *et al*., 2010).With the aid of aseptic manipulations, 100 µl of sterile deionised water was introduced into each well of a 96 well micro-titre plate. In order to test the antimicrobial properties of SA propolis samples, 100 µl of propolis extracts at a starting concentration of 25 mg/ml in acetone were individually transferred into the top row of the micro-titre plate and serial dilutions were performed, all samples were tested in duplicate to ensure accuracy of results. Each assay was conducted in duplicate to ensure accurate, reproducible results.

Before addition to the micro-titre plates, all cultures used were sub-cultured into suitable broth. Culture was diluted until just turbid (0.5 McFarland standard). This was then adjusted to a 1:100 ratio to ensure an approximate concentration of 1 x 10^6(CFU/ml). Hereafter 100 µl of sub-culture was added to all 96 wells. Each plate was subsequently sealed with a sterile adhesive sealing film (AEC Amersham). This was done to prevent evaporation of the test sample. Micro-titre plates were then incubated at 37°C for 24 hours and at 37°C for 48 hours for bacteria and yeasts respectively. After incubation, 40 µl of a 0.04% $^{w}/_{v}$ *p*-Iodonitrotetrazolium (INT) chloride (0.04% w/v) (Sigma-Aldrich) indicator solution was added to each well of the micro-titre plates. When the indicator INT was added, colour change (from colourless to pink) was monitored within the culture control row. 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 (i.e. no visible growth in that well) (van Vuuren *et al.,* 2010). This observable colour change is dependent on the relevant growth

pattern of the pathogen being tested; it could take anything from two hours (e.g. *E. coli*) to 24 hours after re-incubation (e.g. *C. albicans*) (Figure 3.2).

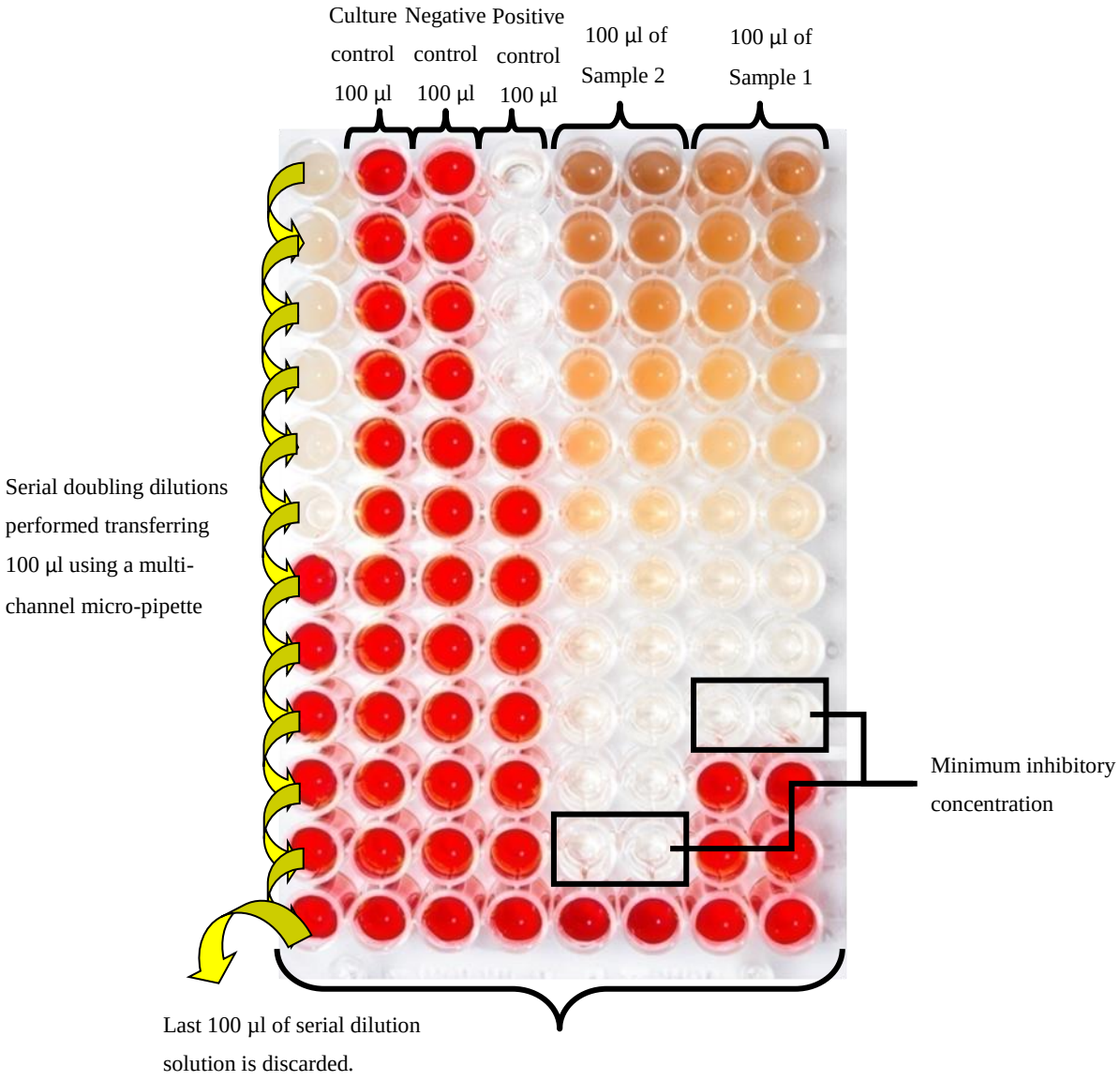


Figure 3.2: Representation of the MIC micro-dilution assay

3.2.4.2. Negative, positive and culture controls

Negative controls of acetone were prepared at 25 mg/ml and were included in order to ascertain if the solvent itself exhibited any antimicrobial effects towards the pathogens tested. Positive controls (conventional antimicrobials) of ciprofloxacin (Sigma-Aldrich) and amphotericin B (Sigma-Aldrich) were used as reference antimicrobial agents to confirm microbial susceptibility. These conventional antimicrobials were selected due to their broad-

spectrum activity against a wide range of pathogens. Positive controls were prepared using sterile deionised water to yield stock concentrations of 0.01 mg/ml (ciprofloxacin) and 0.1 mg/ml (amphotericin B). A culture control was included to verify that the broth was capable of supporting microbial growth. This culture control was also utilized as the standard to read results.

3.2.5. Minimum bactericidal concentration assay (MBC)

The MBC is the lowest concentration of propolis that kills off a micro-organism after sub-culturing 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 TSA plate (Figure 3.3). The plates were incubated at 37°C for 24 hours and 37°C 48 hours for bacteria and yeasts, respectively. Results were recorded as the lowest concentrations of propolis where no growth of the micro-organism was observed.

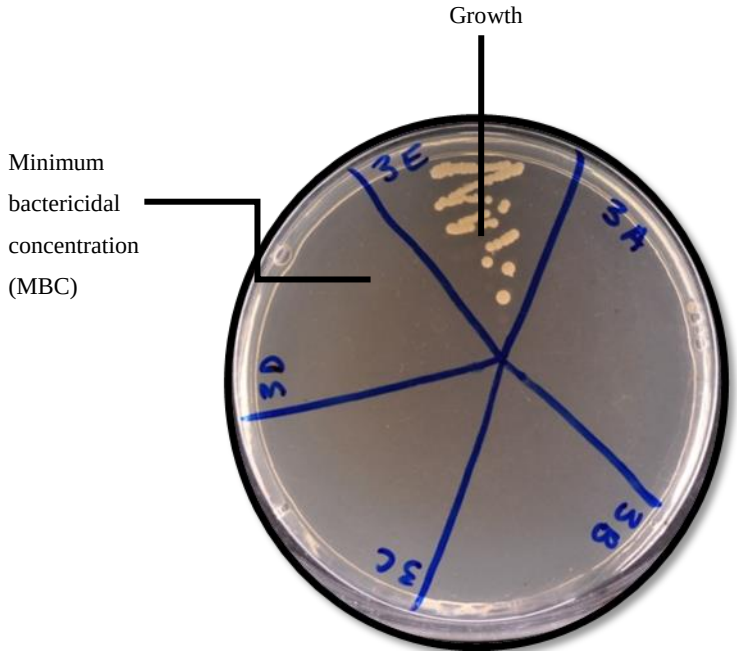


Figure 3.3: Visual representation of minimum bactericidal concentration assay where 3A-E represents decreasing concentrations of propolis; 3A= 6250 µg/ml, 3B= 3125 µg/ml, 3C= 1563 µg/ml, 3D= MBC= 781 µg/ml; 3E=Growth at 391µg/ml.

3.2.6. Interactive efficacy studies with conventional antimicrobials

Ten of the most antimicrobially active propolis samples were investigated for their interactive efficacies, samples were selected based on their promising MIC values correlating to all three

chosen pathogens (i.e. MIC's between 6 - 24 µg/ml against *S. aureus*, MIC's ≤ 195 µg/ml against *P. aeruginosa* and MIC's 12 - 49 µg/ml against *C. neoformans*). These samples were combined with two broad-spectrum commercial antimicrobials specific to the pathogen being tested. Combinations were tested against one Gram-positive bacteria; *S. aureus* (ATCC 25923), one Gram-negative bacteria; *P. aeruginosa* (ATCC 27853), and one yeast; *C. neoformans* (ATCC 14116). Ciprofloxacin and penicillin G were tested against *S. aureus*; ciprofloxacin and gentamicin were tested against *P. aeruginosa* and amphotericin B and nystatin were tested against *C. neoformans.* One Brazilian sample that displayed the lowest MIC value was selected for comparative purposes. The antibiotics were introduced at starting concentrations of 0.01 mg/ml for antibacterials and 0.10 mg/ml for antifungals (van Vuuren and Viljoen, 2006; van Vuuren *et al*., 2010). This MIC assay was carried out as described previously (Chapter 3, Section 3.2.4.1) using a 1:1 ratio (50 µl of propolis and 50 µl of antimicrobial) of propolis to a conventional antimicrobial (i.e. antibacterial/antifungal).

The sum of the fractional inhibitory concentration (ΣFIC) was calculated accordingly and used to determine the interactive correlation between propolis and the conventional antimicrobial. The ΣFIC was calculated using the following equation, where (a) represents the MIC value obtained for the propolis sample and (b) represents the MIC value obtained for the conventional antimicrobial sample (van Vuuren and Viljoen, 2011):

$$\text{FIC (i)} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$\text{FIC (ii)} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

The ΣFIC is then calculated using the following equation; ΣFIC = FIC (i) + FIC (ii). The interactions between the combinations of propolis samples and the conventional antimicrobials were further classified using the sum of the fractional inhibitory concentration (ΣFIC). The interactions were classified as synergistic for ΣFIC values ≤ 0.5, additive for ΣFIC values >0.5-1.0, indifferent for ΣFIC values >1.0 ≤ 4.0, or antagonistic for ΣFIC values >4.0 (van Vuuren and Viljoen, 2011).

3.2.7. Interactive efficacy studies with a natural product derived from the hive

The ten samples tested in combination with conventional antimicrobials were also investigated for their interactive efficacies in combination with a SA honey sample showing the greatest broad-spectrum activity, as identified in a previous study conducted by Khan *et al*. (2014)where a sample was obtained from Mossel Bay (Western Cape province) had a mean MIC % value of 10.42 SD ± 8.27. Combinations were tested against one Gram-positive bacterium; *S. aureus* (ATCC 25923), one Gram-negative bacteria; *P. aeruginosa* (ATCC 27853), and one yeast; *C. neoformans* (ATCC 14116).This assay was conducted on the ten SA propolis samples as tested in the antibiotic: propolis combination component. The honey sample was diluted to a starting concentration of 50% $^{V}/_{V}$ in sterile distilled water. This assay was carried out as described previously (Chapter 3, Section 3.2.4.1) using a 1:1 ratio (50 µl of propolis and 50 µl of honey) of propolis to honey. The ΣFIC was calculated in the same way as in the antibiotic interactive efficacy study with the conventional antimicrobial, and used to determine the interactive correlation between propolis and the honey sample.

3.2.8. Chemometric data analysis to correlate liquid chromatography-mass spectrometry (LC-MS) profiles to antimicrobial activity

In order to correlate the antimicrobial activity to specific chemical constituents in the propolis extracts, a metabolomics approach was applied. Using Simca-P+ 13.0 (Umetrics, Umeå, Sweden) chemometrics software, multivariate data analysis tools were applied to a dataset consisting of both positive and negative liquid chromatography mass spectrometry(LC-MS) profiles of 42 propolis extracts (same dataset used for chemometric analysis in Chapter 2, Section 2.3) and their corresponding MIC values. The propolis extracts were assigned to classes based on the level of antimicrobial activity observed. For the purpose of biomarker identification, extracts with MICs ≤500 µg/ml were classified as having good activity and were assigned to class 1. Conversely, extracts with MICs ≥ 500 µg/ml were considered to be poorly active and assigned to class 2. A dummy Y-variable was assigned to this classification to allow for an orthogonal projection to latent structures (OPLS) and a model was constructed.

Initially, PCA was performed on the X-data (LC-MS) to observe chemical variation within the propolis extracts. The X-data was 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 were performed using mass spectrometry (MS) fragment comparisons, library database searches and literature reviews.

3.3. Results

3.3.1. Antimicrobial assay

The average MICs and MBCs of each of the 39EEP samples collected from various geographical regions of SA as well as the overall average activity across all pathogens tested are reported in Table 3.2 and their percentage yields are reported in Chapter 2, Table 2.1.

Propolis extracts where MIC values are <125 µg/ml are considered as having noteworthy activity, propolis extracts where MIC values are 125 - 500 µg/ml are considered as having moderate activity and propolis extracts where MIC values are >500 µg/ml are considered as having weak activity (Alencar *et al*., 2007; Velazquez *et al*., 2007; Seidal *et al*., 2008). The inhibitory activity of EEP against all pathogens tested ranged from 6 to 1563 µg/ml. According to the criteria defined herewith, 28 (i.e. 71%) propolis extracts displayed noteworthy activity (MIC ≤ 125 µg/ml) against *S. aureus*. Three propolis extracts, GP9, NW2 and WC8, displayed noteworthy activity (MIC ≤ 125 µg/ml) against *E. faecalis*. Two propolis extracts, WC9 and WC10, displayed noteworthy activity (MIC ≤ 125 µg/ml) against *C. albicans*. Thirty-one (i. e. 79%) propolis extracts displayed noteworthy activity when tested against *C. neoformans*. Three propolis samples originating from Honeydew (GP9), Edenvale (GP11), and the southern suburbs of Cape Town (WC8), Gauteng, and Western Cape, respectively were found to display exceptional noteworthy activity with MICs as low as 6 µg/ml against *S. aureus*.

More than half of the SA propolis samples, when tested against *S. aureus,* displayed MIC values lower than the Brazilian propolis extracts which were added as comparative controls. Of the 39 SA samples tested, 30% showed better inhibition than the Brazilian comparator when tested against the Gram-negative *P. aeruginosa* strain. Furthermore, when tested against the yeast species *C. neoformans,* 77% displayed greater inhibition with MIC values

(GP9- 12 µg/ml) lower than that of the Brazilian samples. Against *C. albicans* 64% of the 39 SA EEP samples tested showed better inhibition than two of the Brazilian control EEPs, BR1 and BR3. Of the 39 SA EEP samples, 56% showed inhibitory activity against *E.coli* equivalent to the inhibitory activity of all three Brazilian control EEPs and one sample WC9 from the Southern suburbs of Cape Town in the Western Cape displayed inhibitory activity of 391 µg/ml.

The average antimicrobial activity ranged from 155-1496 µg/ml (Table 3.2). In order to compare against all three Brazilian samples, the average broad-spectrum activity of all three samples was determined. Of the 39SA propolis samples tested it was found that 56% displayed better average inhibitory antimicrobial activity (i.e. against all six pathogens tested) than the three Brazilian control samples tested.

The break point expectation ranges for the conventional antimicrobials used as positive controls in this study; ciprofloxacin and amphotericin B are displayed in Table 3.1. All conventional antimicrobials used as positive controls against each pathogen in this study fell within the break point expectation ranges. This correlation was undertaken in order to ensure that the assay was responding to antimicrobials in a predictable manner and thus confirm methodology accuracy (CLSI, 2012).

Table 3.1: Breakpoint expectation ranges of conventional antimicrobials (µg/ml).

Micro-organism	**Ciprofloxacin**	**Micro-organism**	**Ciprofloxacin**	**Micro-organism**	**Amphotericin**
S. aureus	0.12-0.5	*E. coli*	0.004-0.016	*C. albicans*	-
E. faecalis	0.25-2	*P. aeruginosa*	0.25-1	*C. neoformans*	-

– no values could be found in available literature (CLSI guidelines, 2012; van Vuuren, 2007)

The MBC assays undertaken demonstrated that the propolis demonstrated noteworthy (≤ 125 µg/ml) cidal activity against the Gram-positive *S. aureus* and yeast *C. neoformans* (Table 3.2). 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 against *S. aureus*.

Table 3.2: Antimicrobial activity (µg/ml) of SA propolis against six pathogens.

Code	Source of sample	*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														
EC1	Baviaanskloof - PE	1563	1563	1563	1563	781	1563	391	>6250	781	781	391	391	912
Free State province														
FS1	Bloemfontein	49	49	391	521	1563	>6250	195	6250	391	521	98	98	448
Gauteng province														
GP17	Beaulieu - Midrand	24	24	195	195	1563	>6250	391	>6250	195	293	49	49	403
GP19	Devon - Sedibeng area	49	49	391	391	781	>6250	391	3125	195	293	98	98	318
GP10	Edenvale 1	24	24	391	586	781	>6250	195	>6250	391	586	98	98	313
GP11	Edenvale 2	6	9	195	488	781	>6250	195	6250	391	391	49	49	270
GP9	Honeydew	6	6	49	49	781	2604	391	1563	195	456	12	49	239
GP5	Johannesburg 1	49	49	195	586	781	>6250	391	3125	391	391	49	65	309
GP6	Johannesburg 2	195	456	1563	6250	781	>6250	391	>6250	781	781	24	49	623
GP7	Johannesburg 3	24	37	391	2344	1563	>6250	195	>6250	781	781	49	65	501
GP8	Lakeside/Westlake	98	98	781	2604	1563	>6250	195	>6250	391	521	49	49	513
GP14	Lydiana Gardens 1-Pretoria	98	98	1563	1563	781	1563	391	>6250	781	1953	195	195	635
GP15	Lydiana Gardens 2-Pretoria	24	37	781	1172	1563	>6250	391	6250	781	1953	49	49	598
GP1	Northern Pretoria	391	391	781	1172	1563	>6250	391	>6250	781	781	98	260	668
GP18	President Park –	49	49	391	391	781	3125	391	1563	195	391	24	24	305

Code	Source of sample	*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	
	Midrand													
GP12	Pretoria 1	24	37	781	781	781	>6250	391	>6250	781	781	195	195	492
GP13	Pretoria 2	24	24	781	781	781	>6250	391	3125	391	391	98	98	411
GP3	Springs	24	41	195	195	1563	>6250	391	1563	195	260	49	65	403
GP2	Walkerville (Vereeniging)	98	98	195	195	781	>6250	391	1563	195	391	98	98	293
GP16	Wilgerivier – Bronkhorstspruit	49	98	391	391	781	>6250	391	>6250	391	586	98	98	350
KwaZulu-Natal province														
KZN1	KwaZulu-Natal 1	195	195	781	781	781	1563	391	3125	391	586	195	195	456
Northern Cape province														
NC3	Douglas	49	65	391	2344	1563	>6250	195	6250	391	521	98	98	448
NC2	Northern Cape 1	195	586	781	3646	781	>6250	391	>6250	391	651	98	130	440
NC4	Northern Cape 2	24	24	195	195	781	1563	195	3125	195	293	24	24	236
NC1	Orange River	1563	1563	1563	2344	1563	>6250	391	>6250	391	521	98	130	928
North-West province														
NW2	Christiana 1	49	74	98	147	781	>6250	391	>6250	195	260	98	98	269
NW3	Christiana 2	49	49	195	195	781	>6250	195	>6250	195	260	98	98	252
NW4	Mooinooi	1563	3125	1563	6250	1563	>6250	781	3125	3125	3125	391	391	1496
NW1	North West	391	391	1563	1563	1563	>6250	391	>6250	781	781	195	195	814
Western Cape province														
WC6	Beaufort West	1563	>6250	1563	1563	781	>6250	781	>6250	1563	1172	195	391	1074

Code	Source of sample	*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	
WC4	Botrivier	195	195	781	2084	1563	>6250	391	>6250	781	1042	98	195	635
WC5	Graafwater	391	651	1563	3907	1563	>6250	781	>6250	781	781	195	260	879
WC1	Outeniqua Mountains, Oudtshoorn	12	12	195	293	1563	6250	391	>6250	781	781	98	130	507
WC3	Somerset West	391	521	1563	6250	1563	>6250	391	>6250	391	781	195	195	749
WC8	Southern Suburbs - Cape Town 1	6	6	98	98	781	1563	195	2604	195	488	24	24	217
WC9	Southern Suburbs - Cape Town 2	24	24	195	195	391	1563	195	>6250	98	147	24	24	155
WC10	Southern Suburbs - Cape Town 3	24	49	195	195	1563	>6250	391	>6250	98	147	49	49	387
WC2	Western Cape 1	195	228	781	3125	781	>6250	195	>6250	781	781	195	130	488
WC7	Western Cape 2	24	24	195	195	781	3125	195	3125	195	195	49	49	240
South America														
BR1	Brazil 1	98	195	781	1953	781	>6250	391	3125	781	781	195	195	505
BR2	Brazil 2	98	147	781	781	781	>6250	391	>6250	195	651	195	195	407
BR3	Brazil 3	195	260	781	781	781	>6250	391	>6250	781	781	24	41	492
	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		

* MIC calculated across all six pathogens and averaged; **Ciprofloxacin used for bacterial strains and Amphotericin B for yeast strains

Of the 39 SA EEP samples tested, 67% displayed a cidal activity at the same inhibitory concentration for *S. aureus*. This demonstrates that the majority of the EEP samples not only inhibited the pathogen but also demonstrated cidal effects equivalent to the inhibitory concentrations. Similar effects were noted for *C. neoformans*. For *E. faecalis* and *C. albicans* 41% of all EEP samples displayed cidal effects equivalent to the inhibitory efficacy.

