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An *in-vitro* Analysis of the Antioxidant and Antimicrobial Properties of the Methanol Extract of *Hypoxis hemerocallideacorm* (MEHHC) from Botswana

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Authors' contributions

This work was carried out in collaboration between all authors. All the authors participated in designing the study, wrote the protocol, performed the statistical analysis and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To evaluate the antimicrobial and antioxidant activity of the methanol extract of *Hypoxis hemerocallidea* corm (MEHHC) with different assays.
Study Design: *In vitro* evaluation of antibacterial and antioxidant activities of the MEHHC.
Location and Duration of Study: Department of Biological Sciences (Faculty of Sciences) and Faculty of Health Sciences, University of Botswana from Jan.2015 to Sep. 2016.
Methodology: The MEHHC was prepared and evaluated for total phenolic content (TPC) and antioxidant properties with DPPH and ABTS methods. Phytochemical screening was conducted to

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detect bioactive constituents. Antimicrobial activity was determined with diffusion assays, minimum inhibitory concentration and minimum bactericidal concentration assays.
Results: The MEHHC exhibited antioxidant activity and the ability to scavenge DPPH radicals. A relatively high TPC was observed and there was a strong association between antioxidative activities and phenolic compounds (R=0.871), suggesting that the constituent phenolic compounds may confer the antioxidative activities to the MEHHC. The MEHHC exhibited anti-fungal activity against *C. albicans* and variable antibacterial activity against the Gram positive and Gram negative bacteria. There was more pronounced activity against the tested Gram positive bacteria, particularly *S. aureus* (including the MRSA ATCC 430043 strain) whereas *E. coli, S. typhimurium* and *K. pneumoniae* displayed the lowest susceptibility to the MEHHC.
Conclusion: The results indicate that the MEHHC has considerable antioxidant and anti-microbial properties. Potentially the MEHHC is appropriate for pharmaceutical purposes both as an antioxidant and antimicrobial agent.

Keywords: Antioxidants; antimicrobial; total phenolic content; radical scavenging activity; diffusion assay; minimum inhibitory concentration.

1. INTRODUCTION

Hypoxis hemerocallidea (African potato), particularly the tuberous rootstock/ corm has been used as a traditional medicinal plant for many years in Southern Africa and certain European countries [1-3]. Its extracts, powders, infusions and decoctions are used for the management of diverse ailments, including vomiting, the common cold, dizziness, burns, wounds, urinary tract infections, polyarthritis, hypertension, anxiety, nervous disorders and immune-related illnesses. It has been scientifically investigated extensively and its aqueous and alcohol solvent extracts have been shown to exhibit antimicrobial, antioxidant, anti-inflammatory, antinociceptive, anticonvulsant and antidiabetic properties [1,2,4,5]. Currently there are *H. hemerocallidea* commercial products that are available for immune modulation and amelioration of benign prostate hypertrophy [6,7].

The bioactive constituents of *H. hemerocallidea* include antioxidants, antimicrobial components and phytosterols. One of earliest bioactive phytochemicals isolated is a diglycoside termed hypoxoside and its active derivative rooperol. The latter is derived by hydrolysis of hypoxosideby a beta-glucosidase, in the gastrointestinal tract [8,9]. Several studies have shown that rooperol is a powerful antioxidant and has inhibitory effects on mouse BL6 melanoma cell growth [2,10]. There are indications that certain malignancies can be treated with hypoxoside as a putative, non-toxic prodrug. Furthermore both hypoxoside and rooperol exhibit antimicrobial properties by causing leakage in bacterial cell membranes and consequently inhibition of bacterial cell growth [11]. In certain countries *H. hemerocallidea* is recommended for HIV/AIDS treatment as there are suggestions that hypoxoside and rooperol may be beneficial in the management of HIV-infection. However there is some indication that these affect cytochrome P450 and when taken with antiretroviral agents, they may increase the risk of antiretroviral treatment failure and or drug toxicity [12,13].

H. hemerocallidea is also rich in phytosterols i.e. β-sitosterol which has been shown to have anti-cancer; anti-inflammatory; antioxidant; antidiabetic; cholesterol-lowering and immune modulating properties [14-17]. Crude extracts and β-sitosterol from several *Hypoxis* species (i.e. *H. hemerocallidea*, *H. stellipilis*, *H. sobolifera*) have been shown to significantly increase phagocytic activity in pretreated differentiated U937 cells and increase production of nitric oxide and reactive oxygen species which are microbicidal [1]. It is plausible that the production of immune modulating phytochemicals such as antioxidants and phytosterols in *Hypoxis* species including *H. hemerocallidea* may trigger immune system pathways that inadvertently have an antimicrobial effect during an infection [1]. The present study investigated the main classes of phytochemicals present in the methanol extract of *H. hemerocallidea* corm (MEHHC) and examined a correlation between the antioxidant potential and the total phenolic content of the MEHHC. *H. hemerocallidea* is extensively used for the treatment of infections, thus the present study also investigated the antimicrobial activities of the MEHHC against several bacteria and fungus species.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plants

H. hemerocallidea was collected from local traditional herbalist sellers within Gaborone. Species identification was done at the University of Botswana Herbarium and the voucher number given was: G2016/, A01.

