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Antimicrobial and antioxidant activities of different solvent extracts from fermented and green honeybush (*Cyclopia intermedia*) plant material

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ABSTRACT

Ethnopharmacological relevance: Cyclopia intermedia is indigenous to South Africa and used to prepare honeybush herbal tea. This aromatic herbal tea has been associated with numerous health benefits, mostly based on anecdotal evidence and with very few studies reporting on the antimicrobial activities.
Aim: The inhibitory effect on the growth of important nosocomial microorganisms and possible association with the antioxidant capacity/content of various solvent extracts of green/unfermented and fermented honeybush plant material were determined in the current study.
Materials and methods: The agar disk diffusion assay was used as a screening assay for the antimicrobial activity of the various honeybush extracts, while the minimal inhibitory concentration (MIC) values, using the broth micro-dilution method, were determined against *Streptococcus pyogenes*, *Staphylococcus aureus* and *Candida albicans*. Active antimicrobial compounds were then shown using thin layer chromatography bioautography. Total antioxidant capacities and -content were also determined for each extract, while the main phenolic compounds were quantified using HPLC.
Results: Six of the eight solvent extracts of honeybush showed antimicrobial activity, with the fermented and green methanol extracts being most effective against *S. aureus* and *C. albicans* respectively, whilst the green chloroform extract was most potent against *S. pyogenes*. Thin layer chromatography-bioautography acknowledged the existence of active antimicrobial fractions within these different solvent honeybush extracts. Regardless of the assay, the green honeybush extracts generally exhibited the highest antioxidant capacity when compared to the fermented honeybush. The total polyphenols were also observed to be highest in green extracts when using water and methanol as solvents. In general the mangiferin and hesperidin contents were higher in the green than the fermented extracts of honeybush plant material.
Conclusions: The inhibitory activity of the various extracts against specific microorganisms was observed to be linearly proportional to the extract concentration. Although the different solvent extracts can only be considered weak antimicrobial agents, three compounds showed specific activity and should be further elucidated in future. It appears the antioxidant capacity of the various solvent herbal extracts did not relate to the antimicrobial activities; however, further work will be required to ascertain this observation. The current data also suggested that the various extracts of honeybush can be considered a good source of a unique blend of natural phytochemical antioxidants and antimicrobial compounds and should be further elucidated.

1. Introduction

The Honeybush plant (*Cyclopia intermedia*) is endemic to the South African fynbos region and the herbal tea brewed from the plant material has been consumed widely because of its unique aroma and taste (Kamara et al., 2003; Joubert et al., 2008, 2011). It has been associated with a variety of bioactivities which include anti-diabetic (Muller et al., 2013; Chellan et al., 2014), anti-cancer (Marnewick et al., 2009; Sissing et al., 2011; Magcwebeba, 2013), anti-obesity (Dudhia et al., 2013; Pheiffer et al., 2013), antioxidant (Hubbe and Joubert, 2000; Joubert et al., 2008) and antimicrobial (Coetzee et al., 2008) activities. However, considering its steady increasing popularity as a health beverage, studies of its possible bioactivities are currently lagging. The antimicrobial activity of honeybush is one of the activities that have been overlooked by the research community.

Abbreviations: HPLC, High-perfomance liquid chromatograpy; MIC, Minimum inhibitory concentration; TLC, Thin layer chromatography.

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The antimicrobial and antioxidant activities have been concurrently examined in some medicinal plants including *Camellia sinensis*, the traditional tea plant. These studies revealed contradictory observations; one reporting a direct relation meaning high antioxidant activity is associated with strong antimicrobial effects (Chan et al., 2011), whilst the other observed an inverse relation entailing the expectation of a weak antimicrobial activity if a high antioxidant capacity has been displayed (Yildirim et al., 2000). The current study aims at contributing novel data to the existing knowledge pool pertaining to the antimicrobial and antioxidant activity of the honeybush herbal tea and possibly lay a foundation for future studies that will confirm any relationship that may exist between the two activities within the honeybush herbal tea extracts.

The rising morbidity and mortality of oxidative stress-related conditions like cancer and drug resistant *P. aeruginosa*, *S. aureus*, *S. pyogenes* and *C. albicans* infections in Africa including South Africa are alarming. As it has been said 'prevention is better than cure', preventative measures of these conditions are still being discovered. Ideally these should be affordable, non-toxic, easily accessible and efficient. Honeybush herbal tea fits the criteria and hence makes a suitable candidate. With the outcomes of this study, the community will acknowledge the health benefits of a consistent daily consumption of honeybush herbal tea. It is important to note that the phytochemical constituents present in honeybush herbal teas are different to those present in the other highly prevalent indigenous herbal tea, rooibos. For the current study, it is therefore crucial to assess the antimicrobial and antioxidant activities of the various honeybush extracts and not to assume it will be similar to that of rooibos.

2. Methods and materials

2.1. Chemicals and apparatus

Ampicillin (10 μg), blood agar, chloroform, ciprofloxacin (5 μg), cooked blood agar, dichloromethane (DCM), dimethyl sulfoxide (DMSO), ethyl acetate, fluconazole (25 μg), hydrochloric acid (HCL), methanol, Mueller hinton agar, Mueller-Hinton broth, Sabouraud dextrose 4% agar, sodium acetate, yeast peptone broth and vanillin were all purchased from Merck (Johannesburg, SA). 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2′- azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) ferric chloride ($FeCl_3$), fluorescein sodium salt, Folin Ciocalteu's reagent, gallic acid, $K_2S_2O_8$ (potassium-peroxodisulfate), L-ascorbic acid, nitrotetrazolium blue chloride (NBT), potassium phosphate (KH_2PO_4), sodium carbonate, sulphuric acid (H_2SO_4), 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Johannesburg, SA). All solvents purchased and used through the study were of analytical reagent grade. Sterilized water was used throughout the study. Greiner crystal clear 96-well flat bottom, Costar 96-well UV flat bottom and Nunclon black 96-well flat bottom microplates were supplied by Sigma-Aldrich (Johannesburg, SA).