3.3.1 Interactive efficacy studies

Interactive efficacy studies were conducted on ten of the most active SA propolis samples and one Brazilian comparator, against conventional antimicrobials. Of the 60 combinations (EEP + ciprofloxacin/gentamicin/penicillin G/amphotericin B/nystatin) tested, nine combinations displayed synergism and 16 combinations displayed additive activity (Table 3.3). A sample obtained from Springs in Gauteng, displayed enhanced synergistic interactive efficacy when combined with gentamicin (ΣFIC of 0.19) against *P. aeruginosa* (Table 3.3). Synergism was also noted with samples from Northern Cape and Western Cape (ΣFIC of 0.31) and Honeydew (ΣFIC of 0.37) in combination with gentamicin against *P. aeruginosa*. When propolis was combined with ciprofloxacin, more than half of the combinations displayed additive activity against *P. aeruginosa*, however, three samples, from Outeniqua mountains (WC1), Oudtshoorn, Springs and Honeydew displayed synergism (ΣFIC 0.31-0.37). Two samples, from Honeydew and Southern suburbs of Cape Town displayed synergism (ΣFIC of 0.50) when combined with ciprofloxacin against *S. aureus*. Combinations of propolis with antifungals yielded mainly non-interactive efficacies. The Brazilian sample tested during interactive efficacy studies showed only additive and non-interactive interactions, and was found to be the only sample to display antagonism when combined with amphotericin B and nystatin, against *C. neoformans.*

Interactive efficacy studies were also conducted on ten of the most active SA propolis samples and one Brazilian comparator, against an antimicrobially active SA honey sample (Khan *et al*., 2014). Of the 30 combinations (EEP + honey) tested, two combinations displayed synergistic activity (ΣFIC of 0.25 and 0.5) and 16 combinations displayed additive activity (Table 3.4). A sample obtained from the southern suburbs of Cape Town, Western Cape, displayed enhanced synergistic activity with a ΣFIC as low as 0.25 against *C. neoformans* (Table 3.4).

Table 3.3: Interactive efficacy of SA propolis with conventional antimicrobials.

Location	*S. aureus* (ATCC 25923)				*P. aeruginosa* (ATCC 27853)				*C. neoformans* (ATCC 14116)			
	Ciprofloxacin		Penicillin G		Ciprofloxacin		Gentamicin		Amphotericin B		Nystatin	
	ΣFIC	Interpretation	ΣFIC	Interpretation	ΣFIC	Interpretation	ΣFIC	Interpretation	ΣFIC	Interpretation	ΣFIC	Interpretation
Outeniqua Mountains, Oudtshoorn	1.02	Non-interactive	2.54	Non-interactive	**0.37**	**Synergistic**	0.75	Additive	1.49	Non-interactive	1.24	Non-interactive
Springs	1.05	Non-interactive	3.02	Non-interactive	**0.37**	**Synergistic**	**0.19**	**Synergistic**	1.25	Non-interactive	1.13	Non-interactive
Johannesburg	0.52	Additive	1.52	Non-interactive	1.25	Non-interactive	1.25	Non-interactive	2.49	Non-interactive	1.13	Non-interactive
Brazil	0.62	Additive	2.45	Non-interactive	0.75	Additive	1.50	Non-interactive	*4.56*	*Antagonistic*	*8.65*	*Antagonistic*
Honeydew	**0.50**	**Synergistic**	0.56	Additive	**0.31**	**Synergistic**	**0.37**	**Synergistic**	1.06	Non-interactive	0.52	Additive
Edenvale	1.01	Non-interactive	2.25	Non-interactive	0.56	Additive	0.62	Additive	1.25	Non-interactive	1.13	Non-interactive
Northern Cape	1.05	Non-interactive	1.52	Non-interactive	0.56	Additive	**0.31**	**Synergistic**	2.29	Non-interactive	2.17	Non-interactive
Western Cape	0.52	Additive	1.52	Non-interactive	0.56	Additive	**0.31**	**Synergistic**	1.25	Non-interactive	1.13	Non-interactive
Southern Suburbs-Cape Town	**0.50**	**Synergistic**	2.25	Non-interactive	0.62	Additive	0.62	Additive	0.56	Additive	1.08	Non-interactive
Southern Suburbs-Cape Town	2.10	Non-interactive	1.52	Non-interactive	0.62	Additive	0.62	Additive	2.29	Non-interactive	2.17	Non-interactive

synergistic interactions highlighted in bold; antagonistic interactions highlighted in italics

The Brazilian sample tested displayed additive and non-interactive properties, and was found to be the sample to display the greatest antagonism when combined and tested against *C. neoformans* with a ΣFIC as high as 8.21. One SA sample from the Outeniqua mountains (WC1), Oudtshoorn in the Western Cape was found to display antagonism against *S. aureus* with a ΣFIC of 4.09.

Table 3.4: Interactive efficacy of SA propolis with honey.

Location	Honey					
	S. aureus		***P. aeruginosa***		***C. neoformans***	
	(ATCC 25923)		**(ATCC 27853)**		**(ATCC 14116)**	
	ΣFIC	**Interpretation**	**ΣFIC**	**Interpretation**	**ΣFIC**	**Interpretation**
Outeniqua Mountains, Oudtshoorn	*4.09*	*Antagonistic*	1.06	Non-interactive	2.06	Non-interactive
Springs	2.05	Non-interactive	0.53	Additive	1.02	Additive
Johannesburg	1.02	Additive	2.07	Non-interactive	1.02	Additive
Brazil	1.03	Additive	1.06	Non-interactive	*8.21*	*Antagonistic*
Honeydew	1.00	Additive	0.53	Additive	**0.50**	**Synergistic**
Edenvale	2.00	Non-interactive	1.03	Additive	1.02	Additive
Northern Cape	2.05	Non-interactive	0.52	Additive	2.06	Non-interactive
Western Cape	1.02	Additive	0.52	Additive	0.51	Additive
Southern Suburbs - Cape Town	2.00	Non-interactive	1.03	Additive	0.25	**Synergistic**
Southern Suburbs - Cape Town	1.02	Additive	0.52	Additive	2.06	Non-interactive

synergistic interactions highlighted in bold; antagonistic interactions displayed in italics

3.3.3. Chemometric data analysis correlating LC-MS profiles with antimicrobial activity

The correlation between chemical data and antimicrobial activity was investigated using multivariate data analysis tools. Two OPLS models were created for the two Gram-positive bacteria (*E. faecalis* and *S. aureus*) and *C. albicans* where higher antimicrobial activities were observed.

Figure 3.4 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 *E. 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 *E. faecalis*.

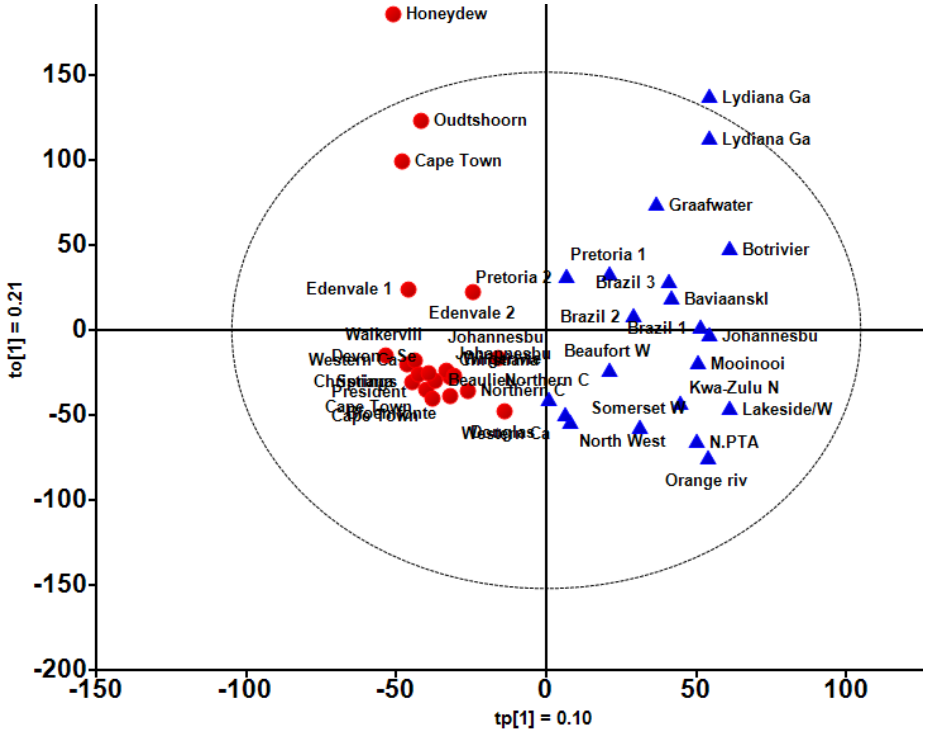


Figure 3.4: OPLS scores plot showing separation of active propolis (●) and poorly active propolis (▲) extracts against *E. faecalis*.

For illustration purposes, a detailed explanation of the model developed for *E. faecalis* is provided whilst a summary of the model statistics and the biomarkers identified for all three micro-organisms is provided in Table 3.5. Using the filtered retention times (min) in combination with the mass-scan number combinations, it was possible to identify the active constituents in propolis for the three models (Table 3.5). Table 3.5 lists the active constituents in propolis as chrysin, pinocembrin,

galangin and pinobanksin-3-*O*-acetate.To investigate the bioactive markers within the propolis extracts, an S-plot of covariance and correlation was constructed for the X-variables (Figure 3.5). 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.

Figure 3.6 is the UPLC-ESI$^+$-MS chromatograph of the ethanol 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 can be concluded that extracts containing higher levels chrysin, pinocembrin, and galangin are more likely to possess higher antimicrobial activity when compared to the others.

Table 3.5: Model statistics and identified biomarkers contributing to good activity of propolis extracts against selected micro-organisms.

Organism	Model statistics	Retention time (min) (Biomarkers)	Mass	Identity
E. 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
S. 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
C. 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

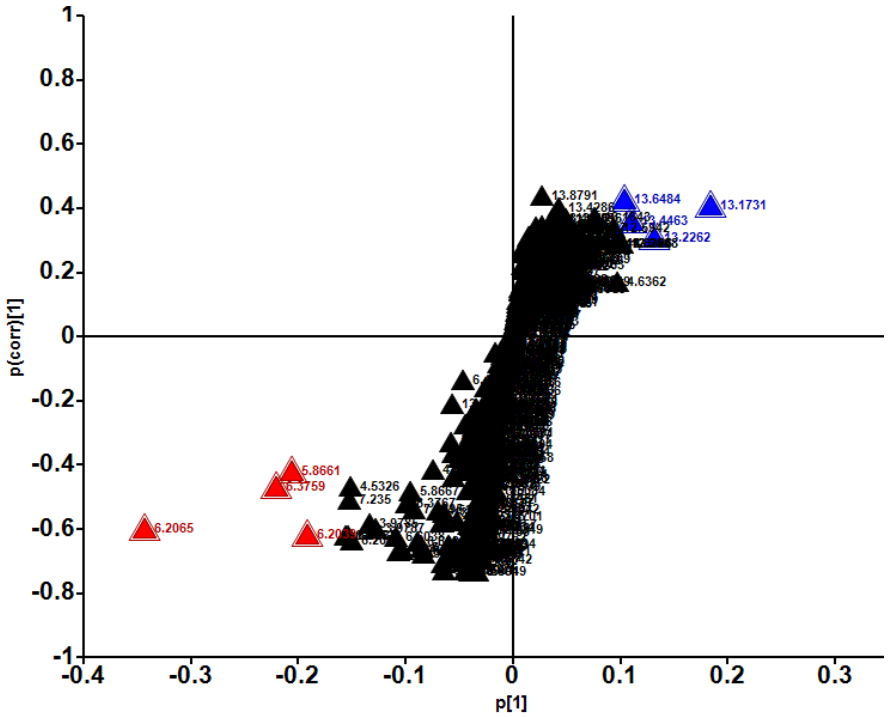


Figure 3.5: An S-plot showing putative biomarkers that have been identified as contributing noteworthyly to good activity (▲left bottom quadrant) and poor activity (▲top right quadrant) against *E. faecalis*.

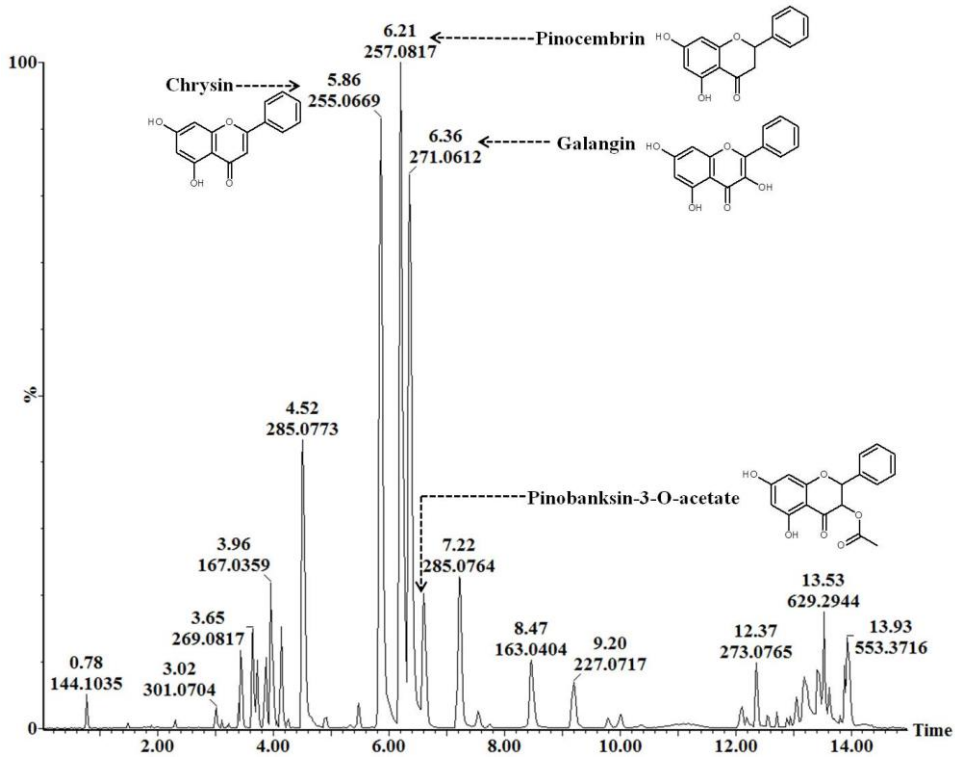


Figure 3.6: UPLC-ESI^{+}-MS chromatogram of propolis ethanol extract showing the biomarkers contributing noteworthyly to the antimicrobial activity as identified in the S-plot.

3.4. Discussion

As seen in Chapter 2, Table 2.1, a majority of samples had higher yields. These samples were noted to display better antimicrobial activity with the exception of an outlier (WC2), which had a low yield of 7.27% but displayed a noteworthy antimicrobial activity of 6 and 24 µg/ml against *S. aureus* and *C. neoformans*, respectively. Highlighted by this study in Chapter 1, Figure 1.3, is the fact that propolis from Africa and South Africa in particular is severely understudied, this study reported EEP samples from SA as displaying activity better than the vastly studied "gold standard" Brazilian propolis (South America), and European propolis samples (Turkish, Italian, Iranian, etc).

Propolis from regions of Argentina have previously been reported to exhibit better Gram-positive than Gram-negative activity with MICs as low as 15.30 µg/ml against *S. aureus* (Nieva Moreno *et al*., 1999). The antimicrobial activity of propolis from various regions of Anatolia, Turkey, reported low MICs against Gram-positive *S. aureus* (8 - 16 µg/ml), Gram-negative *P. aeruginosa* (32 - 256 µg/ml) *E. coli* (16 - 128 µg/ml), and the yeast *Candida albicans* (4 - 32 µg/ml) (Uzel *et al*., 2005). Furthermore, another study conducted on propolis from Turkey also reported a low MIC against Gram-positive *S. aureus* (9 µg/ml) (Keskin *et al*., 2001).Propolis from Oman was found to display MICs ranging from 42 - 169 µg/ml against *S. aureus* and 169 - 356 µg/ml for *E. coli* (Popova *et al.*, 2013). In another study conducted on Brazilian red propolis from Maceió and the state of Algoas, the growth of *S. aureus* was reported to be inhibited by very low concentrations (exact concentration is not reported), MICs of 256 µg/ml were reported against *P. aeruginosa* and *C. albicans* and 512 µg/ml against *E. faecalis* and *E. coli* (Righi *et al.*, 2011).

In comparison to these previous studies on the inhibitory efficacy of propolis (Nieva Moreno *et al.*, 1999; Uzel *et al.*, 2005; Righi *et al.*, 2011; Popova *et al.*, 2013), this study demonstrated that three SA propolis samples, GP9, GP11 and WC8 (Table 3.2) displayed greater inhibitory efficacy than Brazilian propolis. Furthermore, activities from the SA samples were higher than propolis samples from other regions of the world (Suleman *et al*., 2015). It was noted that the majority of these whole propolis extracts, when tested against Gram-positive *S. aureus*, and the yeast species *C. neoformans*, demonstrated activities comparable to antimicrobial activities displayed by isolated bioactive compounds(van Vuuren, 2008), thus suggesting a natural product with superior activity. Predominant cidal activity against Gram-positive bacteria and yeasts was also observed. Keskin *et al*. (2001), tested EEP samples from Turkey and reported an MBC against *S. aureus* of 16 µg/ml. In comparison, this current study reported that three EEP samples from SA, namely; WC8, GP9 and

GP11displayed better cidal activity than that reported in literature with MBCs of 6 µg/ml (WC8 and GP9) and 9 µg/ml (GP11) against *S. aureus*.

It was noted that all propolis extracts tested were distinctive in colour, the colour of samples ranged from colourless/whitish-grey → light yellow-brown/golden → dark reddish-brown/maroon (Figure 3.7). A majority of samples (74% against *S.* aureus; 28-33% against *P. aeruginosa, C. albicans* and *E. faecalis* respectively; 93% against *C. neoformans*) ranging in colour from light yellow-brown/golden → dark reddish-brown/maroon were the samples that displayed exceptional inhibition with MICs ≤ 195 µg/ml. Dark reddish-brown/maroon extracts (e.g. GP9 and WC8) were found to display greater inhibition against Gram-positive *S. aureus* and the yeast *C. neoformans* than colourless/whitish grey extracts (e.g. WC6 and NW4) (Table 3.2).Although seasonality and age of propolis has been mentioned and studied previously (Bonvehí *et al*., 2000; Teixeira *et al*., 2010; de Souza *et al*., 2014; Schmidt *et al*., 2014), extract colour has not been explored or reported as a possible factor correlating to biological activity.

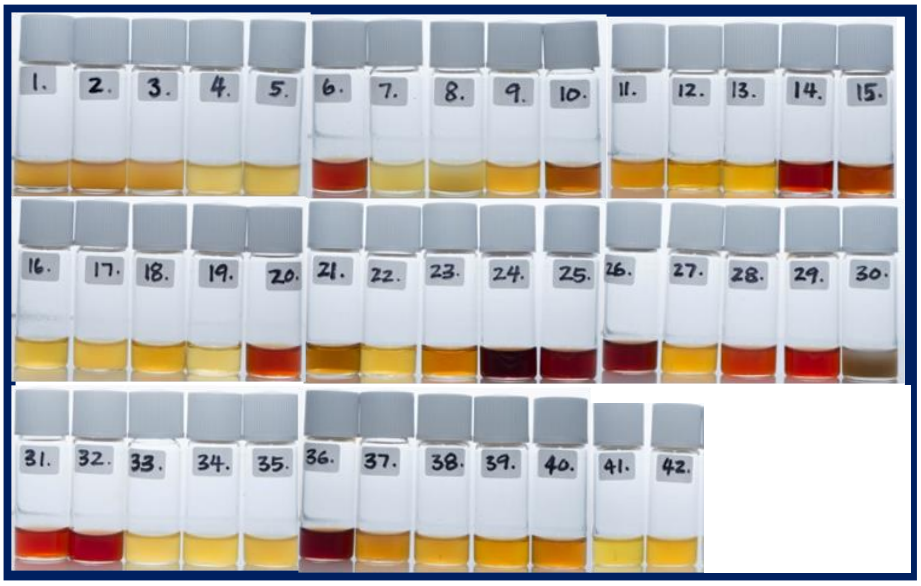


Figure 3.7: Visual representation of colour variances between EEP samples. (colourless/whitish grey (e.g. sample 30) → light yellow-brown/golden (e.g. samples 19, 22 and 23) → dark reddish-brown/maroon (e.g. samples 14, 20 and 24))

The use of propolis independently, as well as in combinations with other natural products and conventional antimicrobials has shown a dramatic increase in popularity (Miguel and Antunes,

2011). Resistance to current conventional antimicrobials is of a growing concern in the industry of medical health. Hence, scientists are relentlessly looking for an unconventional means of inhibiting microbial growth as well as enhancing the potencies of currently available conventional antimicrobials. Propolis has also previously been reported to displaying synergism with conventional antimicrobials such as clarithromycin, ampicillin, ceftriaxone, doxycycline, amikacin, nystatin, ciprofloxacin and more (Chapter 1) (Stepanović *et al.*, 2003; Fernandes *et al*., 2005; Orsi *et al*., 2006; Onlen *et al*., 2007; Rahman *et al*., 2010; Helaly *et al*., 2011; Naher *et al*., 2011; Orsi *et al*., 2012). This study found that nine combinations displayed synergism with conventional antimicrobials, including ciprofloxacin and nystatin. Mantovani *et al*. (2008) postulated that synergistic interactions may be linked to the flavonoid content of the sample.

Hübsch *et al*. (2014) reported that most combination studies found in the literature emphasized mainly synergistic interactions; however, the reporting of antagonism has been greatly neglected. In the current study an antagonistic interaction was identified when Brazilian propolis was combined with antifungals, amphotericin B and nystatin against *C. neoformans.* Also, when Brazilian propolis was combined with another natural product of the hive, honey, against *S. aureus* and *C. neoformans*, an antagonistic interaction was seen. In light of this finding, combinations of propolis (against yeasts) with antifungals and other natural products should always be administered with caution and in a controlled environment, such as a hospital or clinic environment where any allergic reaction or anaphylaxis can be easily treated.

A study conducted in Nigeria by Adewumi and Ogunjinmi (2011), also combined propolis with honey as was done in the current study. The honey and propolis were combined in a lotion and applied to the septic wounds of patients, three times a day. The study reported a 100% healing rate by the end of the 15th day, however, the study used no control sample (e.g. known lotion used for wound healing). As suggested by the aforementioned study, this current study also found that some EEP samples displayed synergistic activity when combined with an antimicrobially active SA honey sample. Propolis has also been combined with other natural products such as; Aloe vera, myrrh, garlic, essential oils and minerals like calcium hydroxide and zinc (in the treatment of otitis media in children) (Lofty *et al*., 2006; de Rezende *et al*., 2008; Bertolini *et al*., 2010; Marchisio *et al*., 2010; Probst *et al.*, 2011; Maekawa *et al*., 2013, Moreno-Cruz *et al*., 2014).

As elaborated previously in Chapter 1, the chemical composition of propolis is largely variable. 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 (Hernández *et al.,* 2005; Lotti *et al.,* 2010; Righi *et al.,* 2011). Our earlier study (Kasote *et al.,* 2014a) 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 compound, a derivative of *p*-coumaric acid, 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 (Hegazi and Abd El Hady, 2001; Bosio *et al.,* 2000; Cushnie and Lamb, 2005). Due to the high flavonoid content, propolis is noted to have good activity against dermatophytes as well as some *Candida* spp. (Cafarchia *et al.,* 1999; Cushnie and Lamb, 2005). Chrysin, a flavonoid 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.,* 2014b).