2.2 Preparation of the Methanol Extract

The corm was cut into small pieces and dried at room temperature in a biosafety level 2 cabinet for three days at room temperature, when it was completely dry. The dried corm pieces were ground with a laboratory pestle/ mortar and an electric blender. Three hundred grams (300 g) of the ground corm was soaked in 500 mL of 70% methanol for three days to allow for optimal extraction of the phytochemicals into the methanol. The extract was then passed through Whatman filter paper No. 1 and concentrated using a Rotary vacuum evaporator at 40℃/ 902 mbar for at least 45 min. The extract was then placed in biosafety level 2 cabinets for up to 3days to allow it to dry completely. The dry plant extracts were then sealed and stored at 2-8℃ in a laboratory refrigerator for further analysis. A working stock solution (0.2 g/mL) of the methanol *H. hemerocallidea* corm (MEHHC) extract was prepared in 10% DMSO.

2.3 Chemicals

Analytical grade chemicals were used: DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent (Fluka Chemicals, Steinheim, Germany); Folin-Ciocalteau reagent (Rochelle Chemicals, South Africa);ascorbic acid and anhydroussodium carbonate (Unilab, South Africa); gallic acid (Sigma Chemicals, Steinheim, Germany). Ready-made Thin-layer chromatography (TLC) sheets were aluminium backed and coated to a thickness of 0.25 mm with silica gel 60 F254.

2.4 Phytochemical Screening

The phytochemical tests were carried as previously described [18]. Briefly, the following was conducted to screen for phytochemicals:

2.4.1 Flavonoids

The extract (1 mL) was added to concentrated sulphuric acid (0.2 mL) and 0.5 g of Magnesium. The development of a pink or red colour that disappeared on standing (3 min) indicated the presence of flavonoids. Alternatively, lead acetate (10%) solution drops were added to the extract (1 mL) and the formation of a yellow precipitate indicated the presence of flavonoids.

2.4.2 Tannins

The MEHHC (1 mL) was mixed with 2 mL of sterile distilled water and drops of dilute ferric chloride (0.1%) solution were added. The development of a green or blue-green (cathechic tannins) or a blue-black (gallic tannins) colour were indicators of the presence of tannins.

2.4.3 Saponins

The MEHHC (1 mL) was vigorously shaken with sterile distilled water. The development of a froth that persisted for 20 min was a positive indicator for saponins.

2.4.4 Coumarins

Sodium hydroxide (2 mL, 10%) was added to 1mL of the MEHHC and the formation of a yellow color indicated the presence of coumarins.

2.4.5 Terpenoids

Drops of concentrated sulphuric acid were added to a mixture of the MEHHC (2 mL) and acetic anhydride (2 mL). The formation of blue-green rings indicated the presence of terpenoids.

2.4.6 Fatty acids

The MEHHC (0.5 mL) was mixed with 5mL of ether then transferred onto filter paper and allowed to evaporate on the filter paper. The appearance of a transparent area on the dried filter paper indicated the presence of fatty acids.

2.4.7 Phenols

Drops of ferric chloride (5%) solution were added to the MEHHC (1 mL) and the formation of a deep blue or black colour indicated the presence of phenols.

2.4.8 Amino acids and proteins

Drops of the ninhydrin (1%) solution were added to the MEHHC (1 mL) and the mixture was then placed in a boiling water bath for 2 min. The formation of a purple colour was a positive indicator for amino acids and proteins.

2.4.9 Quinones

Drops of concentrated hydrochloric acid were added to the MEHHC (1 mL) and the formation of yellow precipitate indicated the presence of quinones.

2.4.10 Oxalates

Drops of glacial acetic acid were added to the MEHHC (2 mL) and the development of a greenish black colour indicated the presence of oxalates.

2.5 Antioxidant Activity Assays

2.5.1 TLC and semi quantitative DPPH assay

This procedure was adapted and revised from the methods previously described by Juma & Majinda (2004). DPPH (0.2%) solution was prepared in methanol and kept at 2-8°C for further use. On the aluminum based TLC sheet, a grid comprising 1.0 cm^2grid spaces was marked. A series of dilutions of the MEHHC stock solution and the standard (ascorbic acid and gallic acid) were prepared ranging from 0.35 µL to 0.005 µL. The TLC grid sheet was labeled with the MEHHC on the horizontal axis, whereas the concentrations of the MEHHC and the respective standards (ascorbic acid and Gallic acid) were labeled on the vertical axis. The dilutions of the MEHHC and the standards were then plotted on the TLC sheets, as a series of spots. Care was taken to maintain an equal volume of the extract dilutions in order to maintain the same size for the spots on the TLC sheet. The spots were allowed to dry for at least 2 hr. To detect the antioxidant activity, the TLC chromatograms were sprayed with 0.2% DPPH solution and the development of yellow spots indicated the presence of antioxidant activity.