2.2. Plant material

Fermented and green/unfermented honeybush (*C. intermedia*) (Family: Fabaceae; Tribe: Podalyrieae), plant material was supplied by Rooibos Limited, Clanwilliam. The plant material was similar to the form in which it is being used commercially to brew a cup of herbal tea.

2.3. Extraction

The different extracts were prepared by macerating the plant material in solvents of differing polarity. Water extracts (as it is traditionally consumed) of the fermented and green/unfermented honeybush were prepared by adding 1000 ml of boiling distilled water to 100 g samples of the fine honeybush plant material. The ethyl acetate, methanol and chloroform extracts were prepared by adding 1000 ml of the respective solvent to 100 g plant material sample at room temperature. The mixtures were left for 24 h at room temperature on a magnetic stirrer, after which the various extracts were filtered through cotton wool, to remove the residual plant material. The extracts were then filtered using Whatman no. 4 mm paper. The water extract filtrate was frozen and lyophilized using a freeze-drier. The organic (ethyl acetate, methanol and chloroform) extract filtrates were concentrated to a fifth of the initial volume using a rotary evaporator. These were finally dried under a fume hood to yield crude extracts. All dried extracts were weighed and stored at 4 °C in dark, sterile sealed containers for further use.

2.4. Antimicrobial studies

2.4.1. Microorganisms

The microorganisms selected; Gram-positive *Staphylococcus aureus* (ATCC 25923) and *Streptococcus pyogenes* (ATCC 19615), Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and yeast *Candida albicans* (ATCC 10231); represent the three main groups of microorganisms. *S. aureus* and *P. aeruginosa* were cultured on prepared blood agar (BA), *S. pyogenes* on cooked blood agar (CBA) whilst *C. albicans* on Sabouraud dextrose 4% agar (SDA) and all were kept viable by sub culturing.

2.4.2. Agar disk diffusion assay

The agar disk diffusion assay has been used as a preliminary screening for antimicrobial activity of plant extracts (Salie et al., 1996; Ncube et al., 2008). The dried extracts were dissolved in their respective extracting solvents, yielding a stock solution of 1 g/ml from which various extract concentrations were prepared by dilution. Sterile 9 mm disks were impregnated with 50 μl of plant extracts and incubated at 37 °C for 24 h to dry. Each extract was tested in triplicate. Negative control disks contained 50 μl of sterile extracting solvent. The positive control substances used for *S. pyogenes* and *S. aureus* was the broad spectrum antibiotic ampicillin, for *P. aeruginosa* was ciprofloxacin and that for *C. albicans* was fluconazole. The dried paper disks were then placed carefully onto the surface of the agar inoculated with microbial culture. The agar plates were then incubated at 37 °C for 24 h (*P. aeruginosa*, *S. aureus*, *S. pyogenes*) and 48 h (*C. albicans*). The inhibition zones were measured in millimeters (mm) from the circumference of the disk to that of the growth-free zones around the disk and recorded (Salie et al., 1996).

2.4.3. Minimum inhibitory concentration (MIC)

The MIC for each sample displaying antimicrobial activity in the agar disk diffusion assay was determined using the micro-titer plate method (Eloff, 1998). The samples were selected according to the results obtained from the disk diffusion assay. Microbial suspensions in Mueller-Hinton (bacteria) and Yeast Peptone (fungus) broths were prepared and incubated for 24 h at 37 °C, from which a McFarland No. 0.5 standard (approximately 1×10^8 CFU/ml) was prepared. The plant extracts were dissolved in 10% dimethyl sulfoxides (DMSO) (Salie et al., 1996; Langfield et al., 2004). The organic dried honeybush extracts, chloroform and methanol, were dissolved to starting concentrations of 200 mg/ml and 300 mg/ml, respectively, and the aqueous extracts to a starting concentration of 600 mg/ml. Those extracts that were difficult to dissolve were agitated using a vortex. The positive control substances used (to confirm microbial susceptibility) for *S. pyogenes* and *S. aureus* were the antibiotic ampicillin, and that for *C. albicans* was fluconazole and the respective extraction solvents and 10% DMSO were used as the negative controls.

The 96-well micro-titer plates were aseptically prepared in a horizontal laminar air flow cabinet. In each well 100 μl of 10% DMSO was added, and then 200 μl of the extracts was added in the first wells

from which serial dilutions (two-folds) were made to the desired minimum concentration. The microbial cultures (100 μl) were added to the wells, the whole plate was then placed on a shaker (500 rpm) for 30 s. Soon after shaking, the plates were read at 620 nm wavelength with a multi-well plate reader to obtain the first reading ($t = 0$ h), a sterile film was used to cover the plates to prevent evaporation then incubated at 37 °C for 24 h. After incubation the absorbance reading at the same wavelength were recorded for each plate. The MIC determined by the spectrophotometric method was defined as the concentration at which there was a sharp decline in the absorbance value after incubation (Salie et al., 1996; Devienne and Raddi, 2002). All MIC values were tested in triplicate.