In our previous study, the chemical profiling techniques demonstrated that SA samples were chemically distinct from the Brazilian comparator samples (Kasote *et al.,* 2014a). LC-MS analysis found that pinocembrin, chrysin, galangin and pinobanksin are the major constituents of SA propolis, whilst the major constituent of the Brazilian propolis samples tested was artepillin C. Pinocembrin and chrysin are flavanones, while galangin is a flavonol and pinobanksin-3-*O*-acetate a dihydroflavonol (flavonol). 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 (250 µg/ml)

and 3.40 mg/ml (3400 µg/ml) against *S. aureus*, and 0.10 mg/ml (100 µg/ml) and 0.05 mg/ml(50 µg/ml) against *C. albicans*. The activity of 23 EEP samples tested in the current study against *S. aureus* (6 - 49 µg/ml) and 15 EEP samples tested against *C. neoformans* (12 - 49 µg/ml) displayed antimicrobial activities lower than the activities displayed by the single bioactive compound pinocembrin found by Melliou and Chinou (2004).

Furthermore, Pepeljnjak and Kosalec (2004) reported on the antimicrobial activity of galangin against MRSA, *Enterococcus* spp. and *P. aeruginosa*. The study reported that galangin inhibited the growth of MRSA, *Enterococcus* spp. and *P. aeruginosa* with MICs of 0.16 mg/ml (160 µg/ml), 0.24 mg/ml (240 µg/ml) and 0.17 mg/ml (170 µg/ml), respectively. Of the 39 SA EEP samples tested in the current study, 14 samples tested against *E. faecalis* an *Enterococcus* spp. displayed lower antimicrobial activities (98 - 195 µg/ml) than the activity displayed by the single bioactive compound galangin found by Pepeljnjak and Kosalec (2004).

3.5. Conclusion

In conclusion, this study demonstrated that a wide range of SA propolis displayed noteworthy (< 125 µg/ml) as well as bactericidal activity. In alignment with literature, Gram-positive bacteria displayed greater sensitivity towards SA propolis than Gram-negative bacteria. *Staphylococcus aureus* was found to be inhibited and killed by low concentrations of propolis (6 µg /ml), thus making SA propolis a possible and valuable future alternative in anti-infective therapy.

Chapter 4: Conclusions and future recommendations

4.1. Overview

Despite the vast number of studies conducted worldwide (Figure 1.3), especially on South American (Brazil and Argentina) and European (Turkey and Italy) propolis, studies on African propolis and more specifically SA propolis have been severely neglected. Brazilian propolis was notably utilized as the "gold standard" for comparison in a vast majority of these studies (Appendix G). The antimicrobial efficacies and chemical composition of 39 SA propolis samples and three reference Brazilian samples was investigated by undertaking a number of objectives as outlined in Chapter 1. These objectives included; the preparation of extracts of propolis using absolute ethanol as an extraction solvent, investigating the antimicrobial activity of EEP using the MIC and MBC methods described in Chapter 3. Investigating the interactive efficacy of EEPs with conventional antimicrobials and conducting chemical profiling of propolis samples using HPTLCUPLC-PDA-qTOF-MS/MS. Furthermore, chemical characteristics were correlated with geographical variances using chemometric analysis.

4.2. Chemistry

As elaborated in Chapter 2, the chemical composition of propolis is extremely variable and dependant on a number of factors (Markham *et al.,* 1996; Righi *et al.,* 2011). The chemical analysis of propolis in this study was principally conducted in order to explore the chemical composition of SA propolis samples collected from various regions in the country. Optimal chemical methods such as HPTLC, UPLC-ESI-MS and chemometric modelling were used in order to map the possible geographical patterns and to compare SA propolis to Brazilian propolis. This study detailed a comprehensive report, published on the chemical profiling of propolis samples from various regions in SA (Appendix A). The antimicrobial properties of propolis have been reported as being attributed to its high flavonoid content, with galangin, chrysin and pinocembrin being the most potent bioactive flavonoids.

The SA propolis samples were found (using LC-MS) to be rich in flavonoids with the main constituents being, pinocembrin, galangin, chrysin, myricetin and pinobanksin and is chemically distinct from Brazilian propolis which contained only artepillin C, a derivative of *p*-coumaric acid (Kasote *et al.,* 2014a). Although this study conducted a comprehensive analysis of propolis

compositions, propolis volatiles were not investigated and the chemical composition was not linked to the plant origin of active samples as undertaken in other studies (Bankova *et al*., 2000; Kumazawa *et al*., 2003; Salatino *et al*., 2005; Toreti *et al*., 2013). Further chemical analysis could possibly include propolis volatiles.

4.3. Antimicrobial properties

A total of 39 SA propolis samples and three Brazilian control samples were collected, extracts were prepared accordingly and screened for antimicrobial activity (both inhibitory (MIC) and bactericidal (MBC) activity) against two Gram-positive bacteria, two Gram-negative bacteria and two yeasts. It was observed that, SA propolis displayed greater antimicrobial activity, both inhibitory and cidal, than the Brazilian samples used as comparators (Chapter 3, Table 3.2). Three SA propolis samples (i.e. GP9 (Honeydew), GP11 (Edenvale) and WC8 (southern suburbs- Cape town 1)) demonstrated noteworthy (MIC ≤ 125 µg/ml) activities with MICs as low as 6 µg /ml (Table 3.2, Chapter 3). More than 50% of SA propolis samples displayed average MIC values which were lower than the control Brazilian propolis extracts. In addition 53% of SA propolis samples were found to display better average antimicrobial activity (i.e. against all six pathogens tested) than the three Brazilian comparators. This study also detailed a comprehensive report, published on the antimicrobial of propolis samples from various regions in SA (Appendix B).As discussed in Chapter 3, studies on propolis from regions of Argentina, Turkey and Oman have previously reported MICs as low as 15.30 µg/ml, 8-16 µg/ml and 42-169 µg/ml respectively against *S. aureus* (Nieva Moreno *et al.*, 1999; Uzel *et al.*, 2005; Popova *et al.*, 2013). In comparison to these previous studies, the current study on SA propolis demonstrated superior inhibitory activity against *S. aureus* than the samples from these regions, with MICs as low as 6 µg/ml. In alignment with available antimicrobial literature (Appendix G) this study reports that Gram-positive bacteria (*S. aureus*) and yeast spp. (*C. neoformans*) were the most sensitive pathogens.

The cidal activity of propolis was predominantly observed against Gram-positive bacteria, *S. aureus* and yeast, *C. neoformans*. Of the 39 SA EEP samples tested, 67% of samples demonstrated predominant cidal activity equivalent to the inhibitory concentration for *S. aureus* and *C. neoformans*. For *E. faecalis* and *C. albicans* 41% of SA EEP samples displayed cidal effects equivalent to the inhibitory efficacy observed.

A review by van Vuuren *et al.* (2008) on the antimicrobial activity of SA plants reported that whole extracts having activities where MIC values are ≤ 8 mg/ml are considered as having some

antimicrobial activity whilst natural products with MIC values ≤ 1 mg/ml are considered noteworthy. This study reports SA propolis extracts as having remarkable noteworthy activity with MICs ≤ 6µg/ml. Overall, SA propolis displayed superior inhibitory, cidal as well as average antimicrobial activity in comparison to the Brazilian gold standard tested as controls.

A study conducted by Melliou and Chinou (2004) reported that pinocembrin and chrysin displayed respective MIC values of 0.25 mg/ml (250 µg/ml) and 3.4 mg/ml (3400 µg/ml) against *S. aureus*. The activity of 23 EEP samples tested in this study displayed MICs of 6 - 49 µg/ml against *S. aureus*. It was found that the antimicrobial activities of these whole extracts were lower than those activities displayed by the single bioactive compounds pinocembrin and chrysin as reported by Melliou and Chinou (2004). This demonstrates that the compounds within the propolis samples may be acting synergistically to enhance activity (Melliou and Chinou, 2004; Mantovani *et al*., 2008; Oldoni *et al*., 2011). Future studies should identify if this is apparent.

One needs to take into account that even though some noteworthy activities (both inhibitory as well as cidal) were obtained, the limitation exists that these results like many others show *in vitro* activity only and further *in vivo* studies are recommended on SA propolis. Future studies should also include other relevant disease specific pathogens (pathogens in nosocomial infections for example), as these may yield interesting results. Antimicrobial resistance is currently of growing concern globally and therefore further studies detailing the effects of SA propolis on resistant and clinical strains should also be considered.

4.4. Interactive antimicrobial properties

Combination studies were conducted in order to investigate the possible antimicrobial interactions between propolis, in a 1:1 combination ratio with conventional antimicrobials (ciprofloxacin, gentamicin, penicillin G, nystatin and amphotericin B) and an antimicrobially active SA honey, respectively. Of the 60 combinations tested with conventional antimicrobials, nine combinations displayed synergism. An ΣFIC of 0.19 was noted against *P. aeruginosa*, and 16 combinations displayed additive activity (Chapter 3, Table 3.3). This study noted that SA propolis enhanced the Gram-negative activity of gentamicin, whilst adding to the effects of penicillin G (Chapter 3, Table 3.3). The comparative Brazilian sample was found to be the only sample to display antagonism when combined with antifungals, amphotericin B (ΣFIC of 4.56) and nystatin (ΣFIC of 8.65), against *C. neoformans.* The combination of propolis - with the antimicrobially active honey sample, resulted in 30 combinations. Two combinations displayed synergistic activity against *C.*

neoformans and 18 combinations displayed additive activity (Chapter 3, Table 3.4). The Brazilian sample tested in combination with this honey sample displayed additive and non-interactive interactions, and was once more found to be the sample displaying the greatest antagonism when combined against the yeast species *C. neoformans* with a ΣFIC of 8.21. It is important to note that SA propolis did not display any antagonism when combined with the conventional antimicrobials tested in this study. Other propolis samples have previously been reported to display synergism with various conventional antimicrobials including ciprofloxacin and gentamicin (Fernandes *et al*., 2005; Orsi *et al*., 2006; Rahman *et al*., 2010; Helaly *et al*., 2011; Naher *et al*., 2011; Orsi *et al*., 2012), this is in keeping with what has been uncovered in this study.

Although conventional antimicrobials were tested in 1:1 ratios with SA propolis, one needs to consider what the possible results would be if varied ratio studies were conducted. Furthermore, the interactive efficacy of combinations of the major compounds (i.e. pinocembrin, chrysin, pinobanksin, galangin, etc) identified in SA propolis should be studied in order to determine which compounds or combinations of compounds are responsible for the observed efficacy of SA propolis. Even though SA propolis was tested here in combination with honey, other natural products and minerals (e.g. essential oils and other plant extracts) could also be considered for further investigation.

4.5. Other aspects

Distinct colour variations in SA propolis extracts were noted by this study. Extracts varied from colourless/whitish grey → light yellow-brown/golden → dark reddish-brown/maroon. Further analysis of the antimicrobial results lead to the hypothesis that dark reddish-brown/maroon extracts (e.g. GP9 and WC8) displayed greater inhibition against Gram-positive *S. aureus* and the yeast *C. neoformans* than colourless/whitish grey extracts (e.g. WC6 and NW4) (Chapter 3, Table 3.2). This study additionally established that a majority of samples (74% against *S. aureus* and 93% against *C. neoformans*) ranging in colour from light yellow-brown/golden → dark reddish-brown/maroon displayed the highest inhibition with MICs ≤ 195 µg/ml. No other studies have reported such differences in antimicrobial activity in relation to the colour of their extracts. Thus, another future recommendation made by this study is the chemical analysis of samples in order to ascertain if darker samples contain more of a certain active flavonoid (e.g. pinocembrin) thus lending to its greater antimicrobial activity, than those samples with lighter colour and lesser antimicrobial efficacy.

The toxicity of SA propolis has never been studied. However, Burdock (1998) tested the toxic effects of propolis on rats and cats and reported that by employing a safety factor of 1000 to account for the lack of chronic toxicity studies a safe dose could be calculated for humans. A safe dose for humans would therefore be 1.4 mg/kg of body weight/day, which is equivalent to approximately 70 mg/day. Owing to the lack of toxicity information available on SA propolis it is recommended that future toxicity studies be investigated.

Not many studies have been conducted on determining the quality of propolis and Bankova *et al*. (2005a) discusses the problems with standardisation of propolis due to chemical diversity at length. However, Bonvehí (2000) and Kosalec (2003) postulate that the quality of propolis can be determined by a measurement of its flavonoid content thereby leading to the provision of grading of propolis samples into groups according to "quality". Cui-ping *et al*. (2014) recently stated that the quality grading of poplar type propolis be made by measuring the content of specific compounds namely; pinobanksin, pinocembrin, 3-*O*-acetylpinobanksin, chrysin, and galangin. The quality of SA propolis has not been studied here and therefore, it is the recommendation of this study that future quality control studies be conducted on SA propolis. In addition, the effects of age of sample and seasonality on SA propolis have not been studied. Future studies on the effects of age (old vs. fresh) of samples and seasonality (collection during summer vs. winter, etc) on the antimicrobial activity of SA propolis may be worthy of investigation.

Although many studies on SA propolis are still required, this study is the first of its kind detailing the antimicrobial activity, interactive efficacy and chemical profiling of SA propolis using a large sample size (n=39). This study concludes that SA propolis displays superior antimicrobial activity, both inhibitory and cidal activity (6 µg/ml for selected samples), in comparison to the control "gold standard" Brazilian propolis samples (98 µg/ml) included as comparators in this study. Furthermore, many synergistic as well as additive interactions were observed when SA propolis samples were combined with conventional antimicrobials. This could possibly be due to synergistic interactions between compounds present in SA propolis and could lead to an improvement in current empirical treatments. Furthermore, six compounds namely; quercetin, galangin-5-methyl ether, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-butyrate or isobutyrate, pinobankin-3-*O*-pentanoate or 2-methylbutyrate and pinobanksin-3-*O*-hexanoate were identified for the first time in SA propolis by this study. This study concluded that SA propolis demonstrated not only superior activity in comparison to the gold standard but also possessed superior antimicrobial activity in

comparison to other vastly studied propolis from Argentina, Turkey, Italy and Oman (i.e. South America, Europe and Asia) against the tested pathogens.

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Chemical profiling and chemometric analysis of South African propolis

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ABSTRACT

UPLC-ESI-MS analysis of 39 South African propolis samples was undertaken to report on the chemical composition and variability of South African propolis and to compare the chemical profiles to Brazilian samples ($n = 3$). Chemo-geographical patterns within South African propolis were further analysed by chemometrics. South African propolis samples displayed typical UPLC-ESI-MS fingerprints, which were different from their Brazilian counterparts. UPLC-PDA-qTOF-MS/MS was used to identify marker compounds from representative groups and 15 major phenolic acids and flavonols from common South African propolis were identified. Chemometric analysis of the UPLC-ESI-MS data revealed two distinct clusters among the South African samples and also confirmed that the South African propolis was chemically distinct from the Brazilian propolis. The majority of the samples were phytochemically congruent with propolis from the temperate regions.

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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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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,

Appendix C:Oral presentation abstract, Postgraduate Symposium, University of Johannesburg (UJ), 2013

INVESTIGATING THE ANTIMICROBIAL AND CHEMICAL PROPERTIES OF SOUTH AFRICAN PROPOLIS.

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Propolis is a sticky resin collected from various flora by bees (*Apis Mellifera*) and is masticated and mixed with salivary enzymes. It is used by the bees to seal off holes in the hive from intruders, prevent putrefaction, as well as prevent infections of the colony. Studies on a global scale have reported on the broad-spectrum antimicrobial activity of propolis. The chemical diversity of propolis is also clearly apparent and extensively studied. However, chemical and antimicrobial properties of propolis samples from South Africa, have not yet been extensively researched. This study investigated the antimicrobial activities of 42 propolis samples from regions of South Africa, using the minimum inhibitory concentration and minimum bactericidal concentration assays. Also investigated were the chemical fingerprinting profiles using high performance thin layer chromatography (HPTLC), ultra performance chromatographic-photodiode array detector-quadrupole-time of flight- mass spectrometry (UPLC-PDA-qTOF-MS). Propolis samples were found to be highly antimicrobially active with efficacies as low as 3 µg/ml. This study also concluded that South African propolis is clearly chemically different from the Brazilian standard. Furthermore, South African propolis demonstrates noteworthy antimicrobial activity in comparison with other natural products.

Appendix D: Abstract for 2014 Research Day oral presentation at the School of Therapeutic Sciences, University of the Witwatersrand.

South African Propolis: Antimicrobial Activity, Chemical Properties and Interactive Efficacy.

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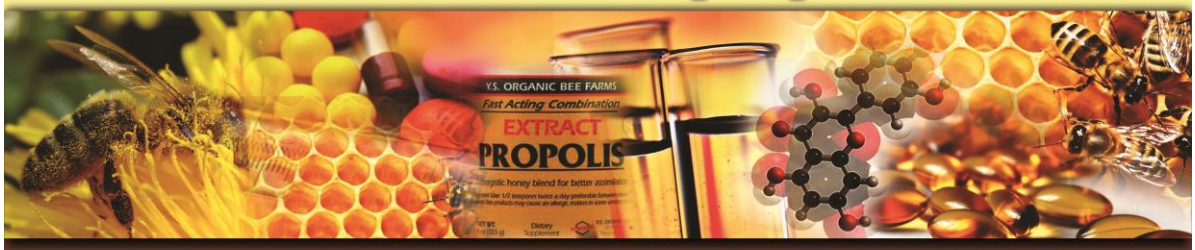
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Propolis is a sticky resin collected from various flora by bees (*Apis Mellifera*). It is used by bees to seal off holes in the hive, prevent putrefaction and prevent infections of the colony. Studies on a global scale have reported on the broad-spectrum antimicrobial activity of propolis. The chemical diversity of propolis is also clearly apparent and has been extensively studied. However, chemical and antimicrobial properties of South African (SA) propolis, has only been briefly researched. Therefore, this study investigated the antimicrobial activities of 46 propolis samples from the various provinces of SA, using the minimum inhibitory concentration and minimum bactericidal concentration assays. Chemical fingerprinting profiles of 42 samples using high performance thin layer chromatography (HPTLC) and ultra performance chromatographic-photodiode array detector-quadrupole-time of flight- mass spectrometry (UPLC-PDA-qTOF-MS) were studied. Propolis samples were found to display noteworthy antimicrobial activity with concentrations as low as 6µg/ml. Interactive efficacy studies with ciprofloxacin revealed an FIC value of 0.4 (synergistic activity) against *P. aeruginosa*. SA propolis is clearly chemically different when compared to the Brazilian samples.

Key words: Propolis, Antimicrobial, Chemistry, Interactive efficacy.

Appendix E: Chemical profiling of South African propolis

Chemical profiling of South African propolis



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INTRODUCTION

Propolis is a resinous material (beeswax and plant exudate) produced by honeybees (*Apis mellifera* L.). It is a valuable therapeutic agent used in the treatment of various ailments. The application of propolis in pharmaceutical and food preparations is on the increase due to its broad range of biological and pharmacological activities, such as anti-oxidant, anti-inflammatory, antibacterial, antifungal, antiviral, immunomodulatory, hepatoprotective, antiallergic, wound healing, antitumor and antidiabetic properties (Monzote et al., 2012; Navarro-Navarro et al, 2013). The chemical composition of propolis has been reported to be extremely complex and is highly variable due to geographical and seasonal effects (Salatino et al., 2011). The vegetation in the immediate vacinity of the beehives is a key factor impacting on the chemical composition of propolis. Propolis from various parts of the world are reported to contain over 300 different types chemical compounds such as polyphenols, esters of phenolic acids, flavonoids, sequiterpenes, diterpenes, triterpenes, lignans, prenylated benzophenones, aldehydes, steroids, coumarins, etc. (Monzote et al., 2012).

South African propolis (Fig.1) has been reported to exhibit a range of biological activities such anti-oxidant, antimicrobial and anti-inflammatory (Du Toit et al., 2009). However, the chemical composition of South African propolis remains unexplored. The objectives of this study were thus to:

1. Develop HPTLC and UPLC-ESI-MS fingerprints of South African propolis and to compare these profiles to Brazilian propolis.
2. Determine the chemical composition of a representative South African propolis sample using UPLC-qTOF-MS/MS.

MATERIALS AND METHODS

Forty-two samples were obtained from geographically distinct areas of South Africa and extracted with 80% methanol. The resultant extract was filtered through a 0.22-µM syringe filter and further used for fingerprinting and chemical profiling. Commercial standards of common propolis constituents were prepared in methanol. HPTLC analysis of South African propolis samples in comparison with Brazilian propolis was carried out on a CAMAG semi-automated system. Each sample (2 µL) was applied to the silica gel pre-coated aluminum plates and the separation was carried out by using chloroform-methanol-formic acid, 47:3.5:2.5 (v/v) as the mobile system. The chromatogram was developed for 70 mm from the base of the plate. After development, the plate was dried and visualized at 254 and 365 nm before and after derivatization with natural product reagent in order to capture chromatographic profiles. Preparative HPTLC was carried out to isolate marker compounds.

Ultra Performance Liquid Chromatography-Photodiode Detector-Quadrupole-Time of Flight-Mass Spectrometry (UPLC-PDA-qTOF-MS) analysis was performed using a Waters ACQUITY™ UPLC™ system. Each sample (1 µL) was injected and separation was achieved on a C18 BEH column with a flow rate 0.45 mL/min. The binary mobile phase consisted of 0.1% formic acid in water (phase A) and acetonitrile (phase B). The UV spectra were recorded by a PDA detector between 200-400 nm. The mass spectra acquisition was carried out in the negative mode by electrospray ionization (ESI). The prominent ions were selected for MS/MS fragmentation analysis.



Fig.1 South African propolis samples.

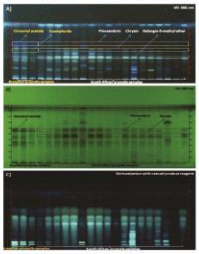


Fig. 2 HPTLC fingerprints of South African propolis samples in comparison to Brazilian propolis samples: A) under wavelength 254 nm; B) under wavelength 366 nm; C) After derivatization with natural product reagent.

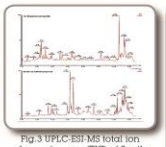


Fig.3 UPLC-ESI-MS total ion chromatogram (TIC) of South African sample and Brazilian propolis sample.