2.5.2 DPPH spectrophotometric method

The DPPH radical is a model of a lipophilic radical initiated by lipid autoxidation. The radical scavenging activity of the MEHHC was measured using the DPPH method adapted from [19]. A 500 µM DPPH solution in methanol was prepared. Different concentrations of the MEHHC and the standards (ascorbic acid and gallic acid) were prepared ranging from 0.001-0.05 mg/mL in methanol. Two milliliters (2 mL) of the MEHHC and the standard solutions were added to separate equal volumes of the DPPH solution, making a total reaction volume of 4 mL. A negative control reaction mixture was prepared comprising 2 mL methanol without extract and an equal volume of the DPPH solution. The test tubes were shaken vigorously and placed in a dark cupboard for 30 min. The absorbance of each solution was then measured at 517 nm, with methanol used as the blank for baseline correction. This was repeated after 2hr and finally after 24 hr. The percentage inhibition of DPPH, (I %) was calculated using the following formula:

> %inhibition = [(Absorbance of control– Absorbance of test sample)/Absorbance Control] x100.

The concentration of the MEHHC required to inhibit the DPPH radical activity by 50% (IC_{50}) was determined from the inhibition curves (I % versus sample concentration µg/mL) and with non-linear regression equations that best fitted the curves. The experiment was carried out in triplicate and the IC_{50}values were reported as an average µg/ mL + the standard deviation.

2.5.3 2, 2-Azobis-3-ethyl benzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity

The ABTS radical scavenging activity assay was adapted from Pellegrini et al. (1999). Varying concentrations (2.5 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml) of the MEHHC (50 µl) were added to 950 µl of ABTS working solution. The absorbency of the solution was then recorded immediately at 734 nm. This was also repeated for Gallic acid at different concentrations (1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml and 16 µg/ml) which was used as reference standard. The percent inhibition was calculated using the following equation:

> %inhibition = [(Absorbance of control– Absorbance of test sample)/Absorbance Control] x100.

2.5.4 Total phenolic content

The total phenolic content (TPC) of the MEHHC was determined using the Folin-Ciocalteu reagent method adapted from [19]. Five concentrations of the standard (gallic acid in methanol) and the MEHHC were prepared ranging from 0.01 to 0.05 mg/mL. A volume (5 mL) of 90% aqueous methanol and 0.5mL Folin-Ciocalteu reagent were added to 0.5 mL of each of the standard solutions and to 0.5 mL of each MEHHC solution. After 3 min, 1 mL of 2% Na_2CO_3 was then added to each test-tube and

the mixture was shaken vigorously for 2 min and left to stand for 2 hours at room temperature. The absorbance of the supernatant solution was determined at 725 nm using 90% aqueous methanol as a blank. A Gallic acid standard curve was prepared and the equation derived by linear regression i.e. y =36.84 x+0.1069 was used to determine the TPC of each extract in mg of gallic acid equivalents/g of extract (mg GAE/g). The experiment was performed in triplicate and TPC was reported as the average value of 3 trials ± the standard deviation.

2.6 Antimicrobial

2.6.1 Microbial cultures

The ATCC strains of the microorganisms tested in the antimicrobial assays were obtained from Department of Microbiology, School of Allied Medical Sciences, University of Botswana, Botswana. These were Gram positive cocci: *Staphylococcus aureus* (ATCC 25923); Methicillin resistant *Staphylococcus aureus* (ATCC 43300); a clinical *Staphylococcus aureus* isolate; *Staphylococcus epidermidis* (ATCC 12228) and *Streptococcus agalactiae* (ATCC 27956). The Gram positive bacilli species was: *Listeria monocytogenes.* The Gram negative bacilli were *Escherichia coli* (ATCC 10536); *Klebsiella pneumonia* (ATCC700803); *Proteus mirabilis* (ATCC 25933); *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC43300). The fungus was *Candida albicans* (ATCC 90028). The microorganisms were grown as pure cultures on nutrient agar plates (OXOID).