2.4.4. Thin layer chromatography (TLC)—bioautography

Dried extracts of the most potent antimicrobial active extracts were dissolved in ethanol to a concentration of 10 mg/ml. 20 μl of the extracts (aqueous and organic) was applied onto pre-coated duplicate plates of silica gel 60 F_{254} (Merck, Germany). Visualization of the TLC plates was done by observing the bands after development under UV at λ254 nm and at λ366 nm using a UV lamp (CAMAG, Switzerland), followed by spraying with the detection agent, vanillin-sulphuric acid. Chemical profiling of the bands was done based on the color produced after viewing. The solvent system used for the TLC development of the honeybush (*C. intermedia*) extracts fractions is DCM-methanol (95:5). The duplicate plates were used for the bioautographic agar overlay assay. A suspension of the test microorganisms (approximately 1×10^8 CFU/ml) was sprayed onto the developed TLC plates. The bioautograms were then incubated at 37 °C for 24 h in humid conditions. Nitrotetrazolium blue chloride (NBT, sigma), the microbial indicator was used as a growth detector (Silva et al., 2005). It was sprayed onto the plates, which were re-incubated at 37 °C for 3–4 h (Dilika et al., 1997; Runyoro et al., 2006). Clear zones on the bioautogram indicated inhibition of growth i.e. antimicrobial activity of the extract components.

2.5. Antioxidant capacity studies

2.5.1. Oxygen radical absorbance capacity (ORAC) assay

Antioxidant capacity of the various solvent honeybush extracts was measured using the ORAC assay according to Ou et al. (2001). 2, 2′-Azobis (2-methyl-propanamide) dihydrochloride (AAPH) is thermally decomposed to a peroxyl radical which oxidizes a fluorescent probe [fluorescein (FL)], resulting in a loss of fluorescence intensity. The decrease in fluorescence intensity reflects on the concentration of free radicals and a delay of this loss signifies the presence of antioxidants in the test sample, counteracting the activity of the oxidative species. The ORAC results were expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol of TE/g). The reagents and standards (FL, AAPH, Trolox) were prepared using the ORAC phosphate buffer (75 mM at pH 7.4). 138 μl of fluorescein solution (14 μM concentration), 12 μl of sample (controls and extracts) and 50 μl of AAPH solution (500 μM) were dispensed and mixed in a 96-well black plate prior to reading the plates. Trolox controls were prepared within a range of 0–417 μM. The fluorescence readings were measured with 485 and 538 nm as excitation and emission wavelengths, respectively using a fluoroskan ascent plate reader (Thermo Fisher Scientific, Waltham, Mass, U.S.A.). The final antioxidant activity results were given in Trolox equivalents. All samples were assessed in triplicate.

2.5.2. Ferric reducing ability of plasma (FRAP) assay

The FRAP assay is used as a novel method for assessing "antioxidant power" of biological and pure samples (Benzie and Strain, 1996). The principle of this spectrophotometric method is based on the antioxidants' ability to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), in an acidic environment. The antioxidant capacity of the samples is evaluated by the ability of the antioxidants to donate electrons during a redox (reduction/oxidation) reaction. At acidic pH, the oxidant ferric chloride hexahydrate $Fe_3(TPTZ)_2Cl_3$ (TPTZ = 2,4,6, Tripyridyl-s-triazine) prepared by mixing acetate buffer (300 mM), TPTZ (10 mM), $FeCl_3$ (20 mM) and distilled water is reduced by antioxidants in samples to give an intense blue colored ferrous tripyridyltriazine complex. The color develops in the presence of electron donating antioxidants and is monitored by a spectrophotometer measuring the change in absorption maximum at 593 nm (Benzie and Strain, 1996; Gupta et al., 2009). L-Ascorbic acid was used in the preparation of the antioxidant standard solutions ranging between 0 and 1000 μM. 10 μl of the standards/extracts followed by 300 μl of the FRAP reagent was added to a 96-well plate and incubated at 37 °C for 30 min before reading. Samples were each ran in triplicates.

2.5.3. Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay measures the total antioxidant capacity of a given substance. The procedure was previously described by Re et al. (1999). The ABTS reagent (2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) was prepared by mixing 5 ml (7 mM) ABTS and 88 μl (140 mM) $K_2S_2O_8$ (potassium- peroxodisulfate) and allowed to react for 24 h in the dark at room temperature. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard with concentrations ranging from 0 to 500 μM. After 24 h, the ABTS reagent was diluted with absolute ethanol to read an initial absorbance of approximately 2.0(±0.1), which is the control value. 25 μl of standards/extracts and 300 μl of ABTS mix were added and mixed in 96-well plates and were allowed to react at room temperature in the dark for 30 min. The plates were then read at 734 nm at 25 °C using the plate reader and results were expressed as μM Trolox equivalents per milligram dry weight of sample (μM TE/g). All samples were run in triplicate.

2.6. Phytochemical assays

2.6.1. Total polyphenols

Total polyphenols content (TPC) of the honeybush extracts was determined by spectrophotometry, using the Folin–Ciocalteu method described by Singleton and Rossi (1965) with gallic acid as a standard. The reagents (10% ethanol, Folin–Ciocalteu (0.1 g/ml), sodium carbonate (0.075 g/ml) were all prepared using distilled water except the gallic acid standards (give an absorbance of 0.509 ± 0.010 at 280 nm) which were diluted in 10% ethanol. The solvent extracts were diluted to a concentration of 15 mg/ml. 25 μl of standards/extracts were added to 125 μl of Folin–Ciocalteu in the 96-well plates. After 5 min, 100 μl of sodium carbonate was then pipetted into each well. The plates were incubated at room temperature for 2 h before being read with the multiskan plate reader at an absorbance of 765 nm. Total polyphenol results were expressed as milligram Gallic acid equivalents per gram of extract (mg GAE/g). The concentration of polyphenols in extracts were derived from a standard curve of gallic acid standards ranging from 10 to 50 μg/mL (Pearson's correlation coefficient: $r^2 = 0.9996$). Samples were analyzed in triplicate.