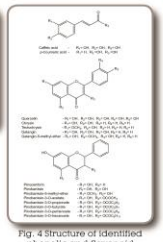


Fig. 4 Structure of identified phenolic and flavonoid compounds from of South African propolis

Table 1 Identification of phenolic and flavonoid compounds in South African propolis by UPLC-qTOF-MS

Peak No.	t_R (min)	UV_{max}	Pseudo-molecular ion $[M-H]^-$ (m/z)	MS/MS fragmentation of $[M-H]^-$	Compound
1	1.91	238,285, 291,308	179	177, (133)	Caffeic acid[a]
2	2.29	229,262, 277,306	163	163,143,(117),115	p- comaric acid[a]
3	3.26	291	301	(301),299,176,149	Quercitin[a]
4	3.43	284	285	283,265,(258),337, 222,163, 136	Pinobanksin-5-methyl ether[b]
5	3.86	289	271	(269),251,223,195, 149,125, 105	Pinobanksin[b]
6	4.53	259,350	283	281,266,(237),209	Galangin-5-methyl ether[b]
7	5.92	266, 313	253	(251),207,179, 149,105	Chrysin[b]
8	6.27	288	255	(253),211,185, 169,149 105	Pinocembrin[b]
9	6.44	264,357	269	(267),211,195, 167	Galangin[b]
10	6.72	291	313	313,269,(251)	Pinobanksin-3-O-acetate[b]
11	7.32	264	283	281,266,(237),209	Tectochrysin[b]
12	9.37	291	327	325,269,(251)	Pinobanksin-3-O-propionate[b]
13	12.42	291	341	339,269,(251)	Pinobanksin-3-O-butyrate or isobutyrate[b]
14	13.07	290	355	251	Pinobanksin-3-O-pentanoate or 2-methylbutyrate[b]
15	13.42	290	369	269,(251)	Pinobanksin-3-O-hexanoate[b]

[a]Confirmed with standard; [b]Confirmed with literature

RESULTS AND DISCUSSION

The observed HPTLC chromatograms (Fig 2.) shows that South African propolis samples are different from Brazilian propolis samples. South African propolis samples showed characteristic colored florescent bands (Rf 0.4-0.6) under 366 nm before and after derivatization with natural product reagent which confirmed the presence of flavonoids and phenolic acids. Bands isolated by preparative HPTLC were tentatively identified by using UPLC-PDA-qTOF-MS as pinocembrin, chrysin and galangin-5-methyl ether which could be marker compounds for most of the South African propolis samples. Cinnamyl acetate and kaempferide were detected in Brazilian propolis but not in South African propolis (Fig. 2A and 2B).

UPLC-ESI-MS total ion chromatogram (TIC) of South African propolis in comparison with Brazilian propolis is shown in Fig. 3. The profile is totally different when compared to that of Brazilian propolis. UPLC-PDA-qTOF-MS/MS in negative mode was used to identify phenolic and flavonoid compounds from South African propolis. Identification of compounds was achieved by matching retention time, maximum UV absorption, pseudomolecular ion mass and MS/MS fragmentation pattern with authentic compounds and /or published in literature. Fifteen major phenolic and flavonoid compounds were identified in South African propolis (Table 1 and Figure 4).

CONCLUSIONS

- Using HPTLC and UPLC-ESI-MS chromatographic profiles have been produced for the first time for South African propolis samples.
- South African propolis is clearly different from Brazilian propolis which is generally regarded as the "golden standard" for propolis.
- UPLC-PDA-qTOF-MS/MS data allowed for the identification of 15 phenolic and flavonoid compounds in South African propolis.

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Appendix F: HPTLC, UPLC-TOF-MS profiling and chemometric analysis of South African propolis

HPTLC, UPLC-TOF-MS PROFILING AND CHEMOMETRIC ANALYSIS OF SOUTH AFRICAN PROPOLIS



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Introduction

Propolis is resinous material produced by honeybees and is mostly composed of beeswax and plant exudates. Propolis displays a wide range of bioactivities such as antioxidant, antiinflammatory, antimicrobial, immunomodulatory and hepatoprotection, among others (Monzote *et al.*, 2012; Navarro-Navarro et al, 2013). The chemical composition of propolis is highly complex and variable depending on the region, season and type of vegetation surrounding the beehives. (Salatino *et al.*, 2011). Numerous studies on chemical profiling of propolis have been conducted but little is known about the chemistry of South African propolis. The current study investigates chemo-geographical patterns of South African propolis from differences regions and also in comparison to poplar Brazilian propolis using high performance thin layer chromatography (HPTLC) and ultra performance liquid chromatography coupled to mass spectrometry (UPLC-ESI-MS) in combination with chemometric algorithms.

Materials and Methods

39 propolis samples were obtained from different regions of South Africa and Brazil (Fig. 1). Extraction was performed using 80% methanol. The extracts were filtered through 0.22-µM syringe filter and analysed on the UPLC-MS instrument. The standards were prepared in methanol. Ultra performance liquid chromatography-photodiode detector-quadrupole-time of flight-mass spectrometry (UPLC-PDA-qTOF-MS) fingerprinting and chemical profiling analysis of the samples was performed using Waters ACQUITY™ UPLC™ system. 1 µL of each sample was injected and separation was achieved on C18 BEH column with flow rate 0.45 mL/min. The binary mobile phase consisted of 0.1% formic acid in water (phase A) and acetonitrile (phase B). The UV spectra of elute were recorded by using PDA detector between 200-400 nm. The mass spectra acquisition was carried out at the negative modes by electrospray ionization (ESI). The prominent ions were selected for MS/MS fragmentation analysis. Chemometric analysis of the UPLC-ESI-MS data was performed in an untargeted approach using MarkerLynx v 4.1 for spectral alignment and SIMCA-P+ 13.0 for classification and discriminant analysis. HPTLC analysis of propolis was carried out on a CAMAG semi-automated HPTLC. 2 µL of each propolis sample was applied onto the silica gel pre-coated aluminum plates and the separation was carried out by using chloroform-methanol-formic acid, 47:3.5:2.5 (v/v). The chromatogram was developed up to 70 mm. After development, the plate was dried and images were captured under ultraviolet light at 254 and 365 nm before and after derivatization with natural product reagent in order to record fingerprinting profiles. Preparative HPTLC experimentation was carried in order to identify marker compounds.



Fig. 1: Distribution map of South African propolis.

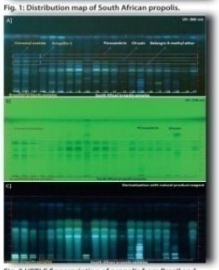


Fig. 2 HPTLC fingerprinting of propolis from Brazil and South Africa observed under A) 254 nm; B) 366 nm and C) after derivatization.

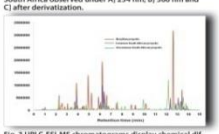


Fig. 3 UPLC-ESI-MS chromatograms display chemical differences between Brazilian and South African propolis.

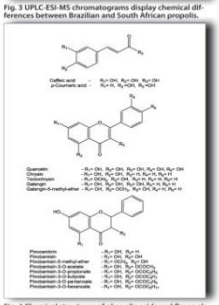


Fig. 4 Chemical structures of phenolic acids and flavonols in South African propolis

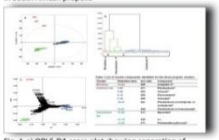


Fig. 4. a) OPLS-DA score plot showing separation of propolis samples, which corresponds to the branching in the dendrogram (b). The X-variables correlated to separation of the samples are displayed in the loadings plot (c) and the identities of these are listed in Table 1.

Results and Discussion

HPTLC fingerprinting displayed distinct chemical profiles of Brazilian propolis when compared with South African propolis at 254 nm (Fig. 2A), 366 nm (Fig. 2B) and after derivatization with natural product reagent (Fig. 2C). The distinct chemical profiles of propolis were also observed on the UPLC-MS chromatograms where South African propolis displayed two distinct chemotypes (common and uncommon South African propolis) (Fig. 3). Orthogonal projections to latent structures–discriminant analysis (OPLS-DA) displayed three clusters of propolis in the scatter plot (Fig. 4a). Brazilian propolis formed a separate cluster (red) while South African propolis confirmed the occurrence of an uncommon (green) (Gauteng and Western Cape provinces) and common chemotype (blue) (majority of SA samples) in both the scatter plot and the dendrogram (Fig. 4b). The corresponding loadings plot (Fig. 4c) shows the variables that were correlated to separation of the samples and these were identified and listed in Table 1. Pinocembrin, chrysin and galangin-5-methyl ether were identified as possible marker compounds for South African propolis while cinnamyl acetate and Artepillin C were identified for Brazilian propolis (Fig. 4c and Table 1).

Conclusions

The present study demonstrates distinction of Brazilian and South African propolis based on HPTLC and UPLC-MS untargeted analysis. Fifteen major phenolic acids and flavonols from common South African propolis were identified and chemometric data analysis revealed two chemotypes within South African propolis. The majority of South African propolis were phytochemically congruent with propolis from the temperate regions.

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Appendix G: Overview of antimicrobial studies conducted on propolis, globally.

Origin	Results of study	References
France	At a dilution of 1:20 in nutrient agar, propolis completely inhibited the growth of *Staphylococcus aureus* (including strains of MRSA), *S. epidermidis*, *Enterococcus* spp., *Branhamella catarrhalis*, *Corynebacterium* and *Bacillus cereus*. A strain of *Mycobacterium tuberculosis* was completely inhibited at a propolis concentration of 1:320 and partially inhibited at propolis concentrations of 1:640.	Grange and Davey , 1990
United Kingdom - bee health Ltd.	Reported on the concentration-dependant inhibitory action of propolis against *E.coli* and *Bacillus subtilis*. The concentration of propolis that inhibited *B. subtilis* by 50% was 200 µg/ml and the concentration that inhibited the growth of *E.coli* by 50% was 450 µg/ml.	Mirzoeva *et al.*, 1997
Brazil	Water and ethanolic extracts of propolis were tested against *S. aureus.* Water extracts exhibited no inhibition, however, 70% EEP displayed the best inhibition of *S. aureus* with a ZOI of 1.5 mm.	Park and Ikegaki, 1998
Brazil	Against *S. mutans* zones of inhibition ranged 0 to 3 mm.	Park *et al*., 1998
Brazil	Antibacterial and antifungal activity of EEP tested against *S. aureus* and *C. albicans.* Study reported ZOIs of 10.5±0.5 mm and 15±1 mm, respectively.	Bankova *et al*., 1999
Bulgaria (Bg) **Albania (Alb)** **Mongolia**	Ethanolic extracts of propolis (EEP) were tested against *S. aureus*, *E. coli* and *C. albicans*. EEPs were found to display inhibitory zones as follows: Bg = 13.7 ± 0.3 mm, Alb = 13.8 ± 0.6 mm, Mong = 16.2 ± 0.3 mm, Egypt = 15.3 ± 1.5 mm, Br 1-4 = 11.0 - 12.0 mm ± 0.8 -	Kujumgiev *et al.*, 1999

Origin	Results of study	References
(Mong)	1.0mm , K1-2 = 17.3 - 29.0 mm ± 0.7 - 1.2 mm against	
Egypt	*S. aureus* and Bg = 17.7 ± 1.2 mm, Alb = 17.0 ± 1.0	
Brazil (Br1-4)	mm, Mong = 18.00 ± 1.0 mm, Egypt 17.3 ± 0.4 mm,	
Canary Islands (K1-2)	Br1-4 = 14.3 - 18.2 ± 0.3 – 1.2 mm, K1-2 = 17.0 – 18.0 ± 0.7 - 1.0 mm.	
Turkey	The effect of water extracts of propolis (WEP) was tested against moulds; *Aspergillus parasiticus, A. niger, Alternaria alternata, Botrytis cinerea, Penicillium digitatum and Fusarium oxysporum.* After a seven day incubation period the study found that higher concentrations of propolis (i.e. 4%) were more effective at inhibiting the growth of all moulds with *P. digitatum* being the most sensitive with a percentage inhibition of 76.7%.	Özcan, 1999
Argentina	Tested EEP against *S. aureus, E. faecalis, S. pyogenes, S. agalactiae, K. pneumoniae, S. marcescens, Acinetobacter* spp, *Stenotrophamonas maltophilia, E. coli* and *Pseudomonas aeruginosa*. All samples tested were found to display antimicrobial activity only against Gram-positive bacteria. Propolis obtained from El Molino displayed MICs as low as 15.30 µg/ml against *S. aureus* and propolis samples from La Banda Oeste displayed MIC values > 50 µg/ml against *S. agalactiae.*	Nieva Moreno *et al*., 1999
Brazil	EEP tested against *Actinobacillus actinomycetemcomitans*, *Fusobacterium* spp. and *Bacteroides fragilis*. Against *A. actinomycetemcomitans* MICs ranged 0.05 to 0.5%. Fusobacterium found to be more susceptible MICs 0.05 to 0.25%.	Santos *et al*., 1999
Uruguay	EEP tested against *E. coli, B. subtilis* and *S. aureus.*	Bonvehí and

Origin	Results of study	References
and China	Reported MICs of 800-1000 µg/ml against *E. coli* and 80-130 µg/ml against *S. aureus* and *B. subtilis*, respectively.	Coll, 2000
Italy	Study concluded that 50% of *S. pyogenes* strains were killed by solutions containing 58.5 µg/ml of propolis. All strains however, were killed by solutions containing 234 µg/ml concentrations of propolis.	Bosio *et al.*, 2000
Brazil	Study reported MIC values between 25-400 µg/ml against *S. mutans, S. sobrinus and S. cricetus.*	Koo *et al.*, 2000
Brazil	Tested the seasonal effects of propolis on *S. aureus*, *P. aeruginosa*, *E. coli* and *Salmonella typhimuruim*. No significant difference in antimicrobial activity found.	Sforcin *et al*., 2000
Brazil	Antimicrobial activity of EEP tested against *E. coli, S. aureus* and *C. albicans.* Study reported weak inhibitory activity against *E. coli* and *C. albicans* with ZOIs 0-13mm and 11-13.3 mm, respectively. Study further reported *S. aureus* as being the most sensitive test micro-organism with ZOI 10-18.3 mm.	Velikova *et al*., 2000
Egypt	Against *S. aureus,* MICs ranged from 1000 - 8400 µg/ml, against *E.coli* MICs ranged from 1400 - 6400 µg/ml and against *C. albicans* MICs ranged from 1400 - 6400 µg/ml.	Hegazi *et al.*, 2001
Turkey	EEP samples were tested against pathogens such as *S. aureus, E. coli and P. aeruginosa.* The EEP sample obtained from Istanbul showed greater activity against *S. aureus* with an MIC of 9 µg/ml and MBC of 16 µg/ml, whereas the EEP sample obtained from Balikesir displayed MIC and MBC activity against *S. aureus* of 175 and 310 µg, respectively. Both samples displayed poorer activity against the Gram-negative bacterial strains *E.coli* and *P. aeruginosa*	Keskin *et al*., 2001
El Salvador	EEP antimicrobial activity tested against *S. aureus,*	Popova *et al*.,

Origin	Results of study	References
	E.coli and *C. albicans*. No activity reported against Gram-negative *E. coli,* ZOIs of 11 and 12 mm reported for *C. albicans* and *S. aureus*, respectively.	2001
Egypt	EEP tested against *C. albicans, E. coli* and *S. aureus.* Reported MICs of 1320 µg/ml, 1200 µg/ml and 2400 µg/ml against *C. albicans*, *E. coli* and *S. aureus*, respectively.	Abd El Hady and Hegazi, 2002
Korea	Antifungal activity of propolis was tested against *C. neoformans* and *C. albicans. C. neoformans* was reported as being the most sensitive with MICs 2-4 mg/ml whilst *C. albicans* was reported as being less sensitive with MICs of 16 mg/ml.	Chee, 2002
Brazil	The inhibitory activity of propolis extracts was tested against bacteria that cause periodontitis. Reported that all bacterial strains tested against showed susceptibility to propolis with MICs between 64 and 1024 mg/ml.	Santos *et al.*, 2002
Bulgaria	Inhibitory effects of EEP were tested against *H. pylori* and *Camphylobacter* spp. Study reported mean ZOIs of 21.4 mm against *H. pylori* and 13.6 mm against *Camphylobacter* spp.	Boyanova *et al*., 2003
Italy	Tested against 44 strains of *S. aureus.* All strains showed susceptibility, 42 of the 44 strains displayed MICs 0.09 - 0.21 mg/ml.	Dolci and Ozino, 2003
Turkey	Different ethanolic extracts of propolis displayed antibacterial activity against; *S. aureus* (9 - 11mm), *S. epidermidis* (10 - 12mm), and *B. subtilis* (9 - 11mm). The ethanolic extracts of Kazan propolis displayed antibacterial activity against *C. diphtheriae* (10 - 12mm), *B. catarrhalis* (8mm), and *C. albicans* (8 - 10mm). However, when tested against *S. pyogenes, P. aeruginosa, E. coli, K. pneumoniae, and E. faecalis* no antimicrobial activity was found.	Kartal *et al.*, 2003

Origin	Results of study	References
Croatia	EEP tested against *B. subtilis*, reported ZOI of 13-18 mm.	Kosalec *et al*., 2003
Brazil	Against *S. aureus*, the inhibitory activity of propolis produced by *Apis Mellifera* ranged between 0.36 - 3.65 mg/ml and propolis produced by *Tetragonisca angustula* ranged between 0.44 - 2.01 mg/ml.	Miorin *et al.*, 2003
Serbia	Propolis samples tested against different bacterial strains (Gram-positive, Gram-negative and yeasts). MICs for Gram-positive bacteria ranged between 0.078% and 1.25%, Gram-negative bacteria between the ranges of 1.25% - 5% and lastly yeasts ranged from 0.16 - 1.25%. Study concluded that *E. faecalis* was the most resistant Gram-positive bacteria, *Salmonella* the most resistant Gram-negative bacteria and *C. albicans* as the most resistant yeast.	Stepanović *et al*., 2003.
Colombia, Ethiopia, Germany, Italy, Kazakhstan, Poland, Russia and South Africa	Reported that filamentous fungi are less sensitive to the actions of propolis. MIC values of various propolis samples tested lay between 0.005 and 0.5% w/v, with the exception of *E.coli*	Garedew *et al.*, 2004
Northwest Greece	MICs for Gram-positive bacteria 0.50 - 0.80 mg/ml, for Gram-negative MICs 0.65 - 0.90 mg/ml and 0.50 - 0.80 mg/ml against yeast spp.	Melliou and Chinou, 2004
Croatia	Antimicrobial activity of EEP tested against MRSA, MSSA, *P. aeruginosa* and *Enterococcus* spp. Reported ZOIs of 9.3-22.1 mm; MICs of 0.65-14.16 mg/ml and MBCs 1.38-23.44 mg/ml against all micro-organisms tested.	Pepeljnjak and Kosalec, 2004

Origin	Results of study	References
Turkey	Found that the antimicrobial activity of propolis varied depending on not only the propolis sample but also the dosage of propolis used and extraction solvents used. DMSO extracts were more active than acetone extracts. Against *Brucella meliteni*, the acetone extracts showed a greater activity. The study concluded that the most sensitive organism to propolis was *Shigella sonnei* from the Gram-negative group (0.00 -11.2 mm) and *S. mutans* from the Gram-positive group (0.0- 10.2 mm). But the least sensitive to the propolis was *C. albicans.*	Ugur and Arslan, 2004
Brazil	MIC of 90% EEP was shown to be 0.4% v/v.	Fernandes Jr *et al.*, 2005
Brazil	Propolis tested on caries development. Reported MICs of EEP 25 - 400 µg/ml against *S. sobrinus* and *S. mutans.*	Hayacibara *et al*., 2005
Turkey	MIC distributions for samples from Mamak and Kemaliye against methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* were in the range of 7.8 - 31.2 µg/ml, 70.3 - 281.2 µg/ml and 35.1 - 140.4 µg/ml, all samples showed great activity against *MRSA* and *VREF.*	Kilic *et al.*, 2005
Taiwan	Various factors were considered, such as the effects of cell age, incubation temperature, geographical location, concentration and pH on the antibacterial activity of the propolis samples against *S. aureus.* The study reported that the MIC of EEP samples ranged from < 3.75 - 60 µg/ml.	Lu *et al.*, 2005.
Turkey	Tested 30% EEP against *S. aureus* and *E. coli.* Reported *S. aureus* to be more susceptible to lower concentrations (<0.1 - 0.4 ml) of propolis than *E. coli* (0.2- >14 ml).	Popova *et al*., 2005
Lithuania and Czech	Antimicrobial activity of EEP was tested against multiple organisms including; *E. faecalis*, *Bacillus* spp.,	Savickas *et al*., 2005

Origin	Results of study	References
Republic	*S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans*. All samples showed inhibitory activity even after a four times dilution with ZOIs >10 mm.	
Turkey	Study tested propolis produced by three different honeybee species namely *Apis mellifera carnica, Apis mellifera caucasica* and *Apis mellifera anatolica.* Propolis was tested against *S. aureus, E. coli, P. aeruginosa* and *C. albicans.* Growth of Gram-positive bacteria was inhibited better than both Gram-negative and yeast species with both $MIC_{50}$and MIC_{90} ranging from 117 - 7500 µg/ml.	Silici and Kutluca, 2005
Turkey	Propolis tested against oral pathogens. Reported that 10% Turkish propolis displayed no significant activity against oral pathogens.	Sonmez *et al*., 2005
Turkey	MIC values of propolis were 2 µg/ml against *S. sobrinus* and *E. faecalis*, 4 µg/ml against *Micrococcus luteus, C. albicans* and *Candida krusei*, 8 µg/ml against *S. mutans, S. aureus, S. epidermidis* and *Enterobacter aerogenes*, 16 g/ml against *E.coli* and *C. tropicalis* and lastly 32 µg/ml against *P. aeruginosa* and *S. typhimuruim*.	Uzel *et al*., 2005
Turkey	Tested antimicrobial activity of propolis against 15 plant pathogenic bacteria. Reported ZOIs ranging 6 mm to 12 mm.	Basim *et al*., 2006
Bulgaria	Propolis samples were found to be highly active against anaerobic bacteria. Samples inhibited > 89% of all strains tested against. Found to be more effective at inhibition of Gram-positive bacteria rather than Gram-negative bacteria and yeasts.	Boyanova *et al.,* 2006
Korea	All samples were shown to inhibit growth of all strains tested against. Inhibitory zones varied between 2.0 - 6.1 mm.	Choi *et al.,* 2006