2.6.2 Antimicrobial susceptibility testing

The MEHHC was dissolved in 10% dimethyl sulfoxide (DMSO) to give a final concentration of 0.2 mg/mL. Antimicrobial susceptibility testing of the test microorganisms was carried out by well diffusion and disc diffusion methods on Mueller Hinton agar plates (MHA) (MAST) adapted from Coyle, M.B (2005). For the well diffusion assay, the base of a sterile glass Pasteur pipette was used to make 6mm-diameter wells in the MHA agar plates. Suspensions of the tests microorganisms were made in Tryptone Soya broth (OXOID) and adjusted to a 0.5 McFarland standard turbidity ($1.5x10^8$ colony forming units per ml (CFU/mL). Each of the test organisms were respectively inoculated (to attain confluent growth) onto the MHA agar plates (in duplicate) containing the wells. One hundred microlitres (100 µL) of the extract which gives a concentration of 20 mg, was added to each well and a well containing only DMSO (100µL) included in each plate as the control.

For the disc diffusion method, 6mm diameter sterile discs were prepared from Whatman903 saver cards. Each of the test organisms were respectively inoculated (to attain confluent growth) onto MHA agar plates (in duplicate). The discs were placed onto inoculated media plates and then saturated with 20 µL (4 mg) of the MEHHC. Ampicillin (10 µg) discs (Mast Diagnostics) were used as the positive control for most of the test microorganisms. Meropenem (10 µg) (Mast Diagnostics) was used for *S. aureus* (ATCC 43300); ceftazidime (30 µg) (Mast Diagnostics) for *P. aeruginosa*; Co-trimoxazole (CTX 25 µg) (Mast Diagnostics) for *K. pneumonia*e and fluconazole (25 µg) (Mast Diagnostics) for *C. albicans*. For the negative control a disc containing 20µL of DMSO was also added to each plate. All the plates for the well and disc diffusion assays were incubated at 37°C in ambient air for 24 hr. The zones of inhibition were measured using vernier calipers.

2.6.3 Minimum inhibitory concentration

The Minimum Inhibitory Concentration (MIC) of the MEHHC against each of the test microorganisms was determined by the micro well dilution method adapted from Coyle M., B (2005). The 96-well plates were prepared by adding 300 µL of the MEHHC (0.2 g/ml) dissolved in 10% DMSO to the first well. Then 150 µLTSB was added to the remaining wells. One hundred and fifty microlitres (150 µL) of the MEHHC from the first well was transferred to the subsequent well (containing 150 µL TSB) to make the initial serial dilution (1:2). Then 150 µL from the 3^{rd} well was transferred to the 4^{th}well and this was repeated until a dilution of 1:512 was attained. Three microlitres (3 µL) of the bacterial (or fungal) suspension was added to all the wells. The last two wells contained a positive and negative controls which were the bacterial suspension (3 µL in TSB) alone and the TSB (150 µL) alone respectively. The plates were covered and incubated at 37℃ for 24 hr, then visually examined for inhibition of growth. The MIC was considered as the lowest concentration of the MEHHC that prevented visible growth.

2.6.4 Minimum bactericidal concentration

The minimum concentration (MBC) assay was adapted from [20]. The MBCs of the MEHHC

were determined by subculturing the total volume of the individual suspensions from all the wells from the 96-well MIC plates on respective MHA plates. These were then incubated at 37°C in ambient air for 24 hr. The MBC was defined as the lowest concentration of the MEHHC that inhibited the growth of microorganisms i.e. no growth of the microorganism on the MHA plate. The tests were done in duplicate.

2.7 Statistical Analysis

All data was expressed as the mean ± standard error. Analysis of variance was performed by one way ANOVA and the significant difference between the means were determined by the Holm-Sidak method. The *p* value ≤0.05 was regarded as significant. In all these cases, Statistical Software Stata 13.1 was used to analyze the data.

3. RESULTS

3.1 Phytochemical Screening of the MEHHC

The MEHHC exhibited the presence of five phytochemicals i.e. flavonoids, tannins, coumarins, fatty acids and phenols (see Table 1). Conversely saponins, terpenoids, amino acids, proteins, quinones and oxalates were not detected in the MEHHC in this study.

Table 1. Results of the phytochemical screening of the MEHHC

Phytochemicals	Result
Flavonoids	+
Tannins	+
Saponins	-
Coumarins	+
Terpenoids	-
Fatty acids	+
Phenols	+
Amino acids	-
Alpha proteins	-
Quinones	-
Oxalate	-

Key: +present; - absent

3.2 Antioxidants- TLC and Semi Quantitative DPPH Assay

The bleaching of the purple colour of the DPPH reagent and resulting yellow spots was an indication of antioxidant activity (see Fig. 1). The MEHHC showed antioxidant activity comparable to the standard (gallic acid). The control had antioxidant activity from the lowest concentration of 0.025 mg/mL in comparison with the MEHHC which started to show antioxidant activity at the slightly higher concentration of 0.05 mg/ mL (see Fig. 1). Indicating that the MEHHC expresses scavenging activity against the DPPH radical, which is comparable to that of gallic acid. This method is also reported as a decolourisation assay applicable to both lipophilic and hydrophilic antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity.