2.6.2. High performance liquid chromatography (HPLC)

The HPLC is a quantitative analysis, detecting and characterizing known and unknown sample components. The Spectra HPLC system P2000 pump was equipped with HPLC column C18 (150 × 4.6 mm), 5 μm particle size (Agilent Zorbax, South Africa) and a Spectra system FL3000 fluorescence detector. The chromatographic conditions included a flow rate of 1 ml/min, 15 min run time, sample injection volume of 20 μl and the mobile phases A (KH_2PO_4 (50 mM)) and B (methanol (pH 5.8)). Solvent extracts were diluted using DMSO to a concentration of 1 mg/ml. The measurements were made at 280 nm for the main polyphenols, mangiferin and hesperidin against the pure compounds. The analytical signals were monitored at 2–20 mV potentials applied.

2.7. Statistical analysis

Antimicrobial experimental data are expressed as mean ± standard error of the mean (SEM) whilst the antioxidant data are expressed as mean ± standard deviation (SD). Statistical analysis of antimicrobial activity of the different honeybush extracts was achieved using the unpaired Student's *t*-test. One-way analysis of variance (ANOVA) was used to analyze the antioxidant results. $P < 0.05$ was considered as significant.

3. Results

3.1. Antimicrobial activity

When considering the agar disk diffusion assay, the water, methanol and chloroform extracts exhibited some level of antimicrobial activity, with the methanol extracts being the most potent against *S. aureus* and *C. albicans*, while none of the ethyl acetate extracts showed any antimicrobial activity (Table 1). The extract concentration was linearly

Table 1
Inhibition of different microorganisms by various green and fermented *Cyclopia intermedia* (honeybush) extracts using the agar disk diffusion assay as screening assay.

Microorganisms	Extraction solvent	Conc (mg/ml)	Average zones of inhibition (mm ± SEM)		Statistical significance (*P*)
			HG[a]	HF [b]	ab
Pseudomonas aeruginosa	Water	–			
	Methanol	–			
	Chloroform	–			
	Ethyl acetate	–			
Positive control	Ciprofloxacin	5 μg/ml	12 mm		
Staphylococcus aureus	Water	200	1.2 ± 0.17	0.2 ± 0.17	0.0132 *
		300	1.8 ± 0.17	0.7 ± 0.17	0.0078 **
		600	2.2 ± 0.17	1.2 ± 0.17	0.0132 *
		900	3.6 ± 0.17	2.7 ± 0.33	0.055
	Methanol	50	0.2 ± 0.17	0.3 ± 0.17	0.5185
		100	0.7 ± 0.17	0.8 ± 0.17	0.5185
		150	1.2 ± 0.17	1.8 ± 0.17	0.0474 *
		200	1.2 ± 0.17	2.8 ± 0.17	0.0078 **
		300	1.8 ± 0.17	4.2 ± 0.17	0.0006 ***
		500	2.2 ± 0.17	5.7 ± 0.33	0.0007 ***
		600	3.7 ± 0.33	8.2 ± 0.17	0.0003 ***
	Chloroform	–			
	Ethyl acetate	–			
Positive control	Ampicillin	10 μg/ml	13 mm		
Streptococcus pyogenes	Water	50	0.2 ± 0.17	0.8 ± 0.17	0.0474
		100	0.7 ± 0.17	1 ± 0	0.1049
		150	0.8 ± 0.17	3 ± 0	0.0002***
		200	1 ± 0	4 ± 0	<0.0001***
		300	1.3 ± 0.17	4.7 ± 0.33	0.0009***
		600	1.5 ± 0.29	5 ± 0.29	0.001**
		900	2 ± 0	5.7 ± 0.33	0.0004***
		50	0.7 ± 0.33	2 ± 0	0.0155*
		100	1 ± 0	3 ± 0	<0.0001***
		150	1.7 ± 0.33	3.7 ± 0.33	0.0132*
		200	2 ± 0	4 ± 0	<0.0001***
		300	3 ± 0	4.8 ± 0.17	0.0004***
		500	4 ± 0	5.3 ± 0.33	0.0169*
	Methanol	600	5.3 ± 0.33	7 ± 0	0.0073**
		50	0.7 ± 0.33	0 ± 0	0.1233
		100	2 ± 0	1 ± 0	<0.0001***
		150	4 ± 0	2 ± 0	<0.0001***
	Chloroform	200	6.7 ± 0.33	3.7 ± 0.33	0.0031**
	Ethyl acetate	–			
Positive control	Ampicillin	10 μg/ml	13 mm		
Candida albicans	Water				
		100	1 ± 0	1.2 ± 0.17	0.4211
		150	2.2 ± 0.17	1.7 ± 0.33	0.2508
		200	2.7 ± 0.33	2.2 ± 0.17	0.2508
		300	3 ± 0	3.2 ± 0.17	0.4211
		600	3.7 ± 0.33	3.7 ± 0.33	1
		900	5 ± 0	4.3 ± 0.33	0.1100
		50	0 ± 0	0 ± 0	1
		100	1.3 ± 0.33	0.7 ± 0.33	0.2302
		150	1.7 ± 0.33	1.3 ± 0.17	0.4216
		200	2.7 ± 0.33	2 ± 0	0.1233
		300	3.7 ± 0.33	2.3 ± 0.33	0.0474*
		500	4 ± 0	2.7 ± 0.33	0.0155*
	Methanol	600	5.3 ± 0.33	4.3 ± 0.33	0.1012
	Chloroform	–			
	Ethyl acetate	–			
Positive control	Fluconazole	25 μg/ml	10 mm		

Abbreviations: HG: honeybush green; HF: honeybush fermented; SEM: standard error of the mean for $n = 3$. Average zones of inhibition in millimeters (mm). –: no zones of inhibition observed in the presence of all honeybush extracts.