Origin	Results of study	References
Brazil	Tested EEP against *S. aureus*. MIC was described as the volume of propolis extract per 100 ml of culture medium (%v). All extracts found to display antimicrobial activity ranging from 0.2 to 3.2 %v	da Silva *et al*., 2006
Brazil	Study tested the effects of two commercial propolis products one ethanolic extract (EEP) and the other without ethanol (EP) against various pathogens including *S. mutans*, *S. aureus*, *E. coli* and *C. albicans*. Both products were found to display ZOIs ranging 6-18 mm for the EEP product and 6-27 mm for the EP product.	de Rezende *et al*., 2006
Brazil	Against *S. aureus* EEP displayed zones of inhibition ranging from 8 to 13 mm and did not display any inhibitory activity against *E. coli*.	Gonsales *et al.*, 2006
Turkey	Tested EEP against various pathogens including *S. aureus, B. subtilis, E. coli, P. aeruginosa* and *C. albicans*. Contrary to previous studies reporting strong Gram-positive inhibition, this study reported that EEP displayed strong antimicrobial activity against Gram-negative bacteria with a ZOI of 19 mm against *E. coli* and 16 mm against *P. aeruginosa*. Good antifungal activity with a ZOI of 16 mm against *C. albicans* was also noted.	Mercan *et al*., 2006
Brazil	Antifungal activity of EEP was tested against various *Candida* spp. *C. parapsilosis* was reported as being the most sensitive of the species tested with MICs 0.63-5.00 x 10^{-2} mg/ml whilst *C. tropicalis* the most resistant with MICs 2.50-5.00 x 10^{-2} mg/ml.	Oliveira *et al*., 2006
Italy	Against 35 strains of *S. aureus* the MIC_{50} and MIC_{90} were both 1.25 mg/ml, against *S. epidermidis* and *Staphylococcus* spp. were 1.25 and 52.5 mg/ml.	Scazzocchio *et al.*, 2006.
Argentina	Tested the antimicrobial effects of propolis against	Quiroga *et al*.,

Origin	Results of study	References
	fungal strains. EEP displayed MICs of 77 µg/ml against *Saccharomyces carlsbergensis* and 349 µg/ml against *Trichoderma spp., Fusarium* spp. and *Penicillium notatum.*	2006
Brazil	Antimicrobial activity tested against *S. aureus* and *S. mutans*, MICs ranged 50 -100 µg/ml.	Alencar *et al*., 2007
Brazil	Tested the antifungal activity of commercial and aqueous extracts of propolis against C*andida* spp. Commercial extracts were reported as displaying better activity than aqueous extracts with ZOIs ranging 12-28 mm against various *Candidal* spp. tested.	Dias *et al*., 2007
Italy	Antimicrobial activity (MIC and MBC) of a new propolis formulation known as "Actichelated" propolis was tested against various microorganisms including *S. aureus, S. pyogenes, Enterococcus spp., E. coli, Proteus mirabilis* and *P. aeruginosa.* Results were compared to the hydroalcoholic extract of propolis. Study reported that for actichelated propolis against *S. pyogenes* MICs and MBCs ranged 0.016-0.125 mg/ml and 2-4 mg/ml versus the hydroalcoholic extract that ranged 0.084-1.34 mg/ml and 10.7-21.4 mg/ml, respectively. Concluded that the new formulation actichelated propolis possessed greater antibacterial action than the usual hydroalcoholic extract tested in most studies.	Drago *et al*., 2007
Bulgaria	Bactericidal, fungicidal (MBCC) activities of EEP were tested against *C. albicans, E. faecalis* and *E. coli.* MBCCs ranged 1502 ± 320.0 µg/ml against E. *faecalis;* 24 306 ± 1706.4 µg/ml against *E.coli* and 1375 ± 255.2 µg/ml against *C. albicans*	Gardjeva *et al*., 2007
Brazil	Tested the antibacterial and antifungal activity of a new adhesive formulation containing propolis against *Candida* spp., *S. aureus, S. mutans* and *E. faecalis.* All	Gomes *et al*., 2007

Origin	Results of study	References
	pathogens were found to be sensitive to the formulation with MICs ranging 1.75-14.0 µg/ml.	
Turkey and Brazil	Reported MIC values ranging from 4 - 512 µg/ml. Concluded that EEP samples were more effective against Gram-positive bacteria than Gram-negative bacteria.	Koru *et al.*, 2007
Iran	Inhibition of growth was observed against all tested microorganisms with the highest activity being against Gram-positive bacteria such as *S. aureus* and *S. epidermidis* with MICs as low as 125 µg/ml.	Mohammadzadeh *et al.*, 2007
Kenya	Antibacterial activity of different concentrations (i.e. 100%, 70%, 50% and 30%) of EEP was tested *E. coli, S. typhimurium, P. aeruginosa, S. aureus* and *B. subtilis.* Gram-positive *S. aureus* and *B. subtilis* were reported as being the most sensitive with ZOIs 10.5 mm and 11.5 mm, respectively, whilst Gram-negatives *E. coli* and *P. aeruginosa* were less sensitive.	Muli and Maingi, 2007
Brazil and Bulgaria	EEP samples were tested against pathogens associated with human infection and the contamination of food (poultry) namely; *S. typhimurium* and *S. enteritidis.* Study found that *S. enteritidis* was more sensitive to the antimicrobial activity of both Brazilian and Bulgarian EEP samples than *S. typhimurium* with MICs ranging from 217.5 to 221 mg/ml	Orsi *et al*., 2007
Various regions of Asia, South and North America and Europe	Antibacterial activity tested, MIC of 250 µg/ml reported against *S. aureus*	Popova *et al*., 2007
Argentina	Antimicrobial activity tested against *E. coli.* Zones of inhibition found were considered negligible.	Tosi *et al*., 2007

Origin	Results of study	References
Mexico	Using broth a microdilution assay EEP was tested against *S. aureus, E. faecalis, Listeria monocytogenes, E. coli* and *P. aeruginosa.* Study found that propolis from Ures displayed strong antibacterial activity of 100 µg/ml, whilst propolis from Caborca displayed only moderate activity of 200 µg/ml against *S. aureus*. No samples were found to display activity against Gram-negative micro-organisms.	Velazquez *et al*., 2007
Brazil	Antimicrobial activity of propolis tested against *Paenibacillus larvae* that cause American Foulbrood (AFB) disease in honeybees. Study found that inhibitory zones varied between 20.5±2.1 mm and 15.5±2.2 mm thereby proving some antimicrobial activity against *P. larvae.*	Bastos *et al.*, 2008
Various countries within tropical and subtropical regions	EEP from Tanzania and South Africa found to display moderate activity with MICs 31.25 - 250 mg/l and 62.5 - 500 mg/l respectively. Propolis from Cameroon and northwestern Tanzania displayed higher activity with MICs 7.81 - 125 mg/l and 15 - 62 mg/l respectively. American samples displayed moderate activity with MICs 31.25 - 500 mg/l. Asian and European samples were also found to display moderate activity with MICs 31.25 - 500 mg/l.	Seidel *et al.*, 2008
Turkey	MICs ranged 0.12 to > 4.00 mg/ml, greater antimicrobial activity observed against Gram-positive microorganisms. *Mycobacterium smegmatis, S. aureus* and *C. albicans* reported as being the most sensitive microorganisms with MICs 0.12-0.25 mg/ml.	Vardar-Ünlü *et al*., 2008
Italy	Antimicrobial activity of EEP was tested against *Camphylobacter* spp. Strains of *C. jejuni* were reported as being the most sensitive with MICs 0.156-0.3125 mg/ml.	Campana *et al*., 2009

Origin	Results of study	References
Argentina	Tested EEP against *S. aureus*. Found that 77% of samples tested displayed zones of inhibition of> 9 mm.	Chaillou and Nazareno, 2009
Brazil	EEP tested against various oral pathogens; fungal and bacterial, including *S. aureus, Candida* spp., *Streptococcus* spp., *Bacteroides fragilis, Tanerella forsynthesis, Fusobacterium* spp., and *Porphyromonas gingivalis*. MICs, MBCs and ZOIs against; *Candida* spp. ranged 20-50 µg/ml, 100-400 µg/ml and 12.3-28.3 mm; against *S. aureus* ranged 25-50 µg/ml, 200-400µg/ml and 16.3 mm; against *Streptococcus* spp. ranged 25-50 µg/ml, 200-400 µg/ml and 18.3-28.6 mm; against *B. fragilis* ranged 25-50 µg/ml, 300-500 µg/ml and 15.3 mm; against *T. forsynthesis* ranged 30-60 µg/ml, 300-500 µg/ml and 14 mm; against *P. gingivalis* ranged 30-50 µg/ml, 200-400 µg/ml and 14 mm; against *Fusobacterium* spp. ranged 30-60 µg/ml, 200-400 µg/ml and 15.2-17.3 mm.	de Paula *et al*., 2009
Brazil	Study found that 1% ethanol extracts inhibited the growth of *M. luteus, S. aureus, E. coli* and *P. aeruginosa.*	Farnesi *et al.,* 2009
Slovakia	Antifungal activity of EEP was tested against *Candida* spp., it was found that *C. krusei* displayed the greatest sensitivity to 70% EEP with ZOI 6.00±2.83 mm and *C. glabrata* the being least sensitive ZOI 3.50±0.77 mm.	Kačániová *et al*., 2009
Central and Southern Greece, Aegean Sea islands and Cyprus	Antimicrobial activity tested against 18 bacterial strains and two fungal strains. Sensitivity of Gram-positive strains varied with *S. aureus, S. epidermidis, B. cereus and Listeria monocytogenes* being the most sensitive.	Kalogeropoulos *et al.,* 2009
Iraq	Large inhibition zones displayed against *S. epidermidis* 22 - 26 mm, 15 - 22 mm against *S. aureus* and 16 - 22	Najmadeen and Kakamand, 2009

Origin	Results of study	References
	mm against *C. albicans*. Gram-negative bacteria found to display much smaller zones (7 - 9 mm).	
Brazil	Inhibitory effects of red and green EEP tested against *Trichophyton* spp. Study reported MICs for red EEP 64-1024 µg/ml and green EEP 256-1024 µg/ml, with the most sensitive micro-organism to both EEP samples being *T. rubrum.*	Siqueira *et al*., 2009
Brazil	Antimicrobial activity tested against 34 *Staph.* coagulase-positive and *Malassezia pachydermatis* causing canine otitis. Both isolates found to be susceptible to propolis with MBCs 10.7 - 21 mg/ml and 2.4 - 5.3 mg/ml.	Cardoso *et al*., 2010
Jordan	Propolis from Al-Hasmeah was found to display high antimicrobial activity with zones of inhibition 24.67 mm against *S. aureus.* Both samples did not display any significant activity against *E. coli.*	Darwish *et al*., 2010
Chile	Commercial Chilean EEP samples were tested against oral *Candida* spp. Study reported MICs ranging 197-441 µg/ml against *C. albicans* and 51-253 µg/ml against *C. glabrata.*	Herrera *et al*., 2010
Serbia	Tested the antibacterial activity of EEP against various pathogens under different pH values, acidic, neutral, and alkaline. All EEP samples exhibited antibacterial activity regardless of the pH of the solution within which they were tested.	Ivančajić *et al*., 2010
Tunisia	Antimicrobial activity evaluated against *E. faecalis* and *Streptococcus* spp. EEP found to display MICs 2 - 64 µg/ml against *Streptococcus.* spp. and >500 µg/ml against *E. faecalis*.	Kouidhi *et al*., 2010
Romania	EEP tested against Gram-positive bacteria, Gram-negative bacteria and yeast. Study found that Gram-positive bacteria were most susceptible with *S. aureus*	Marghitas *et al*., 2010

Origin	Results of study	References
	being the most sensitive with a ZOI of 17 mm, Gram-negative bacteria were less susceptible and yeast species *C. albicans* displayed moderate susceptibility with a ZOI of 10mm.	
Nigeria	The activity of EEP samples were tested against pathogens known to cause upper respiratory tract infections, bacteria found from throat swabs included S. *pneumoniae, K. pneumoniae, S. pyogenes* and *M. catarrhalis. S. pneumoniae* was found to be the most sensitive pathogen with a ZOI of 32 mm, *S. pyogenes* was found to be the least sensitive microorganism with a ZOI of 10 mm.	Ophori and Wemabu, 2010
Nigeria	An antimicrobial effect of varying concentrations of EEP was tested against *S. mutans.* Study reported a ZOI of 24±4 mm at an MIC of 32 µg/ml.	Ophori *et al*., 2010
Turkey	The antimicrobial, both MIC and MBC of EEP samples were tested against eleven different anaerobic bacteria including *Lactobacillus acidophilus, Prevotella intermedia* and *Actinomyces odontolyticus*. *Actinomyces odontolyticus* was reported as the most susceptible microorganism with MICs 0.4-5.8 mg/ml and MBCs of 0.8-11.6 mg/ml.	Özen *et al*., 2010
Kenya	Sample from Mwingi showed no inhibition against *S. aureus,* sample from Voi displayed zone of inhibition of 18 mm against *S. aureus.* Both samples displayed no activity against *C. albicans* and *E.coli.*	Petrova *et al*., 2010
Canada	Tested against *E.coli* and *S. aureus*, reported propolis concentrations of 2.74 - 5.48 mg/ml as having the largest inhibitory zone of 15.0 ± 0.11mm against *S. aureus*. The study concluded that 2.74 mg/ml of propolis is effective against *S. aureus.*	Rahman *et al.*, 2010
Iran	Study reported a ZOI of 21±1 mm against *S. aureus.*	Trusheva *et al*.,

Origin	Results of study	References
		2010
Iran	Effects of EEP tested against various pathogens causing diseases in fish such as; *Aeromonas hydrophilia, Yersinia ruckeri and Streptococcus iniae.* EEP samples inhibited the growth of all bacteria with MICs and MBCs ranging 195.31 µg/ml against *S. iniae* to 781.25 µg/ml against *A. hydrophilia* and 195.31 to 1562.5 µg/ml, respectively.	Tukmechi *et al*., 2010
Turkey	Inhibitory and cidal activity of EEP was tested against *E. faecalis* and *C. albicans.* Against *E. faecalis,* study reported better inhibition with an MIC and MBC of 0.3 and 0.6 mg/ml respectively. Greater inhibition was observed against *C. albicans* with an MIC of 0.075 mg/ml; the cidal activity of EEP against *C. albicans however,* was poorer with an MBC of 0.150 mg/ml.	Arslan *et al*., 2011
Brazil	Tested EEP against *S. mitis, S. mutans* and *S. salvarius* (cariogenic bacteria). Study reported zones of inhibition of 15.8 mm, 7.5 mm and 15.9 mm against *S. salvarius, S. mitis* and *S. mutans* respectively.	de Castro Ishida *et al*., 2011
Brazil	EEP as well as propolis microparticles (PMs) were tested against *Candida* spp. isolated from vulvovaginal candidasis. Study reported that all yeasts were inhibited by EEP at a concentration of 1100 µg/ml, and by PMs inhibition was observed at a concentration of 5570 µg/ml.	Dota *et al*., 2011
Iran	Antifungal activity of EEP was tested against *Epidermophyton flucosum, Trichophyton violaseum* and *Trichophyton tonsorans* in varying concentrations from 20-0.312 mg/ml. At concentrations of 0.625 and 0.312 mg/ml the diameter of colonies of the dermatophyte spp. ranged from 2-17 mm and 3-22 mm at these respective concentrations.	Gavanji *et al*., 2011

Origin	Results of study	References
Iraq	EEP tested against various pathogens including *E. coli, S. aureus, S. typhimurium, K. Pneumonia, C. albicans* and *P. aeruginosa.* EEP was tested at various concentrations, at the lowest concentration of 10% good antimicrobial activity was noted, with S. *aureus* displaying the greatest sensitivity, EEP was found to exert no effect on *C. albicans*	Hendi *et al*., 2011
Iran	Tested the effects of EEP against oral pathogens including; *E. faecalis, S. mutans* and *S. aureus.* Study reported that all three pathogens were inhibited at the same EEP concentration of 250 µg/ml.	Kashi *et al*., 2011
Korea	Tested propolis against foodborne pathogens. Found to display MICs 0.036 mg/µl against *B. cereus* on agar medium. When tested in nutrient broth, displayed MICs 1.8 mg/ml.	Kim and Chung, 2011
Brazil	Propolis extracts displayed inhibitory zones of 10 - 13 mm against *S. mutans* and 9 - 13 mm against *C. albicans.*	Liberio *et al*., 2011
Turkey	Study tested the effects of a triantibiotic mixture (TAM) and calcium hydroxide in comparison to EEP in the treatment of *E. faecalis* infected root canals. EEP was reported as being the only test sample producing the highest reduction in colony counts of *E. faecalis* with a 100% reduction rate after only 2 days of treatment.	Madhubala *et al*., 2011
Brazil	Reported antimicrobial activity against *S. aureus* MICs 62.5 - 125 µg/ml and against *S. mutans* MICs 62.5 - 125 µg/ml	Oldoni *et al*., 2011
Brazil	Study tested the antimicrobial activity of EEP samples against 210 strains of *S. aureus;* MRSA and methicillin sensitive *S .aureus* (MSSA). Reported that MICs ranged from 710 to 2850 µg/ml when tested against both MRSA and MSSA.	Pamplona-Zomenhan *et al*., 2011

Origin	Results of study	References
Malta and the island of Gozo	Maltese propolis displayed no activity against Gram-negative *E.coli.* All samples displayed good activity against *S. aureus* MICs 15 ± 1 mm to 27 ± 1 mm. Only six samples reported to display antifungal activity against *C. albicans* MICs 12 ± 0 mm to 16 ± 0 mm.	Popova *et al*., 2011
Brazil (red propolis)	Propolis extracts inhibited the growth of all microorganisms tested against with MICs as low as 256 µg/ml against *P. aeruginosa, B. subtilis* and *C. albicans.*	Righi *et al.*,2011
Chile	Tested the effects of commercial EEP samples against *L. fermentum. A*ll samples were found to inhibited growth of *L. fermentum* with the greatest inhibition being dilution 1/32.	Saavedra *et al*., 2011
Turkey	Study tested the inhibitory activity of EEP against foodborne pathogens; *S. enteriditis* and *L. monocytogenes*. Samples were tested as dilutions of 1:10 and 1:100 v/v with sterile distilled water. Reported that the higher concentration 1:10 v/v strongly inhibited the growth of both microorganisms, whilst the 1:100 v/v concentration had a minimal effect on tested pathogens.	Temiz *et al*., 2011
Iran	The effects of propolis and nanopropolis were tested against S. *aureus* and *C. albicans*. Study reported ZOIs of 16-18 mm against *C. albicans* and 11-13 mm against *S. aureus* with propolis with larger particle size, study furthermore found that nanopropolis (propolis milled to form nanoparticles) produced greater ZOIs ranging 18-21 mm and 24-26 mm against *C. albicans* and *S. aureus* respectively.	Afrouzan *et al*., 2012
India	Potent antimicrobial activity was observed against Gram-positive and Gram-negative bacteria as well as yeast species. MICs ranged between 1.21 - 9.75 µg/ml, furthermore no strains were found to be resistant to EEP.	Choudhari *et al.*, 2012

Origin	Results of study	References
Portugal	Samples exhibited antimicrobial activity, however, the effect was dependant on the dose and origin of propolis. MIC's ranged from 0.24 ± 0.17 mg/ml to 1.43 ± 0.32 mg/ml against Methicillin resistant *S. aureus.*	Dias *et al.*, 2012
Iran	Study tested the activity of EEP in comparison to calcium hydroxide against *Lactobacillus, C. albicans, E. faecalis and Peptostreptococcus*. In comparison to calcium hydroxide EEP was found to display larger ZOIs of 8.7 mm compared to the ZOI of calcium hydroxide at 7.1 mm.	Kousedghi *et al*., 2012
Turkey	Tested the effects of EEP against *S. aureus, E. faecalis* and *C. albicans*. Study found that EEP the greatest inhibition was against *E. faecalis* with a ZOI of 15.8 mm.	Mattigatti *et al*., 2012
Slovenia	Tested two samples PEE70 and PEE96. All extracts showed to be more effective against Gram-positive bacteria with MICs 0.15 - 1.2 mg Chlorogenic acid equivalents(CAE)/ml. Against Gram-negative bacteria, yeasts and moulds MICs 0.17 - 1.4 mg CAE/ml.	Mavri *et al*., 2012
Cuba	Antibacterial, antifungal and antiprotozoal activities of red, brown and yellow Cuban EEP tested against *S. aureus, E. coli, T. rubrum, C. albicans, Plasmodium falciparum* and *Leishmania.* Study reported S. *aureus* as being the most sensitive microorganism MIC 4.4±1.3 µg/ml, *E. coli* and *C. albicans* displayed the least sensitivity to all Cuban EEP samples with MIC >64 µg/ml. Yellow Cuban EEP reported as having good antiprotozoal activity, particularly against *Plasmodium* spp. with an MIC of 0.2±0 µg/ml.	Monzote *et al*., 2012
Brazil and Bulgaria	Brazilian propolis inhibited the growth of *S. typhimurium* with an MIC of 9.9% v/v, and the MIC of Bulgarian propolis was 10.0% v/v.	Orsi *et al*., 2012

Origin	Results of study	References
Brazil	Using the microdilution assay, tested EEP against three strains of *S. aureus* causing mastitis in cows. Propolis was found to display antimicrobial activity of 0.292 and 0.586 mg/ml against all strains tested.	Santana *et al*., 2012
Portugal	Antimicrobial activity found to be dependent on origin of propolis. *S. aureus* reported as the most sensitive with MIC values 0.59 ± 0.30; 1.36 ± 0.79 and 1.72 ± 0.87 mg/ml. *C. albicans* found to be the most resistant MIC values 13.19 ± 7.21; 13.44 ± 8.23 and 13.90 ± 7.512 mg/ml.	Silva *et al*., 2012
India	Investigated the effects of propolis produced by *Apis mellifera* and *Trigona* spp. against various pathogens including *B. cereus, Burkholderia glumae, Xanthomonas campestris.* The greatest inhibition was noted against *B. glumae* for *A. mellifera* with a ZOI of 17.52±2.04 mm and for *Trigona sp.* with a ZOI of20.0± 0.63 mm.	Surendra *et al*., 2012
Iraq	EEP 50 mg/ml and 100mg/ml produces ZOIs of 30 and 40 mm against *S. typhimurium*, respectively.	Al-safi, 2013
Czech Republic	Antimicrobial (MIC) and the minimum microbicidal concentration (MMC) of EEP was tested against various pathogens including vancomycin resistant *Enterococcus faecium*, MRSA, *S. pyogenes* and various *Candida* spp. Study reported *S. pyogenes* as being the most sensitive pathogen tested with MICs of 0.03-0.13 mg/ml and MBCs of 0.06-0.25 mg/ml; MRSA and *E. faecium* were reported as the least susceptible with MICs and MBCs of 0.13->4 mg/ml and 0.5->4 mg/ml, respectively. MICs and fungicidal concentrations (MFC) against the various *Candida* spp. ranged from 0.15-1.2 mg/ml, and MFCs of 0.6-2.4 mg/ml.	Astani *et al*., 2013