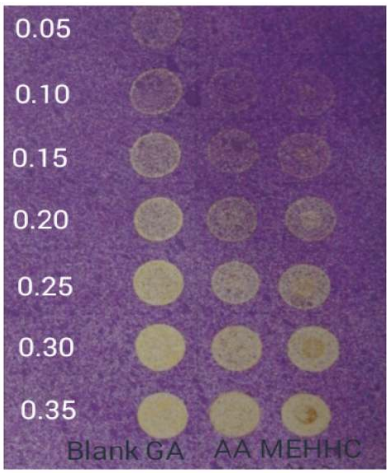

Fig. 1. Semi quantitative TLC-DPPH radical scavenging activity of the MEHHC in comparison to gallic acid and ascorbic acid

3.3 DPPH Spectrophotometric Method

This method has been used extensively to predict the antioxidant activities. There reduction in the absorbance of DPPH is an indication of the degree to which the extract can inhibit the DPPH radical activity. This is associated with the free radical scavenging activities of antioxidants in the tested extract. Fig. 2 shows the % DPPH inhibition by the MEHHC in comparison to two standards i.e. gallic acid and ascorbic acid. As with the standards, the MEHHC showed increasing radical scavenging activity with increasing concentration. Although its scavenging effect was lower than that of Gallic acid, it was highly comparable to ascorbic acid (see Fig. 2). The concentration of the MEHHC required to inhibit the DPPH radical activity by 50% (IC_{50}) was 0.01301 mg/mL which was comparable to the ascorbic acid IC_{50}i.e.0.01325 mg/mL, a further indication of the significant antioxidant activities of the MEHHC.

3.4 ABTS Radical Scavenging Activity

Similar to the results of the DPPH assay, with the ABTS assay the free radical scavenging activities of the MEHHC were concentration-dependent. Fig. 3 shows the % ABTS inhibition by the MEHHC in comparison to two standards i.e. gallic acid and ascorbic acid. Similar to both standards, the MEHHC showed increasing free ABTS radical scavenging activity with increasing concentration, which was comparable albeit slightly lower than both standards in this study (see Fig. 3).

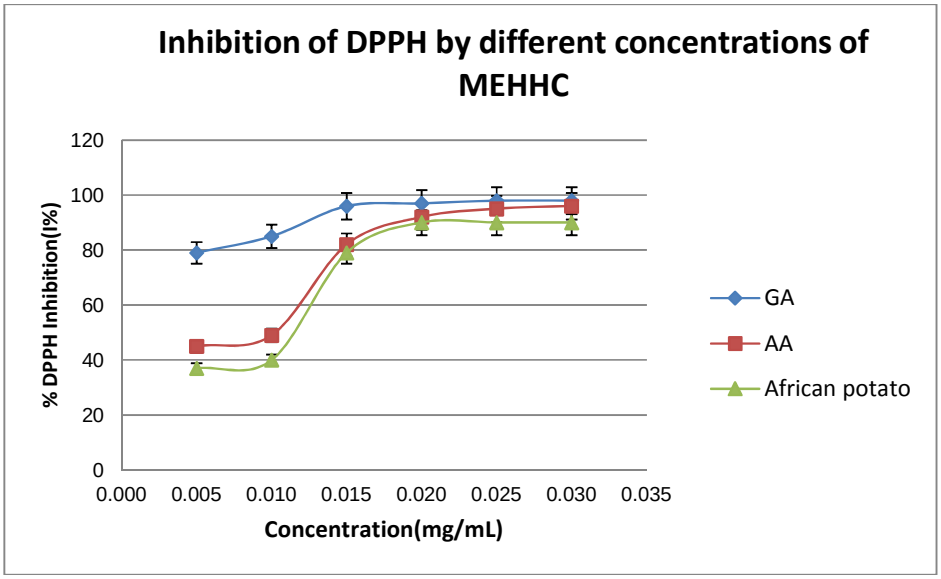

Fig. 2. Free radical scavenging activity of the MEHHC/African potato, Gallic acid (GA) and ascorbic acid (AA) on 1,1-diphenyl-2-picryl-hydrazol (DPPH) radicals