proportional to the inhibition activity of the extracts. The agar disk diffusion assay allowed for the identification of the microbes that were sensitive to the honeybush extracts whilst also assessing effects of fermentation on these sensitivities by statistically comparing the inhibition of the fermented extracts with that of the green extracts. The extracts that exerted antimicrobial activity against the microorganisms were selected to be quantitatively assessed by the determination of the minimum inhibitory concentration (MIC). The MIC of green and fermented methanol extracts against *C. albicans* and *S. aureus* were 37.5 mg/ml and 18.75 mg/ml respectively (Table 2). *P. aeruginosa* displayed insensitivity to all the honeybush green and fermented extracts. Chloroform extracts of green and fermented honeybush exhibited selective antimicrobial activity as they significantly inhibited only the growth of *S. pyogenes*. The MIC of green chloroform extract against *S. pyogenes* was 12.5 mg/ml (Table 2). The most potent antimicrobial extracts were further analyzed using the thin layer chromatography (TLC)—bioautography (Fig. 1) in order to observe for the possible presence of antimicrobial active compounds within these extracts. Zones of inhibition were observed in the presence of the active antimicrobial fractions. The zones corresponded with yellowish compounds on the TLC and had retardation factor values (R_f) of 0.78 (HFM), 0.6 (HGC) and 0.57 (HGM) in the presence of *S. aureus*, *S. pyogenes* and *C. albicans* respectively. The assay reveals the existence of three potential antibiotic compounds within honeybush due to the existence of three fractions with distinct R_f values.

3.2. Antioxidant capacity

The antioxidant capacity of green/unfermented and fermented honeybush extracts were analyzed in triplicate and reported (Table 3). The green/unfermented *C. intermedia* extracts displayed the strongest antioxidant potential in comparison to the fermented forms of the herbal tea infusions. The combination of ORAC, FRAP and TEAC assays allowed the detection of the concentration of antioxidants present that are capable of displaying the specific characteristics under study. The study revealed that the green methanol extracts had the highest ORAC, FRAP and TEAC values, whilst the fermented methanol had the highest antioxidant capacity when comparing the fermented honeybush extracts. This observation may be because methanol has the ability to extract both polar and non-polar compounds with potential antioxidant activities. According to this study, the polar compounds extracted from honeybush green and fermented leaves by water and methanol solvents exhibited higher antioxidant capability in comparison to the chloroform and ethyl acetate extracts.

Table 2
The minimum inhibitory concentration (mg/ml) of selected solvent extracts derived from *C. intermedia*.

Test microorganism	Extract	MIC (mg/ml)
Staphylococcus aureus	HGW	150
	HGM	75
	HFW	300
	HFM	18.75
Streptococcus pyogenes	HGW	150
	HGM	75
	HGC	12.5
	HFW	75
	HFM	37.5
	HFC	50
Candida albicans	HGW	150
	HGM	37.5
	HFW	150
	HFM	75

Abbreviations: HGW: honeybush green water; HGM: honeybush green methanol; HGC: honeybush green chloroform; HFW: honeybush fermented water; HFM: honeybush fermented methanol; HFC: honeybush fermented chloroform; MIC: minimum inhibitory concentration in milligrams per milliliter (mg/ml). $n = 3$.

3.3. Phytochemical analysis

All honeybush solvent extracts showed the presence of the two main flavonoids, mangiferin and hesperidin in varying concentrations (Table 4). The total polyphenolic content ranking of honeybush green and fermented extracts (15 mg/ml) in decreasing polyphenolic concentration was methanol > water > chloroform > ethyl acetate. Ranking of the flavonoid content in the honeybush extracts in decreasing concentrations of mangiferin, was green methanol > fermented methanol > green chloroform > fermented water > green water > fermented ethyl acetate > green ethyl acetate > fermented chloroform and of hesperidin content in decreasing levels was green methanol > green chloroform > fermented methanol > green water > fermented water > fermented chloroform > fermented & green ethyl acetate. When considering the various solvent extracts, the green plant material of honeybush contained higher concentrations of total polyphenols compared to their fermented counterparts, an observation also reported by others, possibly due to the loss of some polyphenolic compounds during the fermentation process of the plant material (Joubert et al., 2008).

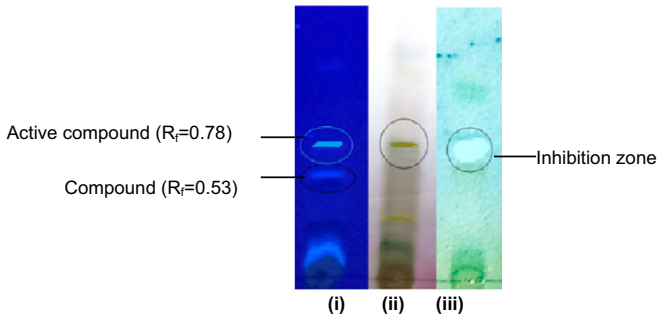
4. Discussion

Regardless of its long history of traditional use, the popularity of honeybush herbal tea is still emerging in the South African herbal tea industry. The scientific interest shown in this herbal tea is lacking, evident by the few experimental studies especially with regards to its antimicrobial and antioxidant bioactivities. A handful of studies have evaluated the antioxidant (Hubbe and Joubert, 2000; Garcia et al., 2003; Leiro et al., 2003; Joubert et al., 2008; Van der Merwe et al., 2012; Malherbe et al., 2014) and antimicrobial (Coetzee et al., 2008) activity of some *Cyclopia spp*. Coetzee and colleagues focused mainly on the anti-fungal aspect of the green honeybush herbal tea, with their main interest in the ethanolic solvent extracts. The lack of scientific evidence was the main motivational drive for this current investigation. The current study did not only focus on exploring the antimicrobial (antibacterial and antifungal) potential of the various honeybush extracts, but also investigated the antioxidant capacity within the various solvent extracts.