Origin	Results of study	References
Chile	Two samples from central Chile displayed the lowest activity with MICs 6.67 and 8.22 µg/ml against *S. mutans* and *S. sobrinus* respectively. Another 2 samples from southern Chile displayed the highest antimicrobial activity with MICs 1.94 and 0.90 µg/ml against *S. mutans* and *S. sobrinus* respectively.	Barrientos *et al*., 2013
Brazil	Using the microdilution assay the study tested propolis produced by honeybees, *Melipona scutellaris*, against *S. mutans*, *S. aureus*, MRSA, *E. faecalis*, *Actinomyces naeslundii* and *P. aeruginosa.* Reported geopropolis as being able to inhibit the growth of *s. mutans, S. aureus* and MRSA with MICs <50 µg/ml and MBCs 25 - 50 µg/ml. No activity was noted against *P. aeruginosa.* Against *E. faecalis* and *A. naeslundii* MICs ranged from 800 to 1600 µg/ml.	da Cunha *et al*., 2013
Brazil	EEP tested against a variety of rumen bacteria. *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* were reported as the most sensitive microorganisms inhabited at an EEP concentration of 500 µg/ml, all other microorganisms tested were less susceptible and all inhabited at an EEP concentration of 1000 µg/ml.	de Aguiar *et al*., 2013
Poland	Mean MICs of EEP against various *S. mutans* spp. ranged 0.78-1.56 mg/ml and against *Lactobacillus* spp. ranged 0.20-1.56 mg/ml. Study also tested the MBC of EEP and found that against *S. mutans* spp. mean MBCs ranged 3.13-12.5 mg/ml and 0.39-12.5 mg/ml against *Lactobacillus* spp.	Dziedzic *et al*., 2013
Portugal	EEP was tested against S. *aureus* and *C. albicans*, study reported ZOI of 24.6±3.8 mm and 32.0±3.2 mm against *S. aureus* and *C. albicans*, respectively.	Falcão *et al*., 2013
India	Antimicrobial activity of EEP was tested against *S.*	Kalia *et al*., 2013

Origin	Results of study	References
Iran	*typhimurium* in varying concentrations, the MIC of EEP was reported as 200 mg/ml and its MBC as 250mg/ml. EEP was tested against 128 various pathogens causing onychomycosis including *C. albicans.* The MIC for various strains of *Candida* spp. ranged from 2-20 µg/ml.	Khosravi *et al*., 2013
Oman	MICs ranged 42 - 169 µg/ml against *S. aureus* and 169 - 365 µg/ml against *E. coli.*	Popova *et al*., 2013
Northern Thailand	30%, 40% and 50% EEP's showed inhibitory zones between 4.11-4.44 mm against Gram-positive bacteria.	Siripatrawan *et al.,* 2013
Poland	The MIC and MBC of Polish EEP samples were tested against MSSA and MRSA. Study reported MICs and MBCs against MSSA of 0.39-0.78 mg/ml and 1.56-3.13 mg/ml, respectively, and against MRSA of 0.39-0.78 mg/ml and 0.78-3.13 mg/ml, respectively.	Wojtyczka *et al*., 2013a
Poland	Antimicrobial activity of EEP tested against *S. epidermidis.* Study reported all strains as susceptible and inhibited at MICs 0.78-1.56 mg/ml.	Wojtyczka *et al*., 2013b
Argentina	Activity of propolis was tested against various *Candida* spp. as well as dermatophytes such as *Trichophyton mentagrophytes* and *T. rubrum.* Study reported that a majority of *Candidal* and dermatophyte spp. were inhibited by EEP with MICs ranging 31.25-125 µg/ml.	Agüero *et al*., 2014
Brazil	Propolis was obtained from bee species *Melipona orbignyi.* MICs ranged 3.1 - 50 mg/ml against *S. aureus* and *C. albicans.*	Campos *et al*., 2014
Brazil	Antibacterial activity tested against *S. aureus*, reported ZOIs 2.6-14.3 mm.	de Souza *et al*., 2014
Egypt	EEP was tested against bacterial pathogens causing mastitis including *E. coli, S. aureus, E. faecalis* and *P. aeruginosa*. *S. aureus* and *E. faecalis* were reported as being the most sensitive approximately 41% of all	Hegazi *et al*., 2014

Origin	Results of study	References
	bacterial strains implicated in mastitis in this study were found to be inhibited by EEP.	
Iraq	EEP samples tested against various pathogens causing keratitis in lab animals such as *E. coli* and *P. aeruginosa. P. aeruginosa* found to be the most sensitive with ZOI range 44-58 mm and *E. coli* less sensitive with ZOI 23-32 mm.	Kadhim and Kadhim, 2014
India	Ultrasonicated EEP samples were tested against various Gram-positive and Gram-negative pathogens including *S. aureus* and *E. coli*, study reported ZOIs of 1+0.03 mm, 4 +0.07 mm and 6+0.2 mm against *S. aureus* and 4 +0.05 mm, 5+0.01 mm and 7 +0.4 mm against *E. coli. Th*ese results were found at propolis concentrations of 500, 750 and 1000µg/ml of EEP respectively.	Kothai and Jayanthi, 2014
Australia	Propolis obtained from bee species *Tetragona carbonaria*. Propolis tested against *S. aureus* and *P. aeruginosa. S. aureus* displayed MIC 6.94 µg/ml.	Massaro *et al.*, 2014
Romania	Inhibitory activity of propolis was tested against organisms known to cause severe infections in animals such as; *S. aureus, E. coli, P. aeruginosa, Salmonella* spp., *Streptococcus suis* and *Pasteurella haemolytica. S. aureus* was found to be the most sensitive pathogen with a ZOI of 27 mm and *P. haemolytica* was noted as the least sensitive pathogen with a ZOI of 15 mm.	Moţ *et al*., 2014
Cameroon	Methanolic extracts of propolis (PME) were tested against *Salmonella spp., E.coli* and *P. aeruginosa.* PME displayed no antimicrobial activity against *Salmonella* spp., however antimicrobial activity was noted against *E. coli* and *P. aeruginosa,* ZOIs of 26 mm and 32.5 mm , respectively, were reported with corresponding MICs of 0.2 mg/ml.	Sakava *et al.*, 2014
Brazil	EEP tested against *S. aureus, E. faecalis* and	Schmidt *et al.*,

Origin	Results of study	References
	Micrococcus luteus; MICs ranged 340-1300 µg/ml, 760-2730 µg/ml and 340-680 µg/ml, respectively. MBCs reported ranged 380-2600 µg/ml, 1500-5480 µg/ml and 380-1560 µg/ml against respective pathogens tested.	2014
Brazil	Antimicrobial properties of geopropolis extracts tested against *S. aureus* and *E. coli.* Reported that only high concentrations of geopropolis extracts inhibited growth with MICs of 1788 µg/ml and 1997 µg/ml against *S. aureus* and *E. coli*, respectively.	Araújo *et al*., 2015
Brazil	EEP was tested against clinical strains of *Candida* spp. in varying concentrations. EEP samples with a concentration of 25% were reported as displaying a ZOI of 26.40±18.00	Bezerra *et al*., 2015
France	Antifungal and antibacterial activity of EEP was tested against various strains of C*andida* spp. and bacteria including *S. aureus, P. aeruginosa*, *E.coli* and *E. faecalis*. Study found that for all extracts of propolis antifungal activity ranged 15.63- >250 µg/ml, MIC against Gram-positive bacteria ranged 57-> 100 µg/ml. Gram-negative bacteria were found to be less susceptible to antimicrobial activity.	Boisard *et al*., 2015
Brazil	The antimicrobial activity of EEP samples were tested against various pathogens including S. *aureus,* E. *faecalis, K. Pneumonia, P. aeruginosa* and *C. albicans*. MICs against Gram-positive bacteria ranged 0.55-1.02 mg/ml, against Gram-negative bacteria ranged 2.25-7.91 mg/ml and against yeast species ranged 7-9.25 mg/ml.	Campos *et al*., 2015
Brazil	Antifungal activity of propolis tested against *C. albicans*. Study reported that all strains were inhibited with MICs 68.35-546.87 µg/ml.	Capoci *et al.*, 2015

Origin	Results of study	References
Lebanon	EEP was tested against extended spectrum -β lacatamase *Klebsiella* (ESBL-*Klebsiella pneumonia*), methicillin resistant *S. aureus* (MRSA) and *Candida albicans.* All samples were found to display antimicrobial activity with the most active sample being from Saffareh-Jezzine, this sample displayed MICs of 6.25 mg/ml against MRSA, 12.5 mg/ml against ESBL *K. pneumonia* and 12.5 mg/ml against *C. albicans.*	Chamandi *et al.*, 2015
Lithuania	Water, oil and ethanolic extracts of propolis were tested against *S. aureus, B. cereus, K. pneumoniae, P. aeruginosa, E. coli* and *C. albicans.* Study reported that some water and oil extracts displayed good antimicrobial activity with ZOIs ranging 16.0-20.5 mm for water and oil extracts against all microorganisms tested. ZOIs for EEP ranged 14.8-17.2 mm against all microorganisms tested.	Kubiliene *et al*., 2015
Brazil	The activity of brown EEP samples was tested against *E. faecalis.* Study reported a percentage inhibition of 36% for samples containing a 40% concentration of Brazilian brown EEP.	Pimenta *et al*., 2015
India	Olive oil and virgin coconut oil (VCO) extracted samples of propolis were tested against *S. aureus, E. coli* and *S. typhimurium* in comparison to EEP extracts. Study reported that oil extracts displayed better antimicrobial activity than all other extracts and an increase in Gram-negative inhibition was also noted. Reported ZOIs of 22 mm and 9 mm against *S. aureus* and *E. coli,* respectively.	Pujirahayu *et al*., 2015
Thailand	Stingless bee (i.e. *Tetragonula laeviceps* and *Tetrigona melanoleuca*) EEP tested against various microorganisms including *S. typhimurium, B. cereus, E. coli, S. aureus, P. aeruginosa* and *S. epidermidis.*	Sanpa *et al*., 2015

Origin	Results of study	References
	Reported MICs of 0.13-16 mg/ml and MBCs of 1-128 mg/ml against all microorganisms tested.	

Appendix H: Compounds identified in propolis worldwide.

Origin	Compounds identified	References
south Bulgaria	Caffeic acid esters and ferulic acid esters.	Bankova *et al*., 1989
Spain	Benzoic acid, 1,2,3-propanetriol, butanedioic acid, glycerol monoacetate, 2-hydroxybutanedioic acid, guaiol, sesquiterpene alcohol, α-glucopyranose, trans-3(3,4-dimethoxyphenyl)propenoic acid, hexadecanoic acid, flenicosane, 3-methyl-3-butenyl trans-4-coumarate, trans-3(3,4-dihydroxyphenyl)propenoic acid, octadecenoic acid, 2-methylpropyl trans-caffeate, tricosane, 2-methyl-2-butenyl trans-isoferulate, 2',6'-dihydroxy-4'-methoxydihydrochalcone, 2-methyl-2-butenyl trans-caffeate, 3-methyl-2-butenyl trans-caffeate, 2',4',6'-trihydroxydihydrochalcone, pinostrobin, 2',6'-dihydroxy-4'-methoxychalcone, pinocembrin, pentacosane, dihydroxymonomethoxyflavanone, pinobanksin, pinobanksin-3-methyl ether, pinobanksin-3-acetate, benzyl trans-caffeate, heptacosane, glyceryl trans-caffeate, galangin-7-methyl ether, chrysin, pinobanksin-3-propanoate, galangin-3-methyl ether, sucrose, galangin, nonacosane, trans-cinnamyl-trans-caffeate, hexacosanoic acid, henitriacontane and octacosanoic acid.	García-Viguera *et al*., 1992
Albania, Bulgaria and	**Ketones, alcohols, phenols:** methoxyacetophenone, 4-phenyl-3-buten-2-one, methoxyacetophenone(isomer), 2-phenylethanol,	Bankova *et al*., 1994

Origin	Compounds identified	References
Mongolia	isoeugenol. **Esters:** Unidentified ester of 2-phenylethanol, benzyl acetate, benzyl benzoate. **Terpenoids:** δ-cadinen, guaiol, α-copaen, caryophylen, β-selinen, α-elemen, calamenen, α-muurolen, γ-muurolen, cadinen (isomer), β-eudesmol, bulnesol. **Hydrocarbons:** 3-methylinden, alkylbenzol, heneicosane (C-21), tricosane (C-23), pentacosane (C-25), heptacosane (C-27), nonacosane (C-29) and hentriacontane (C-31).	
New Zealand	**Dihydroflavonoids:** Pinocembrin, pinobanksin and pinobanksin 3-acetate; cinnamic acid and their esters, including the rare 5-phenyl-*trans*-*trans*-2,4-pentadienoic acid and a new natural product; 5-phenyl-*trans*-3-pentenoic acid.	Markham *et al*., 1996
Canary Islands	Palmitic acid, erytriol, stearic acid, xylitol, oleic acid, inositol, methylmalonic acid, myo-inositol, lactic acid, erytraric acid, malic acid, deoxyerytropentaric acid, dimethyoxybenzoic acid, tetronic acid, phosphoric acid, glucuronic acid, D-ribofuranose, isosesamin, D-xylopiranose, methyl xantoxylol, D-mannopyranose, D-sorbopyranose, D-galactose, D-fructose, β-D-glucopyranose, sucrose, lactose, maltose, melibiose and diterpene acid, myristic acid, aromadendrene, cinnamic acid, ledol, methyl palmitate, spatulenol, ethyl palmitate, isospatulenol, ethyl oleate, palustrol, benzyl benzoate, β-cayophillene, ethyl dihydrocinnamate, α-humulene, benzaldehyde, nonane, piperonal, decane, linalyl propionate, undecane, geraniol, dodecane, nerolidol, tridecane, δ-cadinene, tetradecane, α-muurolene, hexadecane, α-calakorene, heptadecane, T-muurolol, β-selinene, octadecane, germacrene, nonadecane, α-	Bankova *et al*., 1998

Origin	Compounds identified	References
	copaene, henicosane, ledene, docosane, tricosane, 2-methylnaphthalene, m-methylstirol, vanquard BT (pesticide) and dodecaniene-1-ol.	
Brazil	Quercetin, kaempferol, isosakuranetin, sakuranetin, kaempferide, acacetin, isorhamnetin and pinocembrin.	Park and Ikegaki, 1998
Brazil	*p*-Coumaric acid, dihydrocinnamic acid, prenyl-*p*-coumaric acid, diprenyl-*p*-coumaric acid, aromadendrine-4'-methyl ether, kaempferide, β-amyrine and cycloartenol.	Bankova *et al*., 1999
Canary Islands	Sesamin, aschantin, sesartemin and yangambin.	Christov *et al*., 1999
Chile	Viscidone, trementone, 14-hydroxytrementone, 2,2-dimethyl-6-acetyl-2H-chromen, coniferyl-9-*O*-acetate, ferulic acid ethyl ester, coniferyl aldehyde, vanillin, dihydrobenzofuran lignan aldehyde, 9,9'-bisacetyl-olivil, diastereomers of a dimeric coniferyl alcohol and trimeric coniferyl acetate.	Valcic *et al*., 1999
Uruguay and China	Caffeic acid, vanillin, pinocembrin, ferulic acid, naringin, sinapic acid, m-coumaric acid, rutin, 4-hydroxybenzoic, o-cinnamic acid, pinobanksin, quercetin, hesperitin, kaempherol, galangin, apigenin, isorhamnetin, chrysin, acacetin, pinostrobin, tectochrysin and rhamnetin.	Bonvehí and Coll, 2000
Brazil	Benzoic acids, dihydrocinnamic acids, cinnamic acids, long chain caffeates, prenyl caffeates, benzyl and phenethyl caffeates, C-prenylated coumaric acids, diterpenic acids, triterpenic alcohols and sugars.	Velikova *et al*., 2000
Turkey	3-Methyl-2-butenol, ethyl decanoate, ethyl benzoate, diethyl succinate, ethyl decanoate, calamenene, ethyl-3-phenyl propionate, phenylethyl alcohol, (*E*)-ethyl	Keskin *et al*., 2001

Origin	Compounds identified	References
	cinnamate, γ-eudesmol, α-eudesmol, β-eudesmol, ethyl hexadecanoate, decanoic acid and ethyl oleate.	
Brazil	3-prenyl-4-hydroxycinnamic acid, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyrane, 3,5-diprenyl-4-hydroxycinnamic acid and 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran.	Marcucci *et al*., 2001
Brazil	Gallic acid, diterpines, chlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, *p*-coumaric acid, caffeic acid and flavonoids.	Midorikawa *et al*., 2001
El Salvador	Chalcones: 2',3'-dihydroxy-4,4'-dimethoxychalcone and 2',3',4-trihydroxy-4'-methoxychalcone.	Popova *et al*., 2001
Turkey	Alcohols, aliphatic acids, amino acids, aromatic acid esters, aromatic acids, aromatic aldehydes, flavonoids, ketones and terpenoids.	Sorkun *et al*., 2001
Egypt	**Aliphatic acids:** Lactic acid, hydroxyacetic acid, 5-hydroxy-n-valeric acid, 2,3-dihydroxypropanoic acid, pentonic acid- 2-deoxy-3,5-dihydroxy-γ-lactone, pentonic acid- 2-deoxy-3,5-dihydroxy-γ-lactone (isomer), malic acid, succinic acid, 2,3,4,5-tetrahydroxypentanoic acid-1,4-lactone, 2,3,4,5-tetrahydroxypentanoic acid-1,4-lactone (isomer), nonanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, palmitic acid, heptadecanoic acid, linoleic acid, oleic acid, eicosanoic acid, tetracosanoic acid, hexacosanoic acid and 2- hydroxy hexacosanoic acid and stearic acid. **Aromatic acids:** Benzoic acid, 2-phenyl- 2-hydroxy acrylic acid, 4-hydroxy benzoic acid, dihydrocinnamic acid, cinnamic acid, 4-methoxy-cinnamic acid, cis-*p*-coumaric acid, trans-*p*-coumaric acid, 3,4-dimethoxy-cinnamic acid, isoferulic acid, ferulic acid and caffeic acid. **Esters:** Methyl palmitate, ethyl	Abd El Hady and Hegazi, 2002

Origin	Compounds identified	References
	palmitate, stearic acid methyl ester, phthalate ester, benzyl benzoate, benzyl-trans-4- coumarate, cinnamyl-trans-4- coumarate, 3-methyl-3-butenyl isoferulate, 3-methyl-2-butenyl isoferulate, 3-methyl-3-butenyl caffeate, 2-methyl-2-butenyl caffeate, 3-methyl-2-butenyl caffeate, benzyl caffeate, phenylethyl caffeate, cinnamyl caffeate, tetradecyl caffeate, tetradecenyl caffeate, tetradecenyl caffeate (isomer), tetradecanyl caffeate and hexadecyl caffeate. **Di and Triterpenes:** Pimaric acid, dehydroabietic acid, abietic acid, lupeol, cycloartinol, lanosterol, lanosterol with another double bond, α-amyrin, β-amyrin, triterpene of β-amyrin and 3-oxo-triterpenic acid methyl ester (oleanane type). **Flavonoids:** 2',6'-Dihydroxy-4'-methoxychalcone (Pinostrobin chalcone), hexamethoxyflavone, pinostrobin, pinocembrin, pinobanksin, pinobanksin-3-acetate, chrysin, galangin and 5,7- dihydroxy-3-butanoyloxyflavanone.	
Italy, Switzerland and Bulgaria	Pinocembrin, pinobanksin, pinobanksin-3-*O*-acetate, chrysin, galangin, pentenyl caffeates, benzyl caffeate, phenethyl caffeate, phenolic glycerides, ferulic acid and diterpenic acids.	Bankova *et al*., 2002
Uruguay	Pinobanksin 3-hexanoate, pinobanksin 3-butanoate, pinobanksin 3-propanoate, pinobanksin 3-acetate, pinobanksin 3-acetoxy-7-methyl ether (3-acetylalpinone), pinobanksin 5-methyl ether, pinobanksin, pinostrobin, pinocembrin, chrysin, tectochrysin, chrysin 5-methyl ether, galangin, izalpinin, kaempferol , quercetin 3-methyl ether, *p*-coumaric acid, caffeic acid, 3,4-dimethoxycinnamic acid, cinnamylidene acetic acid, 2-methyl-2-butenyl	Kumazawa *et al*., 2002

Origin	Compounds identified	References
	p-coumarate, 3-methyl-3-butenyl ferulate, benzyl *p*-coumarate, benzyl ferulate, phenethyl caffeate, cinnamyl cinnamate, cinnamyl *p*-coumarate, cinnamyl caffeate, cinnamyl isoferulate and cinnamyl 3,4-dimethoxycinnamate.	
Croatia	Galangin, naringenin, chrysin, pinocembrin and caffeic acid.	Kosalec *et al*., 2003
Brazil	Chlorogenic acid, caffeic acid, *p*-coumaric acid, 4,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid methyl ester, 3,4,5-tricaffeoylquinic acid, dihydrokaempferide, 6-methoxykaempferol, drupanin, dihydroconiferyl *p*-coumarate, capillartemisin A, (E)-3-[2,3-dihydro-2-(1-hydroxy-1-methylethyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid, (E)-3-[2,3-dihydro-2-(1-methylethyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid, (E)-3-(2,2-dimethyl-3,4-dihydro-3-hydroxy-8-prenyl-2H-1-benzopyran-6-yl)-2-propenoic acid, artepillin C, (E)-3-prenyl-4-(2-methylpropionyloxy)-cinnamic acid, (E)-3-prenyl-4-(dihydrocinnamoyloxy)-cinnamic acid.	Kumazawa *et al.*, 2003
Brazil	Caffeic acid, *p*-coumaric acid, ferulic acid, dicaffeoylquinic acid isomer, 3,5-diprenyl-4-hydroxycinnamic acid derivative, pinobanksin, dicaffeoylquinic acid isomer, 3-prenyl-4-hydroxycinnamic acid, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, kaempferol derivative, cinnamic acid derivative, 3-prenyl-4-dihydrocinnamoyloxycinnamic acid, 2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid	Cunha *et al*., 2004

Origin	Compounds identified	References
South America, North America, Australia, Europe, Asia and Africa.	Flavonoids including *p*-coumaric acid, flavonoids absent in European propolis samples tested.	Kumazawa *et al*., 2004
Greece	CC: diterpenic and phenolic compounds such as; 7-*O*-prenylstrobopinin, 7-*O*-prenylpinocembrin, pinostrobin, copalol, 13-*epi*-torulosal, pinocembrin, pinobanksin, isoagatholal, sakuranetin, chrysin, pinobanksin 5-methyl ether, 13-*epi*-cupressic acid, benzoic acid, *p*-coumaric acid, isocupressic acid, agathodiol, and caffeic acid. GC-MS: cinnamyl cinnamate, feruginol, benzyl cinnamate, benzyl benzoate, butyl cinnamate and butyl vanillate.	Melliou and Chinou, 2004
Bulgaria, Italy and Swiss	Caffeic acid, *p*-Coumaric acid, Ferulic acid, Kaempferol, Pinocembrin, Phenethyl caffeate , Isopentyl caffeate, Chrysin, Galangin, Pinostrobin and Benzyl caffeate.	Popova *et al*., 2004
Bulgaria	Ethyl hydrocinnamate, hydrocinnamic acid, ethyl indolacetate, 2-indolcarboxylic acid, mannose, inositol, cinnamic acid, hexadecanoic acid, ferulic acid, caffeic acid, oleic acid, 3-ketoadipic acid, acetobutyric acid, pentanedioc acid, pentenoic acid, linoleic acid, pinostrobin, diethyl 2-methylsuccinate, isobutylquinoline, geranyl acetal, patchouli alcohol, menthol, 2-hydroximethyl-3-indolacetic acid, undecanoic acid, glicyrrizic acid, α-amyrin, β-amyrin, 3-methoxy-b-amyrin, β-amyrin acetate and 2-ethylhexanoic acid.	Salomão *et al*., 2004