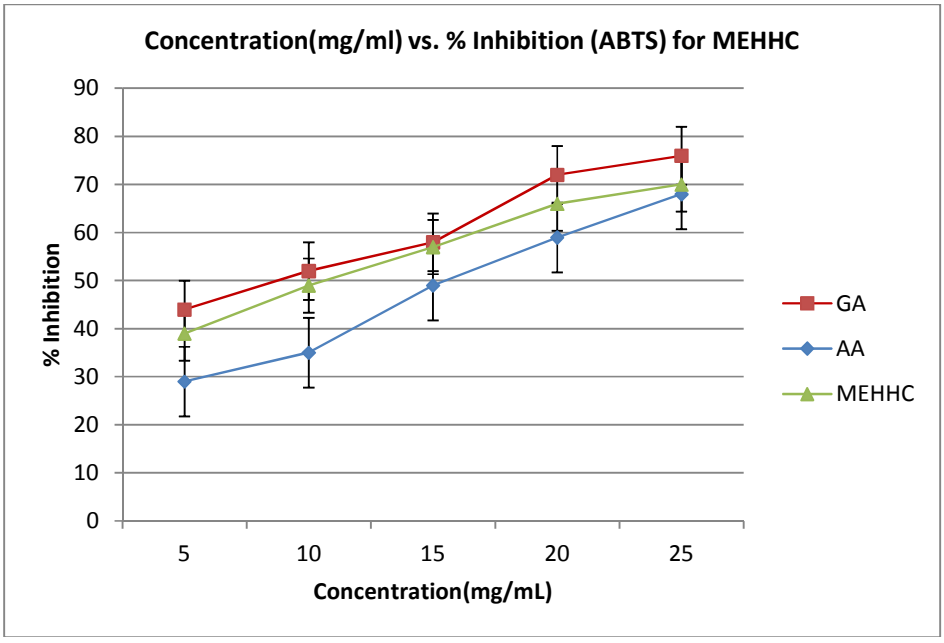

Fig. 3. Free radical scavenging activity of the MEHHC gallic acid (GA) and ascorbic acid (AA) on ABTS radicals

3.5 Total Phenolic Content

Many natural antioxidants are phenolic so the determination of total phenolic content gives valuable information that can be correlated with the antioxidant capacity of the sample. The total phenolic content (TPC) was calculated from the linear regression equation of the standard curve y =0.3042x+70.668. From this equation the equivalent concentration of Gallic acid (mg/mL) was determined for each extract and converted to mg of gallic acid equivalents/g of dry extract (mg GAE/g) (see Table 2). The scavenging ability of the phenols in the MEHHC are shown in Fig. 4.

Table 2. The total phenolic content (TPC) of the MEHHC

Concentration (mg/ml)	TPC (mg GAE/100 g) MEHHC
0.1	17.02
0.2	26.24
0.3	38.30
0.4	49.29
0.5	87.70

Key: Gallic acid equivalent (GAE)

3.6 Antimicrobial Susceptibility Testing

The MEHHC showed antibacterial activity against the tested bacteria. With the well diffusion assay there was a notable difference between the antibacterial activity against the Gram positive compared to the Gram negative bacteria. Generally there was greater activity against the Gram positive bacteria in comparison to the Gram negative bacteria (see Table 3). The highest zones of inhibition and greatest susceptibility to the MEHHC shown with the well diffusion assays were exhibited by the *S. aureus* ATCC 25923 (21 mm) and MRSA ATCC 430043 (23 mm) strains. The inhibition zone diammeters of these strains were relatively comparable to those of the antibiotics which were used as positive controls i.e. *S. aureus* ATCC 25923 and ampicillin (21 mm and 33 mm respectively); MRSA ATCC 430043 and meropenum (23 mm and 29 mm respectively) (see Table 3). The other Gram positive bacteria showed varying degrees of susceptibility to the MEHHC, exhibiting zones of 17 mm and 18 mm. Generally these were larger than most of the Gram negative bacteria (zones ranging from 10 – 15 mm; except *P. mirabilis* – 20 mm).

With the disc diffusion assays the antimicrobial activity against most of the tested microorganisms was more variable. Notably the MEHHC concentration used in the disc diffusion assays was lower (20 µL=4 mg) than that used for the well diffusion assays (100 µL=20 mg). Furthermore there were challenges (i.e. saturation of discs) with loading higher volumes of the MEHHC onto discs. Nonetheless even at a lower concentration with the disc diffusion assay it was still evident that there was more inhibitory activity against the Gram positive bacteria compared to the Gram negative bacteria, except *P. mirabilis* which showed a greater inhibition zone, as was seen with the well diffusion assays (see Table 3). Notably *E. coli* and *S. typhimurium*, had no zone of inhibition, this was similar to the results of the well diffusion assay whereby these same bacteria species had smaller inhibition zones in comparison the other tested bacteria (see Table 3).

The MIC values also indicated that the MEHHC had considerable antibacterial activity against most of the bacteria species investigated. As with the well diffusion assays, the MIC values showed that its antibacterial activity was more evident with the *S. aureus* ATCC 25923 strain i.e. MIC=0.234 mg/mL and similarly effective against a few of the other Gram positive bacteria: *S. agalactiae* (MIC=0.234 mg/mL) and *L. monocytogenes* (MIC=0.234 mg/mL). Also similar to the well diffusion assays, the MIC values indicated that generally the Gram-negative bacteria exhibited slightly less susceptibility to the MEHHC. This was indicated by their higher MICs: *E. coli*, *K. pnemoniae*, *P. mirabilis*, *P. aeruginosa* all had MICs=0.469 mg/mL. Antifungal activity was observed against the MEHHC against *C. albicans* isolate, it exhibited an MIC of 0.938 mg/ml; inhibition zones of 17 mm and 13 mm with the well diffusion and disc diffusion assays respectively (see Table 3).