The agar disk diffusion assay, MIC and thin layer bioautography were used to analyze the antimicrobial activity of green/unfermented and fermented honeybush (*C. intermedia*) extracts against the bacteria *P. aeruginosa, S. aureus, S. pyogenes* and fungus *C. albicans*. *P. aeruginosa,* which is one of the main causes of nosocomial infections, exhibited resistance towards the green and fermented honeybush extracts (Table 1). The basis for its resilience is its impermeable outer membrane surrounding the bacterial cell wall, making pseudomonal infections amongst the most difficult infections to treat (Levinson and Jawetz, 1996; Vardakas et al., 2013). The presence of antioxidants within the tea extracts that scavenged the specific radicals which could impede the viability of the microorganism could further explain this insensitivity (Coetzee et al., 2008). *Aspalathus linearis* (rooibos), another fynbos plant indigenous to the Western Cape Province of South Africa of which an herbal tea is brewed, was observed by Bancirova (2010) to be ineffective against *P. aeruginosa*.

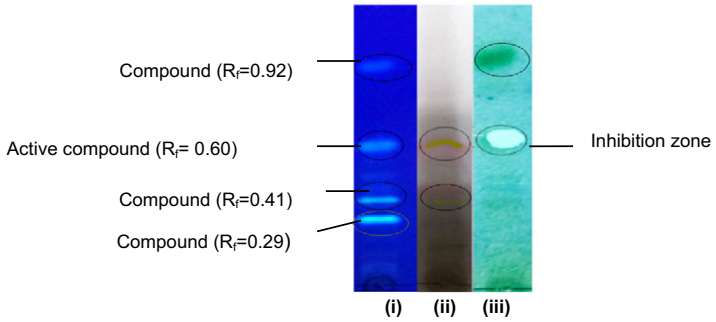
The growth of *S. aureus* and *C. albicans* was inhibited by the methanol and water extracts of green and fermented honeybush, but was resistant to the chloroform and ethyl acetate extracts. *S. aureus* is the causative agent of a wide range of infections, from non-invasive to life threatening systemic infections (Iwamoto et al., 2013), whilst candidiasis (infections caused by *C. albicans*) are known for their increased frequency in HIV/AIDS patients (Conti et al., 2014). The methanol extracts of green and fermented honeybush proved more efficacious in inhibiting the growth of *C. albicans* (green methanol MIC- 37.5 mg/ml) and *S. aureus* (fermented methanol MIC- 18.75 mg/ml), respectively. In spite of the higher hesperidin and mangiferin content in the

Staphylococcus aureus



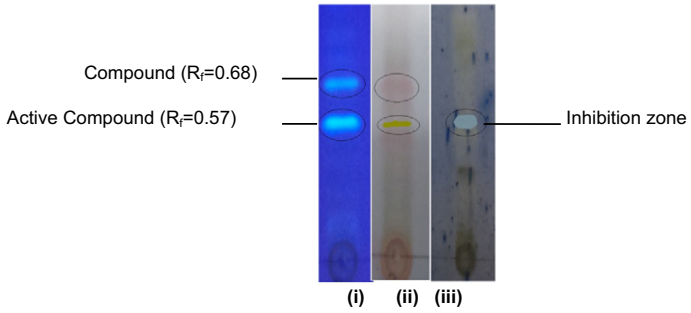
i) Developed TLC plates of the HFM extract under UV at 360nm.
ii) Developed TLC plates of the HFM extract sprayed with vanillin sulphuric acid reagent.
iii) Developed TLC plates of the HFM extract inoculated with *S. aureus* and sprayed with NBT.

Streptococcus pyogenes



i) Developed TLC plates of the HGC extract under UV at 360nm
ii) Developed TLC plates of the HGC extract sprayed with vanillin sulphuric acid reagent
iii) Developed TLC plates of the HGC extract inoculated with *S. pyogenes* and sprayed with NBT

Candida albicans



i) Developed TLC plates of the HGM extract under UV at 360nm
ii) Developed TLC plates of the HGM extract sprayed with vanillin sulphuric acid reagent.
iii) Developed TLC plates of the HGM extract inoculated with *C. albicans* and sprayed with NBT

Fig. 1. The antimicrobial activity of the most potent extracts (HFM, HGC and HGM) against *S. aureus*, *S. pyogenes* and *C. albicans*, respectively *Abbreviations*: TLC: thin layer chromatography; HFM: honeybush fermented methanol; HGC: honeybush green chloroform; HGM: honeybush green methanol R_f: retardation factor; NBT: nitrotetrazolium blue chloride.

methanol green extract, the MIC values observed for the methanol fermented extracts were relatively lower, indicating a better antimicrobial activity, and not linking the increased phytochemical content with the enhanced antimicrobial activity. When focussing on the fermented methanol extract, consideration should be given to the possible extraction of other compounds by the methanol also possessing antimicrobial traits, thus the relatively lower MIC observed against *S. aureus*. The water extracts on the other hand exhibited a weaker inhibitory activity against the *S. aureus* (green water MIC- 150 mg/ml; fermented water MIC- 300 mg/ml) and *C. albicans* (green water MIC- 150 mg/ml; fermented water MIC- 150 mg/ml). Lower concentrations of hesperidin and mangiferin were observed in green (hesperidin- 5.89 mg/g; mangiferin- 1.13 mg/g) and fermented (hesperidin- 3.07 mg/g; mangiferin- 1.57 mg/g) water extracts. Also, the high polarity of the water solvent is associated with a weaker antimicrobial activity (Tian et al., 2009). Both mangiferin (Stoilova et al., 2005; Singh et al., 2009) and hesperidin (Iranshahi et al., 2015) have been shown to play vital roles in the protection against pathogenic microorganisms, confirming the antimicrobial activity associated with these two major honeybush flavonoids.