Origin	Compounds identified	References
Brazil	2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, 6-propenoic-2,2-dimethyl-8-prenyl-2H-1-benzopyranic acid, 3,5-diprenyl-4-hydroxycinnamic acid and kaempferol.	Sawaya *et al*., 2004
Brazil, Bulgaria, Mozambique, England, Finland and North America	*para*-coumaric acid, 3-methoxy-4-hydroxycinnamaldehyde, 2-2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, 3-prenyl-4-hydroxycinnamic acid, chrysin, pinocembrin, 3,5-diprenyl-4-hydroxycinnamic acid and dicaffeoylquinic acid.	Sawaya *et al*., 2004
Nepal	Neoflavonoids, chalcone, (+)-vesticarpan, cearoin, 9-hydroxy-6,7-dimethoxydalbergiquinol,obtusaquinol, , medicarpin, 4-hydroxymedicarpin and 2',4,4'-trihydroxychalcone, (S)-4-methoxydalbergione.	Awale *et al*., 2005
Cuba	Propolone, nemorosone, guttiferone and xanthochymol, garcinielliptone I, hyperibone B, propolone B and propolone D.	Hernández *et al*., 2005
Turkey	**Fatty and aromatic acids:** 9-ocadecenoic acid, 2-propenoic acid and caffeic acid. **Alcohol, ketone, and terpenes:** 2-naphtalenemethanol, 2-propen-1-one, 4H-1-benzopyran-4-one, coumaran-5,6-diol-3-one and benzofran-3-one. **Flavonoids:** chrysin. **Esters:** cinnamyl cinnmate. **Others:** 1-phenathrenecarboxaldehyde, benzeneamine, eicosane, heptacosane and cyclotrisiloxane.	Koc *et al*., 2005
Turkey	Pinocembrin, pinobanksin, pinobanksin 3-*O*-acetate, chrysin, galangin, *p*-coumaric acid, ferulic acid, caffeic aid, pentenyl caffeates, benzyl and phenethyl esters of caffeic, ferulic and *p*-coumaric acid, cinnamic acid, glucose, benzyl cinnamate, oleic acid,	Popova *et al*., 2005

Origin	Compounds identified	References
	dimethylallyl caffeate, dehydroabietic acid, cinnamyl cinnamate, cynnamil caffeate, benzoic acid, vanillin, benzyl *p*-coumarate, benzyl ferulate, coumaroyl glycerol, coumaroyl glycerol (isomer), coumaroyl acetyl glycerol, dicoumarouyl acetyl glycerol, diferuloyl acetyl glycerol, coumaroyl caffeoyl acetyl glycerol, phenylethyl caffeate, dihydroabietic, abietic, isopimaric, hydroxy fatty acids (hydroxypalmitic, hydroxystearic), and triterpenic alcohols.	
Turkey	Naringenin, Chrysin, Acacetin, 9-Octadecanoic acid, Hexadecanoic acid, Decanoic acid, Benzoic acid, Ferulic acid, 3,4-Dimethoxycinnamic acid, 3-Hydroxy-4-methoxycinnamic acid, 4-Pentenoic acid, 1,3-Benzenedicarboxylic acid, 2-Propenoic acid, 3-Hydroxy-4-niethoxycinnamic acid, Benzene acetic acid, 1,3-Benzenedicarboxylic acid, Ethyl acetate, Benzyl benzoate, Benzyl cinnamate, Benzene ethanol, Benzyl alcohol, 4-Vinylphenol, Chrysophanol, 4,5-Dimethoxy-2-phenol, α-Eudesmol, 4-Vinyl-2-methoxy-phenol, α-Cadinol, β-Eudesmol, α-Bisabolol, 2-Naphtalenemethanol, Glycerine, 2-Methoxy-4-vinyphenol, 2-Nonadeeanone, 2-Nonadecanone, 2-Propen-1-one, 4*H*-1-Benzopyran-4-one, 1-Methyl-4-azailuorenone, Propanal, 2,4-Cycloheptadien-l-one, >Nonadecane, 2,3-Dihydro-benzofuran, Heneicosane, 4-Hydroxy-2-methoxycinnamaldehyde, Benzaldehyde, Eicosane, Octadecane, Docosane, Nonadecane, Heneicosane, 2,5-Diethyl-3,6-dimethylpyrazine, Vanillin, Benzene and β-Cadinene.	Silici and Kutluca, 2005
Canada	**Aromatic acids:** benzoic acid, dihydroxycinnamic	Christov *et al*.,

Origin	Compounds identified	References
	acid, Z-cinnamic acid, E-cinnamic acid, 3-phenyl-3-hydroxypropanoic acid, methoxyphenylpropanoic acid, 4-hydroxybenzoic acid, Z-*p*-coumaric acid, E-*p*-coumaric acid, ferulic acid and caffeic acid. **Other aromatics:** benzyl alcohol, 4-hydroxybenzaldehyde, hydroquinone, cinnamyl alcohol and hydroxyacetophenone. **Fatty acids:** oleic acid, stearic acid, palmitic acid. **Esters:** benzyl benzoate, benzyl methoxybenzoate, benzyl hydroxybenzoate, benzyl dihydroxybenzoate, benzyl Z-*p*-coumarate, benzyl E-*p*-coumarate, phenethyl *p*-coumarate, benzyl ferulate, benzyl caffeate, phenethyl caffeate, cinnamyl caffeate, pinostrobin chalcone, pinocembrin, pinobanksin, sakuranetin, isosakuranetin, alpinone, pinobanksin 3-*O*-acetate and galangin. **Dihydrochalcones:** 2' ,6' -dihydroxy-4'methoxydihydrochalcone, 2' ,4' ,6' – trihydroxydihydrochalcone, 2' ,6' -dihydroxy-4',4-dimethoxydihydrochalcone, 2' ,4' ,6' -trihydroxy-4-methoxydihydrochalcone and 2' ,6' ,4-trihydroxy-4'-methoxydihydrochalcone. Others: glycerol, hexoses and sesquiterpenes.	2006
Brazil	Phenolic and flavonoid compounds	da Silva *et al*., 2006
Turkey	Chrysin, apigenin, flavonoids, flavanones, naringenin, ethyl oleate, 3-4-dimethoxy-cinnamic acid and 9-octadecenoic acid.	Mercan *et al*., 2006
Argentina, Italy, Spain, China, Azerbaijan and	Pinocembrin, naringenin, genistein, apigenin, kaempferol, acacetin, galangin and chrysin.	Volpi and Bergonzini, 2006

Origin	Compounds identified	References
Ethiopia		
Brazil	Butanedioic acid, dimethyl ester, hydroxy-butanedioic acid, dimethyl ester, *m*-guaiacol, 1-methoxy-4-(1-propenyl)-benzene, methyleugenol, methyl o-orsellinate, 1,2,3-trimethoxy-5-(2-propenyl)-benzene, methoxyeugenol, hexadecanoic acid, methyl ester, 10-octadecenoic acid, methyl ester, methyl abietate, benzoic acid, homopterocarpin, medicarpin, 2,4,6-trimethylphenol, 4'7-dimethoxy-2'-isoflavonol, 7,4'-dihydroxyisoflavone, 2H-1-benzopyran-7-ol, 2,2,6-*beta*-trimethyl-bicyclo(4.3.0)non-9(1)-en-7.alpha.-ol, 1,1,2-trimethyl-3,5-bis(1-methylethenyl)-,(2.alpha., 3.alpha., 5.beta.)-cyclohexane.	Alencar *et al*., 2007
Cuba	Nemorosone, scrobiculatones A and B, isoliquiritigenin, liquiritigenin, formononetin, biochanin A, vestitol, neovestitol, 7-*O*-metilvestitol, medicarpin, homopterocarpin, vesticarpan, 3,8-dihydroxy-9-methoxypterocarpan and 3-hydroxy-8,9-dimethoxypterocarpan.	Cuesta-Rubio *et al*., 2007
Various regions of Asia, South and North America and Europe	Phenolic acids, flavanones, flavones, flavonols, balsams and dihydroflavonols.	Popova *et al*., 2007
Brazil	Ferulic acid, chrysin, quercetin, medicarpin and 3-Hydroxy-8,9-dimethoxypterocarpan.	Silva *et al*., 2008
Mexico	Flavones, flavonols, flavanones, dihydroflavonols and phenolic compounds.	Velazquez *et al*., 2007

Origin	Compounds identified	References
Brazil	(6aS,11aS)-6a-ethoxymedicarpan, 2-(2',4'-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran, 2,6-dihydroxy-2-[(4-hydroxyphenyl)methyl]-3-benzofuranone, (2R,3R)-3,7-dihydroxy-6-methoxyflavanone, alnusin, alnustinol, (+)-pinoresinol dimethyl ether, (2S)-dihydrooroxylin A, (6aS,11aS)-medicarpan, (6aR,11aR)-3,4-dihydroxy-9-methoxypterocarpan, (2S)-dihydrobaicalein, (6aR,11aR)-4-methoxymedicarpin, (7S)-dalbergiphenol, (2S)-7-hydroxy-6-methoxyflavanone, (6aR,11aR)-3-hydroxy-8,9-dimethoxypterocarpan, 2',4'-dihydroxychalcone, (3S)-7-*O*-methylvestitol, (6aS,11aS)-3,10-dihydroxy-9-methoxypterocarpan, (2S)-7-hydroxyflavanone, (+)-pinoresinol, (6aR,11aR)-3,8-dihydroxy-9-methoxypterocarpan, (3S)-Mucronulatol, (2R,3R)-3,7-dihydroxyflavanone, biochanin A, formononetin, (3S)-ferreirin, 2'-hydroxybiochanin A, (3S)-violanone, pratensein, xenognosin B, (+)-syringaresinol, (3S)-vestitol, Isoliquiritigenin, (3S)-vestitone, (3R)-4'-methoxy-2',3,7-trihydroxyisoflavanone, (2S)-liquiritigenin, calycosin, (2S)-naringenin, garbanzol, 4,4'-dihydroxy-2'-methoxychalcone, (3S)-isovestitol, 2',4,4'-tetrahydroxydihydrochalcone, daidzein.	Awale *et al*., 2008
Romania	Caffeic acid, rutin, quercetin, apigenin, kaempferol and chrysin.	Coneac *et al*., 2008
Brazil	Pinocembrin, rutin, liquiritigenin, daidzein, pinobanksin, pinobanksin-3-acetate, quercetin, luteolin, dalbergin, isoliquiritigenin, formononetin and biochanin A.	Daugsch *et al*., 2008
Turkey	**Fatty and aliphatic acids:** butanedioic acid,	Vardar-Ünlü *et al*.,

Origin	Compounds identified	References
	propanoic acid, decanoic acid, malic acid, D-arabinoic acid, tartaric acid, gluconic acid, α-D-glucopyranuronic acid, octadecanoic acid, hexadecanoic acid, β-D-glucopyranuronic acid, 9,12-octadecadienoic acid, tetradecanoic acid, hexadecanoic acid, octadecanoic acid, pentanedioic acid, glutamic acid, 2,3,4-trihydroxy butyric acid and phosphoric acid. **Aromatic acids:** benzoic acid, caffeic acid, ferulic acid and cinnamic acid. **Alcohols:** glycerol, erythritol, α-cedrol, xylitol, germanicol and stigmast-22-en-3-ol. **Sugars:** D-erythrotetrofuranose, D-altrose, arabinopyranose, d-arabinose, sorbopyranose, α-D-galactopyranose, maltose, α-D-glucopyranoside, D-fructose and D-glucose. **Flavonoids:** pinobanksin. **Esters:** 4,3-acetyloxycaffeate, cinnamic acid 3,4 dimethoxy-trimethylsilyl ester, 3-methoxy-4-cinnamate and cinnamic acid 4 methoxy 3 TMS ester. **Terpene, sesquiterpene alcohols:** farnesol. **Others:** butane, 2(3H)-furanone, L-proline, furan, butane, 2-furanacetaldehyde, 2,5-is-3-phenyl-7-pyrazolopyrimidine, cliogoinol methyl derivative, fluphenazine, 4,8-propanoborepinoxadiborole, 1,3,8-trihydroxy-6-methylanthraquinone, 1-5-oxo-4,4-diphenyl-2-imidazolin-2-yl guanidine, 3,1,2-azaazoniaboratine/piperonal, 3-cyclohexene, 1H-indole, 1H-indole-3-one, 4,8 diphenyl -1-thieno-2-benzazol-1,3-dione, 2-furanacetaldehyde, guanidine, 2(3H)furanone and 1,3,8-trihydroxy-6-meyhylanthraquinone.	2008
China	Flavonoids, lipids, amino acids and long chain alkyl compounds	Wu *et al*., 2008

Origin	Compounds identified	References
China	Rutin, pinocembrin, kaempferol, myricetin, quercetin, apigenin, galangin and chrysin.	Zhou *et al*., 2008
Brazil	Hyperibone A.	Castro *et al*., 2009
Argentina	Quercetin, gallic acid, chlorogenic acid, pinocembrin and kaempferol.	Chaillou and Nazareno, 2009
Brazil	Coumaric acid, artepillin C, kaempferide, cinnamic acid, galangin, quercetin, kaempferol, isorhamnetin, sakuranetin, chrysin and pinobanksin-3-acetate.	de Paula *et al*., 2009
South Africa	Galangin, quercetin and luteolin.	du Toit *et al*., 2009
Myanmar	(22Z,24E)-3-oxocycloart-22,24-dien-26-oic acid, (24E)-3-oxo-27,28-dihydroxycycloart-24-en-26-oic acid, cycloartanes and prenylated flavanones.	Li *et al*., 2009
Chania - Crete	trans-communal, totarol, 13-epi-manool, copalol, trans-communic acid, pimaric acid, 13-epi-torulosal, totarolone (3-oxototarol), isoagatholal, acetylisocupressic acid, mixture of 15-oxolabda-8(17),13E-dien-19-oic acid and 15-oxolabda-8(17),13Z-dien-19-oic acid, 13-epi-cupressic acid, 13-epi-torulosol, junicedric acid, isocupressic acid, agathadiol, isorhamnetin-3-*O*-rutinoside, 14,15-dinor-13-oxo-8(17)-labden-19-oic acid and a mixture of labda-8(17),13E-dien-19-carboxy-15-yl oleate and palmitate, 3,4-seco-cycloart-12-hydroxy-4(28),24-dien-3-oic acid and cycloart-3,7-dihydroxy-24-en-28-oic acid.	Popova *et al*., 2009
Jordan	Pinocembrin, chrysin and pinobanksin-3-*O*-acetate.	Darwish *et al*., 2010
Portugal	**Phenolic acids:** caffeic acid cinnamyl ester, caffeic acid phenylethyl ester, CAPE, caffeic acid benzyl ester, caffeic acid isoprenyl ester, *p*-coumaric acid isoprenyl ester, *p*-coumaric acid methyl ester, 3,4-	Falcão *et al*., 2010

Origin	Compounds identified	References
	dimethyl-caffeic acid, isoferulic acid, ferulic acid, *p*-coumaric acid and caffeic acid. **Flavones and flavonols:** chrysin-6-methyl-ether, kaempferol-5-methyl-ether, chrysin-5-methyl-ether and chrysin. **Flavanones and dihydroflavonols:** 5-methoxy-3-hidroxy-flavanone, pinobanksin-3-*O*-pentanoate or 2-methylbutyrate, pinobanksin-3-*O*-butyrate or isobutyrate, pinobanksin-5-methyl-ether-3-*O*-pentanoate, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-acetate, pinobanksin-5-methyl-ether, pinobanksin, hesperitin-5,7-dimethyl-ether, pinocembrin and pinocembrin-5-methyl-ether.	
Chile	Caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, galangin and caffeic acid phenyl ester (CAPE).	Herrera *et al*., 2010
India	Polyphenols and flavonoids	Laskar *et al*., 2010
Mexico	1-(3',4'-dihydroxy-2'-methoxyphenyl)-3-(phenyl)propane, (Z)-1-(2'-methoxy-4',5'-dihydroxyphenyl)-2-(3-phenyl)propene and 3-hydroxy-5,6-dimethoxyflavan as well as other known isoflavans, pterocarpens and flavonones such as pinocembrin and vestitol.	Lotti *et al*., 2010
Turkey	Aromatic alcohol, alcohols, acids, aromatic acid esters, aromatic esters, aromatic acids, flavanones, hydrocarbons, aliphatic esters, aliphatic acid esters and aromatic hydrocarbons	Özen *et al*., 2010
Kenya	Arylnaphtalene lignans namely; tetrahydrojusticidin, 6-methoxydiphyllin and phyllamyricin. A geranylated flavonol, macarangin and a geranylated stilbene, schweinfurthin.	Petrova *et al*., 2010
The Solomon	prenyl flavanones - propolin H, propolin G, propolin D and propolin C.	Raghukumar *et al*., 2010

Origin	Compounds identified	References
Islands		
Various regions of South America, North America, Europe, Asia and Oceania.	Dihydroxyflavone, chrysin, pinobanksin, pinocembrin, apigenin/galangin, pinobanksin acetate, caffeic acid phenylethyl ester (CAPE) and two prenylated benzophenones identified as markers of Brazilian red propolis tested.	Sawaya *et al*., 2010
Brazil	**Simple phenylpropanoids:** dihydrocinnamic acid methyl ester, dihydrocinnamic acid, *p*-hydroxydihydrocinnamic acid, *p*-coumaric acid, *p*-methoxycinnamic acid, cis--3-methoxy-4-hydroxy-cinnamic acid, trans-3-methoxy-4-hydroxy-cinnamic acid, trans-3,4-dimethoxycinnamic acid, and dihydrocinnamic acid ethyl ester. **Prenylated phenylpropanoids:** allyl-3-prenylcinnamate, 4-hydroxy-3-prenylcinnamic acid, artepillin C, 4-dihydrocinnamoiloxy-3-prenylcinnamic acid, 2,2-dimethylchromene-6-propenoic acid, 2,2-dimethyl-8-prenylchromene-6-propenoic acid, 8-(methyl-butanechromane)-6-propenoic acid and 3-hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid. **Sesqui and diterpenoids:** (-) caryophyllene oxide, farnesol, farnesyl acetate, spathulenol, viridiflorol, dehydrocostus lactone and isocupressic acid derivative. **Triterpenoids and steroids:** squalene, obtusifoliol, bauer-7-en-3b-yl acetate, α-amyrin, α-amyrin acetate, β-amyrin acetate, lupeyl acetate, olean-18-en-3b-yl acetate, taraxer-14-en-3b-yl acetate, urs-18-en-3b-yl acetate and friedooleanan-	Teixeira *et al*., 2010

Origin	Compounds identified	References
Iran	7,12-dien-3b-yl acetate. **Constituents from other classes:** *p*-vinylphenol, *p*-vinyl-o-prenylphenol, quinic acid, 2-hydroxy-7,12-dimethyl-benzanthracene and isomaturnin. **Prenylated coumarin:** Suberosin. **Terpene esters:** tschimgin, tschimganin, ferutinin and teferin.	Trusheva *et al*., 2010
Iran	**Aldehydes**: 2-hydroxy-5-methylbenzaldehyde; **Flavonoids:** 5-hydroxy-7-methoxy flavanone (pinostrobin), 5,7,40-trihydroxy flavanone (naringenin), 5,7-dihydroxy flavone (chrysin), dihydrochrysin; **Aromatic acids:** 3(3,4-dihydroxyphenyl)-2-propenoic acid (caffeic acid); **Sesquiterpenes:** cis-lanceol, caryophyllene oxide, eudesmol, 6-hydroxy-1-oxogermacr-4,10(15),11(13)-trien-12,8-olide; **Triterpenes:** 3,12-oleandione; **Alcohol:** 1-heptatriacotanol; **Aliphatic hydrocarbons:** 1,5,5-trimethyl-6-methylene-cyclohexene, alfaxalone; **Aromatic hydrocarbons:** 2-amino-1-(3-hydroxy-4-methoxyphenyl) ethanone, 1,3,8-trihydroxy-6-methylanthracene-9,10-dione.	Tukmechi *et al*., 2010
Argentina	3'Methyl-nordihydroguaiaretic acid, nordihydroguaiaretic acid, 2-heptanone, tricyclene, camphene, terpinene, *o*-cymene, limonene, *m*-cresol, actophenone, terpinolene, 2-nonanone, *p*-cymenene, linanool, N-nonanal, benzyl acetate, borneol, 4-terpineol, terpineol, N-decanal, (E)-caryophyllene, *cis*-bergamotene, humulene, AR-curcumene, hexadecane, *cis*-guaiene, eudesmol, nonadecane and eicosane.	Agüero *et al*., 2011
Brazil	Gambogenone, aristophenone A, 7-*epi*-clusianone, 7-*epi*-nemorosone, dimethyl weddellianone derivative, propolone derivative, xanthochymol, 18-ethyloxy-17-	de Castro Ishida *et al*., 2011

Origin	Compounds identified	References
	hydroxy-17 and 18-dihydroscrobiculatone B	
Turkey	**Alcohols:** 3-methyl-3-buten-1-ol. **Aromatic alcohols:** phenylethyl alcohol, (E)-11-hexadecen-1-ol, 2-propen-1-ol, 2-naphthalene-methanol, 13-Tetradecy-11-yn-1-ol, olean-12-en-3-ol and benzenemethanol. **Aromatic acids:** 5-phenyl-4-pentenoic acid, benzoic acid, benzenepropanoic acid, 3-phenyl-2-propenoic acid, decanoic acid, 9-octadecenoic acid and octadecanoic acid. **Aromatic acid esters:** benzene acetic acid, 4-hydroxy-3-methoxymethyl ester, octadecanoic acid-methyl ester, 1,2-benzenedicarboxylic acid, bis(8-methyl nonyl) ester, 1,2-benzenedicarboxylic acid, bis(8-methyl propyl) ester, 1,2-benzenedicarboxylic acid, butyl 8-methylonyl ester, benzyl cinnamate and 1,2-benzenedicarboxylic acid diisodecyl ester. **Aldehydes:** benzaldehyde. **Straight-chain acids:** tetradecanoic acid, heptadecanoic acid and n-hexadecanoic acid. **Straight-chain acids esters:** tetradecanoic acid ethyl ester, hexadecanoic acid ethyl ester, and heptadecanoic acid 15-methyl-ethyl ester. **Flavonols**: 4H-1-benzopyran-4-one, 5-hydroxy-7-methoxy-2-phenyl, 4H-1-benzopyran-4-one, 2,3-dihydro-5, 7-dihydroxy-2-phenyl, 4H-1-benzopyran-4-one, 3,5,7-trihydroxy-2-phenyl, chrysin, 5,7-dihydroxy-6-methoxy-3(4'-methoxyphenyl) and 5-hydroxy-6,7-dimethoxy-3(4' -methoxyphenyl). **Hydrocarbons:** cyclotetradecane, heptadecane, 1-heptadecane, 1-nonadecene, 9-tricosene, delta-cadinene, bicyclo(4.4.0) dec-1-ene, 6(Z) 9(E)-heptadecane. **Aromatic esters:** 2-propen-	Duran *et al*., 2011