4. DISCUSSION

Hypoxis hemerocallidea continues to garner scientific interest because of its medicinal properties conferred by its constituent phytochemicals e.g. phenols, flavonoids, tannins, coumarins and fatty acids, which are associated with anti-inflammatory, anti-diabetic, analgesic and central nervous system activities. In this study methanol *H. hemerocallidea* corm extracts (MEHHC) exhibited the presence of slow kinetic antioxidants with the DPPH assay. Similarly the ABTS assay showed the presence

of antioxidant activity which could be applicable to lipophilic and hydrophilic antioxidants. Phenolic compounds have been shown to possess remarkable antioxidant properties because they are effective hydrogen donors and have redox properties which enable them to act as free radical scavengers and inhibitors of lipid peroxidation. In this study the MEHHC had a high phenolic content, this was associated with strong antioxidative activities (R =0.871), suggesting that the phenolic compounds are probably responsible for the antioxidative activities of the MEHHC. Similarly, Yeboah & Majinda [21] also reported that naturally occurring phenolic compounds exhibit antioxidative activity as observed in their study on *Osyris lanceolate*.

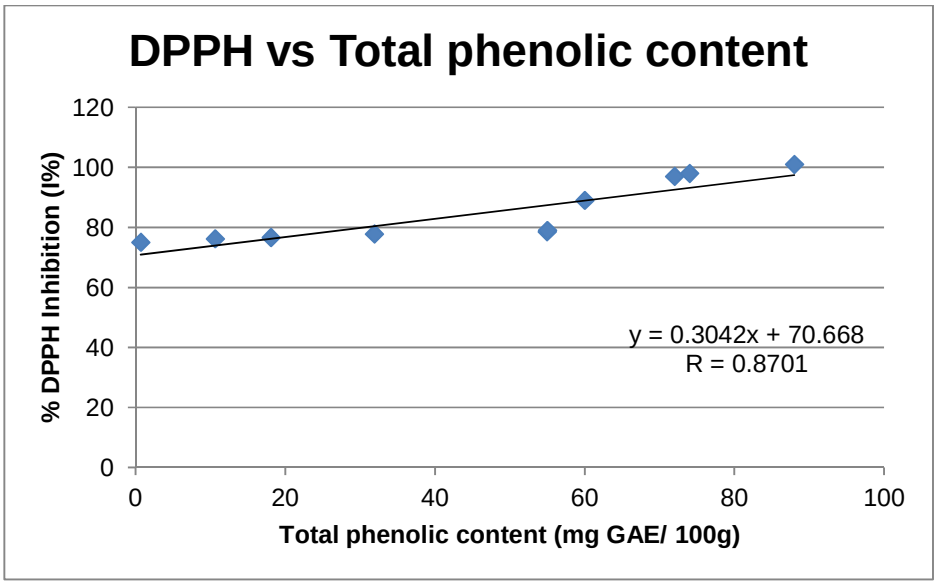

Fig. 4. A comparison of the total phenolic content on the inhibition of DPPH

Table 3. Results of the antimicrobial assays on the tested microorganisms

Microorganisms tested	Zones of diameter (mm)		Ampicillin 10 µg	Concentration (mg/mL)	
	Well diffusion	Disc diffusion		MIC	MBC
Gram positive					
Staphylococcus aureus ATCC 25923	21	7.5	33	0.234	0.469
Methicillin resistant *Staphylococcus aureus* ATCC 430043	23	11.5	29*	0.938	0.938
Staphylococcus aureus (patient sample)	17	12	26	0.938	0.938
Staphylococcus epidermidis (ATCC 12228)	18	17	28	0.938	0.938
Streptococcus agalactiae	17	11	34	0.234	0.469
Listeria monocytogenes	18	11.5	37	0.234	0.234
Gram negative					
Escherichia coli	10	NZ	19	0.469	1.875
Klebsiella pneumoniae	13	6.5	23**	0.469	1.875
Proteus mirabilis	20	15.5	30	0.469	0.938
Pseudomonas aeruginosa	15	7	27***	0.469	0.469
Salmonella typhimurium	14	NZ	24	0.938	0.938
Fungus					
Candida albicans	17	13	25****	0.938	1.875

*Key: *Meropenam 10 ug; **Co-trimoxazole; *** Ceftazidime (30 ug); **** (Fluconazole25 ug); NZ-no zone of inhibition*