S. pyogenes proved most sensitive to the green honeybush chloroform extract (MIC- 18.75 mg/ml) with a lesser extent of inhibition by some of the other extracts. The antimicrobial potential of lipophilic compounds extracted by chloroform and other lipophilic solvents has been previously observed (Kabara et al., 1977; Barros et al., 2013). In addition, the relatively high levels of hesperidin (22.6 mg/g) and mangiferin (2.48 mg/g) present in the green honeybush chloroform extract could further contribute to the antimicrobial capacity of this

Table 3
Comparison of the antioxidant capacities using the ORAC, FRAP and TEAC assays of the various solvent extracts of green/unfermented and fermented honeybush (*C. intermedia*) plant material.

Green honeybush						
Extract conc (mg/ml)	Average ORAC conc (μmol TE/g) ± SD				Statistical comparison	Statistical significance
	HGW	HGM	HGC	HGE		
5	25,245 ± 536	26,960 ± 861	16,448 ± 864	13,230 ± 996	HGW: HGM	ns
					HGW: HGC	<0.05*
					HGW: HGE	<0.01**
					HGM: HGC	<0.05*
					HGM:HGE	<0.01**
					HGC: HGE	ns
15	74,585 ± 4597	85,657 ± 4714	49,532 ± 2513	37,192 ± 3951	HGW: HGM	<0.01**
					HGW: HGC	<0.001***
					HGW: HGE	<0.001***
					HGM: HGC	<0.001***
					HGM: HGE	<0.001***
					HGC: HGE	<0.01**
Fermented honeybush						
Extract conc (mg/ml)	Average ORAC conc (μmol TE/g) ± SD				Statistical comparison	Statistical significance
	HFW	HFM	HFC	HFE		
5	15,430 ± 974	21,731 ± 1779	10,408 ± 1145	11,919 ± 832	HFW: HFM	<0.05*
					HFW: HFC	<0.001***
					HFW: HFE	ns
					HFM: HFC	<0.001***
					HFM:HFE	<0.01**
					HFC: HFE	ns
15	47,504 ± 2204	60,102 ± 6728	29,836 ± 1791	33,082 ± 3385	HFW: HFM	<0.001***
					HFW: HFC	<0.001***
					HFW: HFE	<0.001***
					HFM: HFC	<0.001***
					HFM: HFE	<0.001***
					HFC: HFE	ns
Green honeybush						
Extract conc (mg/ml)	Average FRAP conc (μmol/g) ± SD				Statistical comparison	Statistical significance
	HGW	HGM	HGC	HGE		
5	4790 ± 180	5662 ± 312	3263 ± 140	2947 ± 170	HGW: HGM	<0.05*
					HGW: HGC	<0.001***
					HGW: HGE	<0.001***
					HGM: HGC	<0.001***
					HGM:HGE	<0.001***
					HGC: HGE	ns
15	14,783 ± 265	18,724 ± 823	12,090 ± 381	11,166 ± 376	HGW: HGM	<0.001***
					HGW: HGC	<0.001***
					HGW: HGE	<0.001***
					HGM: HGC	<0.001***
					HGM: HGE	<0.001***
					HGC: HGE	<0.05*
Fermented honeybush						
Extract conc (mg/ml)	Average FRAP conc (μmol/g) ± SD				Statistical comparison	Statistical significance
	HFW	HFM	HFC	HFE		
5	2598 ± 45	2921 ± 140	1851 ± 91	1015 ± 82	HFW: HFM	ns
					HFW: HFC	<0.01**
					HFW: HFE	<0.001***
					HFM: HFC	<0.001***
					HFM:HFE	<0.001***
					HFC: HFE	<0.01**
15	7275 ± 239	10,446 ± 549	4310 ± 180	3789 ± 131	HFW: HFM	<0.001***
					HFW: HFC	<0.001***
					HFW: HFE	<0.001***
					HFM: HFC	<0.001***
					HFM: HFE	<0.001***
					HFC: HFE	<0.05*
Green honeybush						
Extract conc (mg/ml)	Average TEAC conc (μmol TE/g) ± SD				Statistical comparison	Statistical significance
	HGW	HGM	HGC	HGE		
5	3267 ± 174	4437 ± 178	2591 ± 134	1883 ± 60	HGW: HGM	<0.01**
					HGW: HGC	ns

Table 3 (*continued*)

Green honeybush						
Extract conc (mg/ml)	Average TEAC conc (μmol TE/g) ± SD				Statistical comparison	Statistical significance
	HGW	HGM	HGC	HGE		
15	9273 ± 639	14,661 ± 922	6553 ± 226	5813 ± 153	HGW: HGE	<0.01**
					HGM: HGC	<0.001***
					HGM:HGE	<0.001***
					HGC: HGE	ns
					HGW: HGM	<0.001***
					HGW: HGC	<0.001***
					HGW: HGE	<0.001***
					HGM: HGC	<0.001***
					HGM: HGE	<0.001***
					HGC: HGE	ns
Fermented honeybush						
Extract conc (mg/ml)	Average TEAC conc (μmol TE/g) ± SD				Statistical comparison	Statistical significance
	HFW	HFM	HFC	HFE		
5	1525 ± 94	2230 ± 149	1371 ± 61	1129 ± 80	HFW: HFM	<0.001***
					HFW: HFC	ns
					HFW: HFE	<0.01**
					HFM: HFC	<0.001***
					HFM:HFE	<0.001***
					HFC: HFE	ns
15	4826 ± 171	5710 ± 200	3427 ± 127	2848 ± 126	HFW: HFM	<0.001***
					HFW: HFC	<0.001***
					HFW: HFE	<0.001***
					HFM: HFC	<0.001***
					HFM: HFE	<0.001***
					HFC: HFE	<0.001***