Origin	Compounds identified	References
	1-one. **Fatty acid ester:** ethyl oleate, cinnamic acid esters, cinnamyl cinnamate and 3-hydroxy-4-methoxycinnamic acid. **Ketones:** 2(5H)-furanone, 5,5-diphenyl, 2-phenyl-2-tipyl-acenapthenone, 1-(2-vinyl phenyl)ethanone, otarolone, hinokione and 2-heptadecanoate.	
China	Epicatechin, *p*-coumaric acid, morin, 3,4-dimethoxycinnamic acid, naringenin, ferulic acid, cinnamic acid, pinocembrin and chrysin.	Guo *et al*., 2011
Brazil	α-Pinene, β-pinene, sulcatone, myrcene, careen, limonene, eucalyptol, α-ocimene, β-ocimene, acetophenone, linalool, nonanal (E)-4,8-dimethyl-1,3,7-nonatriene, methyl hydrocinnamate, α-cubeben, α-copaene, γ -caryophyllene, aromadendrene, γ -muurolene, viridiflorene, germacrene D, δ-cadinene, β-farnesol, spathulenol, artepillin C, 4-hydroxy-3(E)-(4-hydroxy-3-methyl-2-buthenyl)-5-prenyl cinnamic acid, betuletol, caffeoylquinic acid, 3-prenyl-4-dihydrocinnamoyloxycinnamic acid and dicaffeoylquinic acid.	Nunes and Guerreiro, 2011
Brazil	Vestitol, neovestitol and isoliquiritigenin.	Oldoni *et al*., 2011
Brazil	3-[4-hydroxy-3-(oxobutyl)-phenylacrylic acid, 3-prenyl-3(E)-(4-hydroxy-3-methyl-2-butenol)-5-prenylcinnamic acid, 3-prenyl-4-(2-methylpropionyloxi)cinnamic acid, 3-prenyl-4-dihydrocynamoiloxicinnamic acid, dihydrokaemferide, 3-prenyl-4-hydroxycinnamic acid, caffeic acid, caffeoylquinic acid 1, caffeoylquinic acid 2, caffeoylquinic acid 3, caffeoylquinic acid 4, caffeoylquinic acid 5, cinnamic acid, *p*-coumaric acid, kaempferide, kaempferol,	Pamplona-Zomenhan *et al*., 2011

Origin	Compounds identified	References
	betuletol, 2.2-dimethyl-6-carboxyethenyl-2H-1-benzopirane, 2.2-dimethyl-8-prenyl-2H-1-benzopirano-6-propenoic acid, (E)-3-{4-hydroxy-3-[(E)-4-(2.3)-dihydrocynamoiloxi-3-methyl-2-butenyl]-5-prenylphenyl-2-propenoic acid, 3.4-dihydroxy-5-prenylcinnamic acid and 3,5-diprenyl-4-hydroxycinnamic acid	
Malta	Diterpene acids - isocupressic, communic, pimaric and imbricatoloic acid as well as totarol and 13- *epi*-torulosal. Neoabietic acid and manool oxide: Abietic acid, acetyl isocupressic acid, 1 agathadiol, 1 communal, copalol,1 13-*epi*-cupressic acid, 1 dehydroabietic acid,1 13,14-dehydrojunicedric acid, 14,15-dinor-13-oxo- 8(17)-labden-19-oic acid, 1 ferruginol, 1 ferruginolon, hydroxydehydroabietic acid, 2-hydroxyferruginol, 1 6/7-hydroxyferruginol, isoagatholal, 1 junicedric acid, 1 labda-8(17),12,13-triene,13-epi-manool, 1 sempervirol and totarolon.	Popova *et al*., 2011
Chile	Caffeic acid, myricetin, quercetin, kaempherol, apigenin, pinocembrin, galangin and CAPE.	Saavedra *et al*., 2011
Iraq	Caffeic acid, vanillin, coumaric acid, methyl caffeate, ferulic acid, luteolin, quercetin, sakuranetin, methyl quercetin, cinnamic acid, tectochrysin, apigenin, naringenin, pinobanksin, kaempferol, kaempferide, Bis-methylated quercetin, acacetin, hesperetin, prenyl caffeate, benzyl caffeate, chrysin, pinocembrin, galangin, caffeic acid, phenethyl ester, pinobanksin-3-acetate, isopentyl caffeate, isoprenyl coumarate, isoprenyl ferulate, pinobanksin-3-propionate, clerodane diterpenoid I, clerodane diterpenoid II, pinostrobin, clerodane diterpenoid dihydro, clerodane diterpenoid dehydrated and	Sulaiman *et al*., 2011

Origin	Compounds identified	References
	palmitic acid.	
Kangaroo Island	Prenylated cinnamic acid, prenylated tetrahydroxystilbenes, stilbenes and flavanones	Abu-Mellal *et al*., 2012
Portugal	Caffeic acid, , *p*-coumaric acid, ferulic acid, isoferulic acid, benzoic acid, 3,4-dimethyl-caffeic acid, pinobanksin-5-methyl ether, quercetin, luteolin, cinnamic acid, , *p*-coumaric acid methyl ester, pinobanksin, pinocembrin-5-methyl ether, apigenin, chrysin-5-methyl ether, kaempferol, isorhamnetin, pinobanksin-5-methyl ether-3-*O*-acetate, cinnamyliden acetic acid, galangin-5-methyl ether (isomer), rhamnetin, pinocembrin, chrysin, pinobanksin-3-*O*-acetate, galangin, acacetin, kaempferide, 6-methoxychrysin, pinobanksin-3-*O*-acetate-5-*O*-phydroxyphenylpropionate, pinocembrin-5-*O*-3-hydroxy-4-methoxyphenylpropionate, pinobanksin-3-*O*-propionate, ferulic acid derivative, pinobanksin-3-*O*-pentanoate or 2-methylbutyrate, and pinobanksin-*O*-hexenoate.	Falcão *et al*., 2012
Solomon Islands	Solophenol B, solophenol C, solophenol D, solomonin, prokinawan, nymphaenol A, bonannione A, 6'-geranylpinocembrin, propolin I, sophoraflavanone A, (2S)-5,7-dihydroxy-4'-methoxy-8-prenylflavanone, puyanin, propolin A, nymphaeol B, nymphaeol C, solophenol A, propolin B, propolin E, isonymphaeol B, 3'-geranylnaringenin and gallic acid.	Inui *et al*., 2012

Origin	Compounds identified	References
Honduras	(E,Z)-cinnamyl cinnamate, cinnamic ester derivatives, flavanones, chalcone, triterpenes and aromatic acids.	Lotti *et al*., 2012
Brazil	Phenolic acids - Gallic acid, caffeic acid, *t*-cinnamic acid and hydrocinnamic acid and the flavone - apigenin.	Maraschin *et al*., 2012
Cuba	**Brown Cuban propolis:** Nemorosone, hyperibone, garcinielliptone I, propolone A, propolone B, propolone C and propolone D. **Red Cuban propolis:** Isoliquiritigenin, liquiritigenin, biochanin A, formononetin, vestitol, neovestitol, isosativan, medicarpin, homopterocarpin, vesticarpan, 3,8-dihydroxy-9-methoxy pterocarpan and 3-hydroxy-8,9-dimethoxy pterocarpan and 3,4-dihydroxy-9-methoxy pterocarpan. **Yellow Cuban propolis:** 24-methylene-9,19-ciclolanostan-3β-ol, α-amyrin, α-amyrone, β-amyrin, β-amyrin acetate, β-amyrone, cycloartenol, germanicol, germanicol acetate, lanosterol, lanosterol acetate, lupeol and lupeol acetate.	Monzote *et al*., 2012
China	Caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethylcaffeic acid, quercetin, pinobanksin-5-methyl ether, quercetin-3-methyl ether, apigenin, pinobanksin, isorhamnetin, pinocembrin-5-methyl ether, luteolin-5-methyl ether, quercetin-5,7-dimethyl ether, galangin-5-methyl ether, quercetin-X-methyl ether, quercetin-7-methyl-X-methyl-ether, chrysin, pinobanksin-7-methyl-ether, pinocembrin, caffeic acid isoprenyl ester, galangin, pinobanksin-3-*O*-acetate, caffeic acid phenylethyl ester, hydroxy-cinnamic acid benzyl ester, *p*-coumaric acid benzyl ester, caffeic acid cinnamyl	Shi *et al*., 2012

Origin	Compounds identified	References
	ester, pinobanksin-3-*O*-propionate, chrysin-7-methyl ether, pinocembrin-7-methyl ether, pinobanksin-3-*O*-(pentanoate or 2-methylbutyrate), methoxycinnamic acid and cinnamyl ester	
Korea	(*SS*)-(+)-laserpitin, (*SS*)-(-)-isolaserpitin, (*S*)-(-)-selidin, 4-hydroxyderricin, xanthoangelol and xanthoangelol F	Shimomura *et al*., 2012
India	Fatty acids - 9-octadecenoic acid, decanoic acid, 9,12 hexadecanoic acid and octadecadeinoic acid methyl ester. Alcohols - 1-tetradecanol, octadecanol, 1-dotricontanol and 2,3 epoxy-5,8-hectadecadien-1-ol. Trace amount of quercetin and cyclopentadiene	Thirugnanasampandan *et al*., 2012
Australia	2',3',4'-trimethoxychalcone, 2'-hydroxy-3',4'-dimethoxychalcone, 2',4'-dihydroxy-3'-methoxychalcone, 5,7-dihydroxy-2,3-dihydroflavonol 3-acetate (pinobanksin 3-acetate) and 5,7-dihydroxy-6-methoxy-2,3-dihydroflavonol 3-acetate.	Tran *et al*., 2012
Mexico	Pinocembrin, pinobanksin 3-acetate, chrysin, CAPE, acacetin and galangin.	Valencia *et al*., 2012
Thailand	(7″S)-8-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]-(2S)-pinocembrin and (E)-cinnamyl-(E)-cinnamylidenate.	Athikomkulchai *et al*., 2013
Chile	Quercetin, myricetin, kaempferol, rutin, pinocembrin, coumaric acid, caffeic acid and caffeic acid phenethyl ester	Barrientos *et al*., 2013
Brazil	Neovestitol and vestitol	Bueno-Silva *et al*., 2013
Brazil	6-Acetyl-2,2-dimethyl-3-hydroxychroman, 2-hydroxy-4-methoxychalcon, liquiritigenin, formononetin, medicarpin, biochanin A, retusapurpurin B and hesperetin 7-rhamnoglucoside.	da Silva Frozza *et al*., 2013

Origin	Compounds identified	References
Brazil	Artepillin C, CAPE, chrysin, *p*-coumaric acid, caffeic acid, apigenin and naringenin.	de Aguiar *et al*., 2013
India	Terpenoids, flavonoids, alkaloids, phenols, tannins and saponins.	Kalia *et al*., 2013
East Andalusia	Caffeic acid, *p*-coumaric acid, ferulic acid, pinobanksin, cinnamylden acetic acid, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, cinnamyl caffeate and caffeic acid phenethyl ester (CAPE).	Kumazawa *et al*., 2013
Croatia	Caffeic acid, chrysin, *p*-coumaric acid, ferulic acid, isoferulic acid, kaempferol, apigenin, galangin, naringenin rhamnetin, sakuranetin, tectochrysin, pinocembrin and pinocembrin-7-methyl ether.	Medić-Šarić *et al*., 2013
Portugal	2-Acetyl furan, benzaldehyde, verbenene, 2,6,6-trimethyl cyclohexanone, acetophenone, γ-terpinene, *n*-nonanal, hotrienol, α-campholenal, *trans*-pinocarveol, *trans*-verbenol, pinocarvone, nerol oxide, *p*-mentha-1,5-dien-8-ol, borneol, terpinen-4-ol, octanoic acid, myrtenal, α-terpineol, myrtenol, pinocampheol, *cis*-7-decenal, *n*-decanal, *trans*-carveol, *cis*-ocimenone, carvone, *trans*-ocimenone, nonanoic acid, bornyl acetate, thymol, carvacrol, decanoic acid, aromadendrene, allo-aromadendrene, eremophilene, 1,1,5,6-tetramethyl-1,2-dihydronaphtalene, viridiflorene, 1,1,5,6-tetramethyl-1,2,3,4-tetrahydronaphtalene, *trans*-calamenene, δ-cadinene, α-calacorene, β-caryophyllene alcohol, guaiol, ledol, 1-*epi*-cubenol, δ-cadinol, β-eudesmol, cadalene, *n*-tetradecanol, *n*-heptadecane, benzyl benzoate, ambroxide, 6-acetoxy-11-nor-drim-7-en-9-one, *n*-octadecane, *n*-nonadecane, palmitic acid, *n*-eicosane, *n*-heneicosane, linoleic acid ethyl ester,	Miguel *et al*., 2013

Origin	Compounds identified	References
	ladenol, *n*-docosane, *n*-eicosanol, *n*-tricosane, *n*-tetracosane, *n*-pentacosane and fatty acids.	
Italy	Caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic, 3,4-dimethyl-caffeic acid (DMCA), quercetin, cinnamic acid, pinobanksin-5-methyl-ether, quercetin-3-methyl-ether, apigenin, pinobanksin, kaempferol , isorhamnetin, pinobanksin-5-methyl-ether-3-*O*-acetate, quercetin-7-methyl-ether, quercetin-dimethyl-ether, caffeic acid prenyl ester, chrysin, caffeic acid prenyl ester , caffeic acid benzyl ester , pinocembrin, galangin, pinobanksin-3-*O*-acetate, caffeic acid phenylethyl ester (CAPE), methoxy-chrysin, *p*-coumaric benzyl ester, caffeic acid cinnamyl ester, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-butyrate, *p*-coumaric cinnamyl ester, pinobanksin-3-*O*-pentanoate, pinobanksin-3-*O*-hexanoate, *p*-methoxy cinnamic acid and cinnamyl ester.	Pellati *et al*., 2013
Oman	Sugars, polyols, hydroxy acids, fatty acids, cardanols and cardols, anacardic acids, flavan derivatives, triterpenes, prenylated flavanones and chalcones.	Popova *et al*., 2013
Brazil	Taraxerone, oleanene, caffeic acid 4-*O*-glucoside, quinic acid, dihydroquercetin, caffeoylquinic acid, caffeic acid 4-*O*-arabinoside, caffeic acid 4-*O*-xyloside, caffeoylquinic acid, feruloyl-caffeoylquinic acid, caffeoylquinic acid, caffeoylquinic acid, feruloyl-caffeoylquinic acid, feruloyl-caffeoylquinic acid, methylkaempferol-*O*-rutinoside, naringenin-C-glucoside, apigenin-*O*-rutinoside, feruloylquinic acid, caffeic acid, delphinidin arabinoside, caffeoylquinic acid, catechin arabinoside, apigenin-di-C-glucosyl rhamnoside, apigenin-C-rhamnoside, isoschaftoside,	Righi *et al*., 2013a

Origin	Compounds identified	References
	dicaffeoylquinic acid, caffeic acid-dihydroxy phenyl ethyl ester, dicaffeoylquinic acid, dicaffeoylquinic acid, schaftoside, quercetin-*O*-arabinoside, quercetin-*O*-rhamnoside, dicaffeoylquinic acid, isorhamnetin-glucoside, tricaffeoylquinic acid, tricaffeoylquinic acid, apigenin-*O*-glucuronide, diprenyl chrysin, quercetin, cinnamoyl hexoside, isorhamnetin, rhamnetin, artepillin C, quercetin-dimethyl ether, pentamethoxy flavonol, nobiletin, chrysin rhamnoside, prenyl-trimethoxyluteolin and prenyl-trimethoxykaempferol.	
Brazil	Flavanones, glycosyl flavones, prenylated phenylpropanoids, caffeoylquinic acids, prenylated flavonoids and schaftoside (apigenin-8-C-glucosyl-6-C-arabinose).	Righi *et al*., 2013b
Algeria	Pectolinarigenin, pilosin, ladanein, chrysin, and apigenin.	Segueni *et al*., 2013
Korea	Various chalcones and coumarins.	Shimomura *et al.,* 2013
Poland	Cinnamic acid, *p*-coumaric acid, ferulic acid, gallic acid, caffeic acid, caffeic acid phenethyl ester, pinobanksin, kaempferol, apigenin, pinocembrin, quercetin, chrysin, galangin, acecetin and kampferide.	Wojtyczka *et al*., 2013a
Argentina	Pinocembrin, galangin, chrysin, 3 -methyl-nordihydroguaiaretic acid (MNDGA) and nordihydroguaiaretic acid (NDGA).	Agüero *et al.,* 2014
China	Caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethoxycinnamic acid, cinnamic acid, pinobanksin, naringenin, quercetin, kaempferol, apigenin, pinocembrin, 3-*O*-acetyl pinobanksin, chrysin, CAPE and galangin.	Cui-ping *et al*., 2014

Origin	Compounds identified	References
Brazil	Guttiferone E, oblongifolin A, xanthochymol, pinocembrin, formononetin, biochanin A, daidzein.	Giménez-Cassina López *et al*., 2014
Brazil	(3S)-vestitol, (3S)-neovestitol, isoliquiritigenin, (6aS,11aS)-medicarpin 2-(2′,4′-dihydroxyphenyl), 3-methyl-6-methoxybenzofuran, liquiritigenin, naringenin, (2S)-7-hydroxyflavanone, biochain A, daidzein, formononetin and retusapurpurin A.	Inui *et al*., 2014
Australia	Gallic acid, abietic acid, abietane and flavonoids.	Massaro *et al*., 2014
Germany	Apigenin, ellagic acid, chrysin, pinocembrin, pinobanksin, galangin, kaempferol, quercetin, naringenin and caffeic acid.	Morlock *et al*., 2014
Algeria	Flavonoids and polyphenols.	Nedji and Loucif-Ayad, 2014
Ethiopia	**Triterpenoids:** β-amyrone, α-amyrone, β-amyrin, α-amyrin, β-amyryl acetate, α-amyryl acetate, lupeol, moretenol and moretenyl acetate. **n-Alkanes:** heneicosane, docosane, tricosane, tetracosane, pentacosane, hexacosane, heptacosane, octacosane, nonacosane, triacontane and hentriacontane. **n-Alkenes:** pentacosene, hexacosene, heptacosene, octacosene, nonacosene, triacontene, hentriacontene, dotriacontene, tritriacontene, tetratriacontene and pentatriacontene. **Methyl n-Alkanoates:** methyl dodenoate, methyl tridecanoate, methyl tetradecanoate, methyl pentadecanoate, methyl hexadecenoate, methyl hexadecanoate, methyl heptadecenoate, methyl octadecenoate, methyl octadecanoate, methyl nonadecanoate, methyl eicosanoate, methyl heneicosanoate, methyl docosanoate, methyl tricosanoate, methyl tetracosanoate, methyl pentacosanoate, methyl	Rushdi *et al*., 2014

Origin	Compounds identified	References
	hexacosanoate, methyl heptacosanoate and methyl octacosanoate. **Wax esters:** docosyl hexadecanoate, tetracosyl hexadecanoate, hexacosyl hexadecanoate and octacosyl hexadecanoate.	
Cameroon	Alkaloids, anthraquinones, phenolic compounds, reducing compounds, coumarins, saponins, steroids, triterpenes (25-cyclopropyl-3β-hydroxyurs-12-ene; cycloart-3β-hydroxy-12, 25(26)-diene; lup-20(29)-en-3-one; olean-12-en-3β, 28-diol; lup-20(29)-en-3β-oate and 3β-hydroxylup-20(29)-ene.), tannins and volatile oils.	Sakava *et al*., 2014
Sub-saharan region of Africa	Prenylated flavonoids, flavonoids, diterpinoids, diprenylated flavonoids, geranylated flavonoids, hydroxyl acid, chlorogenic acid, cinnamic esters, triterpenes, triterpenoids, diterpenes, sesquiterpenes, and sesquiterpinoids.	Zhang *et al*., 2014
Brazil	Triterpines, carbohydrates and derivatives thereof, anacardic acid, sugar alcohols and alkylresorcinols.	Araújo *et al*., 2015
France	Pinobanksin-3-acetate, pinocembrin, chrysin, galangin and prenyl caffeate	Boisard *et al*., 2015
Brazil	Benzoic acid, cinnamyl caffeate, benzyl, caffeate, hydrocinnamic acid, hydrocinnamic acid ethyl ester, *p*-coumaric acid, 3-phenyl-*p*-coumaric acid, fructose, glucose, kaurenoic acid, 4-methoxybenzoic acid, retinol, cholesterol and tocopherol.	Campos *et al*., 2015
Lebanon	Alkaloids, flavonoids, phenols, saponins, steroids, tanins, and terepenoids	Chamandi *et al*., 2015
Brazil	Ferulic acid, caffeic acid, cinnamic acid, coumaric acid and 3,4-dihydroxybenzoic acid.	de Lima Silva *et al*., 2015
Sudan	Gallic, b-oh benzoic, caffeic acid, phenol, *p*-coumaric, salicylic acid, ferulic acid, cinnamic acid, quercetin, euganol, chrysin, galangin, pinostrobin,	Elsayed and El-Sarrag, 2015

Origin	Compounds identified	References
	vanillin, 3,5 di methoxy benzyl, pyro gallic, kaempherol, catechine, dadzin, genstin, dadazien and genstein.	
Lithuania	Caffeic acid, naringenin, kaempferol, galangin, trans *p*-coumaric acid and ferulic acid.	Kubiliene *et al*., 2015
Thailand	α-Mangostin, mangostanin, 8-deoxygartanin, gartanin, dipterocarpolde, γ-mangostin, garcinone, dipterocarpol, methylpinoresinole, 3-*O*-acetyl ursolic acid, ocotillone, mixtures of ursolic and oleanolic aldehydes and cabralealactones.	Sanpa *et al*., 2015

Appendix I:Microbial culture ethics waiver

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Medical School Room 10M07, 10th Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-131026-1 26/10/2013

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Prof Sandy van Vuuren, Tasneem Suleman (Student No 302955).

Project title: Antimicrobial and chemical properties of South African propolis.

Reason: This is a laboratory study using microbial cultures including:

Staphylococcus aureus ATCC 25923
Enterococcus faecalis ATCC 29212
Escherichia coli ATCC 25922
Pseudomonas aeruginosa ATCC 27853
Pseudomonas aeruginosa ATCC 27858
Candida albicans ATCC 10231
Cryptococcus neoformans ATCC 14116.

There are no human participants

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2013 -10- 26
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.