In addition to antioxidant activities, *H. hemerocallidea* has also been shown to exhibit antimicrobial properties [1,7,15,22]. In this study the MEHHC showed antimicrobial activity albeit at varying degrees against eleven bacterial species (six Gram positive and five Gram negative bacteria) and one fungal species. The antibacterial activity varied across the microorganisms, particular differences were evident between *S. aureus* compared to other bacteria and generally between the Gram positive and Gram negative bacteria. The *S. aureus* ATCC 25923 and MRSA ATCC 430043 strains exhibited the highest susceptibility to the MEHHC. Their inhibition zones were relatively comparable to those of the positive control antibiotics. This may be an indication that the MEHHC could potentially be as effective as the established antibiotics when treating infections by these strains. Notably *E. coli*, *K. pneumoniae* and *S. typhimurium* showed the lowest susceptibility to the MEHHC with the diffusion assays. This may be an indication that the MEHHC may be less effective in the treatment of infections by these bacteria. The disparity between the antibacterial activity of the MEHHC against the Gram positive and Gram negative bacteria may be due to cell wall differences between these bacteria. The outer membrane of the Gram negative bacteria cell wall is absent in Gram positive bacteria and it comprises extensively of lipopolysaccharides (LPS). The LPS form a tight hydrophilic layer that is also anionic due to acidic proteins; carboxyl and phosphate groups connected to the LPS. The MEHHC in this study had a high phenol content and phenols are hydrophobic. Thus the hydrophilic outer membrane of the Gram negative bacteria would be an inhibitory barrier for efficient penetration of hydrophobic phenolic phytochemicals, enabling the Gram negative bacteria to be less susceptible to the MEHHC.

The more pronounced antimicrobial activity against *S. aureus* in comparison to the other tested bacteria may have been due to differences in the composition of the *S. aureus* cell membrane. Rooperol is a *H. hemerocallidea* phytochemical with strong antioxidant properties and it has been shown to also have antibacterial properties [11]. In particular rooperol has stronger antibacterial activity against *S. aureus* in comparison to *E. coli* [11]. Its postulated mechanism of action involves it binding to specific molecules i.e. phosphatidylglycerol within the bacterial cell membrane, causing leakage and subsequently inhibition of bacterial growth [11]. It has a strong affinity for phosphatidyl glycerol and the *S. aureus* cell membrane is enriched with phosphatidylglycerol in comparison to the *E. coli* cell membrane [11]. Therefore rooperol causes more leakage within the *S. aureus* cell membrane and consequently more pronounced inhibition of bacterial cell growth than in *E. coli* [11]. Pronounced *H. hemerocallidea* extracts antibacterial activity against *S. aureus* in comparison to other bacteria species has also been observed in previously published studies [7,10,15,22]. Katerere & Eloff (2008) showed that ethanol extracts of dried corm exhibited more antibacterial activity against *S. aureus* i.e. MIC=0.31 mg/mL, than *E. faecalis, P. aeruginosa, E. coli*- MICs >1.0 mg/mL [7]. Interestingly the fresh leaves (ethanol extract)of *H. hemerocallidea* showed higher activity than the corm extracts against all the bacteria (MICs ranging from 0.31 – 0.63 mg/mL) [7]. Thus Katerere & Eloff [7] suggested that the *H. hemerocallidea* leaves may have broader antibacterial activity and it may be more rational to use the leaves rather than the corm in the treatment of bacterial infections. Interestingly Ncube et al. [8] showed that the antimicrobial activity of *H. hemerocallidea* corm extracts harvested during different seasons was selective and variable against the various bacteria that they tested (*B. subtilis, E. coli*, *K. pneumoniae, S. aureus* and *C*. *albicans*). However with *S. aureus* the antimicrobial activity was expressed across all the seasons and generally more pronounced in comparison to the other tested microorganisms i.e. MICs=0.39-0.78 mg/mL [8]. Therefore the seasonal variation seemingly affects the expression and accumulation of phytochemicals of *H. hemerocallidea*. Consequently this may also influence the expression of phytochemicals with antibacterial and or anti-fungal properties. This may have been a contributory factor to some of the lower inhibition zones observed for some of the microorganisms tested in this study.

5. CONCLUSION

The methanol extract of *H. hemerocallidea* corm (MEHHC) exhibited antioxidant and antimicrobial activities. The presence of phytochemicals and the high content of phenolic compounds in the MEHHC contribute to its antioxidant and possibly also the antimicrobial activities. Antimicrobial activity was more evident against Gram positive bacteria (particularly *S. aureus*) than Gram negative bacteria. Potentially the MEHHC can be used for the treatment of infections by certain

Gram positive and the fungus *C. albicans*. Future work will involve characterisation of the phenolic compounds and the other phytochemical constituents of the *H. hemerocallidea* corm which have antioxidant and antimicrobial properties.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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