Values in columns = mean ± sd, $n = 3$. *Abbreviations*: conc: concentration; HGW: honeybush green water; HGM: honeybush green methanol; HGC: honeybush green chloroform; HGE: honeybush green ethyl acetate; HFW: honeybush fermented water; HFM: honeybush fermented methanol; HFC: honeybush fermented chloroform; HFE: honeybush fermented ethyl acetate; sd- standard deviation; μmol TE/g: micromoles of Trolox® equivalents per gram; μmol/g: micromoles per gram; ORAC: oxygen radical absorbance capacity; FRAP: Ferric reducing ability of plasma; TEAC: Trolox equivalent antioxidant capacity; mg/ml: milligram per milliliter.

extract. With consideration to the hesperidin (1.42 mg/g) and mangiferin (0.65 mg/g) concentration of the fermented honeybush chloroform extract, its weaker activity against *S. pyogenes* (MIC-75 mg/ml) was not an alarming observation. The resistance of *P. aeruginosa*, *S. aureus* and *C. albicans* to the chloroform extracts could be as a result of the characteristic differences within the individual microbial strains (Chan et al., 2011).

Considering the high MIC values observed for the honeybush extracts against *S. aureus*, *S. pyogenes* and fungus *C. albicans*, the various solvent extracts of honeybush cannot be considered significant antimicrobials, as a noteworthy antimicrobial agent exhibits a MIC value against a specific microorganism of less than 1.00 mg/ml (Rios and Recio, 2005; Ncube et al., 2008; Van Vuuren, 2008). Despite this fact, the antimicrobial activity shown by the honeybush extracts should be acknowledged. Bioautography allowed the localization of three highly active antimicrobial compounds in each of the potent extracts on a silica gel plate. These were noted as zones of inhibition on the bioautograms (Fig. 1). The TLC assay permitted the confirmation of the fractions observed in the fermented honeybush methanol, green honeybush chloroform and green honeybush methanol extracts under UV at 366 nm as those exhibiting antimicrobial activity against *S. aureus*, *S. pyogenes* and *C. albicans* respectively. The identification and further isolation of these active compounds using nuclear magnetic resonance (NMR) and mass spectrometry (MS) will be ideal for future investigatory purposes, as these individual compounds are likely to be more active than the whole plant extracts (McGaw et al., 2013).

Table 4
Total polyphenols (by the Folin- Ciocalteu method using 15 mg/ml crude extract), mangiferin and hesperidin (by HPLC using 1 mg/ml crude extract) concentrations of the various *C. intermedia* extracts.

Extracts	Total polyphenols (mg GAE/g)	Mangiferin (mg/g)	Hesperidin (mg/g)
HGW	200.9 ± 9.02	1.13	5.89
HGM	224.8 ± 13.89	30.66	24.32
HGC	118.8 ± 7.84	2.48	22.60
HGE	79.7 ± 8.15	0.78	0.51
HFW	106.8 ± 6.252	1.57	3.07
HFM	178.5 ± 10.45	3.59	14.51
HFC	93.9 ± 7.76	0.65	1.42
HFE	58.1 ± 5.09	0.83	0.51

Values in total polyphenol column = mean ± sd, n = 3. HGW-honeybush green water; HGM- honeybush green methanol; HGC- honeybush green chloroform; HGE- honeybush green ethyl acetate; HFW- honeybush fermented water; HFM- honeybush fermented methanol; HFC- honeybush fermented chloroform; HFE- honeybush fermented ethyl acetate; sd- standard deviation; mg/g- milligrams per gram; mg GAE/g- milligram Gallic acid equivalents per gram; mg/ml- milligram per milliliter.

The current study also further adds to the little existing data available on the antioxidant capacity of honeybush, especially with focus on various solvent extracts (McKay and Blumberg, 2007; Joubert et al., 2011). In general, the results obtained from the various antioxidant assays represent the concentration of antioxidants present that are capable of displaying the specific characteristics under study. According to this study, the polar compounds extracted from honeybush green and fermented leaves by water and methanol solvents exhibited higher free radical scavenging ability compared to chloroform and ethyl acetate extracts. An antioxidant is defined as a compound when present can significantly retard or prevent the oxidation of oxidizable molecules, scavenge oxygen derived compounds or prevent the formation of reactive oxygen species (Halliwell, 1995). By this definition generally all the honeybush extracts displayed antioxidant traits, evaluated by the different assays ORAC, FRAP and TEAC, though at differing levels and rankings.

Green form of methanol honeybush extract contained higher levels of the flavonoids mangiferin and hesperidin compared to those of water, chloroform and ethyl acetate. This observation was in agreement with studies that identified the polar methanol as the ideal solvent in the extraction of polyphenols from whole plants (Bonilla et al., 1999; Erol et al., 2009). Additionally the high total polyphenol concentration within this polar solvent extract demonstrates a probable relation between the total polyphenol content and individual flavonoid concentrations in the extracts. This relation has been investigated and documented since flavonoids are classified as a group of phenolic compounds (Verzelloni et al., 2007; Daglia, 2012).

5. Conclusion

The results of this study indicate that the green/unfermented and fermented honeybush solvent extracts possess a relatively weak antimicrobial response, while displaying high antioxidant capacity. Due to these antioxidant and antimicrobial effects demonstrated by the various extracts of green and fermented honeybush, the constant intake of these herbal tea forms could be considered a good source of a unique blend of natural phytochemical antioxidants and antimicrobial therapeutics, leading to the enhancement of the general wellbeing of humans. With further characterization of the various extracts and elucidation of the bio-activities, these extracts can be developed into dietary supplements, henceforth contributing to improving the health of the general public. Furthermore honeybush could be utilized in the food industry as a natural preservative whilst maintaining the flavor quality of the food (Almajano et al., 2008). The acknowledgement of specific antimicrobial fractions within these solvent extracts may provide the research community with new leads in the on-going pursuit for novel antimicrobial drugs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2016.10.010.

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