

Research Article

Antiuropathogenic and antioxidant activities of *Hypoxis hemerocallidea* Lam. extracts, and compounds from its taxonomically related species

Kokoette Bassey^{1*} and Sekelwa Cosa²

¹Pharmaceutical Sciences Unit, School of Pharmacy, Sefako Makgatho Health Sciences University, Ga-Rankuwa, South Africa

²Division of Microbiology, Department of Biochemistry Genetics and Microbiology, University of Pretoria, Pretoria, South Africa

More Information

*Address for Correspondence: Kokoette Bassey, Pharmaceutical Sciences Unit, School of Pharmacy, Sefako Makgatho Health Sciences University, P.O-Box, 60, Medunsa, Ga-Rankuwa, Pretoria, 0204, South Africa, Tel: +27792836751; Email: Edward.bassey@smu.ac.za

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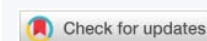
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ORCID: orcid.org/0000-0001-8178-1221

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Keywords: Antibacterial; Antioxidant; Galpinoside; Hemerocalloside; Uropathogen



Abstract

Hypoxis hemerocallidea Lam is one of the 43 *Hypoxis* species in South Africa, marketed extensively as over the counter herbal product for the management of several diseases. The plant commercial products link efficacy to hypoxoside 4, its aglycone (rooperol), or β -sitosterol. This study investigated antibacterial and anti-oxidant activities of four other molecules and two extracts from the *Hypoxis* plant. Visual antioxidant limit of detection and free radical scavenging activities of test samples were determined using 20 mM hydrogen peroxide, 0.4 mM DPPH or ferric reducing antioxidant power assay. Quantitative free radical was determined by spectrophotometric method while antibacterial activity was evaluated using MICs against uropathogens: *S. aureus* (ATCC25923), *S. marcescens* (ATCC 14041), *P. aeruginosa* (ATCC 9721), *P. mirabilis* (ATCC 33583) and *E. coli* (ATCC 10536).

Anti-oxidant activities visual limit of detection of 0.06 mg/mL and percentage free radical scavenging activity ($IC_{50} = 0.048 - 0.032$ mg/ml) for pure compounds 1 - 4 and ($IC_{50} = 0.037 - 0.039$ mg/ml) for extracts were obtained. The reducing power IC_{50} ranged between 0.15 - 0.23 mg/ml for extracts and 0.11-0.35 mg/ml for standards. Antibacterial potentials show a noteworthy to moderate MIC values of 0.20 - 1.56 mg/ml against *S. aureus* (ATCC 25923), *S. marcescens* (ATCC 14041), *P. aeruginosa* (ATCC 9721), *P. mirabilis* (ATCC 33583) and *E. coli* (ATCC 10536). Galpinoside 5, hemerocalloside 3 and curculigoside C 2 are the other active compounds in *Hypoxis* plant.

Introduction

Hypoxis hemerocallidea Lam. (*H. hemerocallidea*) of the Hypoxidaceae genus is a perennial herbal and medicinal plant that is widely distributed in sub-Saharan Africa, in particular the Southern part of Africa. Anecdotal evidence accentuates the infusions and decoctions of the plant in South African traditional health care system. Worthy of note is the use of the plant corm polar extracts in the management of disease such as urinary tract infections, common cold, flu, nausea [1], prostate hypertrophy [2]. In addition to its traditional uses, *in vitro* and *in vivo* studies have indicated the plant extracts to possess anti-oxidant and antidiabetic [3], anticonvulsant, antibacterial, antidiarrhoeal, immunomodulatory, uterolytic, bronchorelaxant, CD4 count reduction pharmacological

properties [4]. Several phytochemicals including but not limited to hypoxoside 4, dehydroxy hypoxoside, bis-dehydroxy hypoxoside [5], nyasoside, nyasicoside, mononyasine A, mononyasine B and nyaside [6], interjectin, geraniol 1, obtuside A, obtuside B [7], zeatin and its glycoside [8], have been isolated from the plant. Bassey and co-workers [9] reported seven other compounds including galpinoside 5, orcinol glycoside, geraniol glycoside 1 and curculigoside C 2 from taxonomically related *H. colchicifolia* and *H. galpinii*. However, the acclaimed medicinal properties of the polar extracts of the plant are linked to the prodrug hypoxoside 4, its aglycone-rooperol or β -sitosterol only. Hence, the present study investigated the antioxidant activities of galpinoside 5, hemerocalloside 3, curculigoside C 2, and geraniol glycoside 1, the other molecules present



in *H. hemerocallidea* or related species. The antibacterial activities of these molecules against selected uropathogens were also investigated. A number of bacterial pathogens associated with UTIs include *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Enterobacter cloacae*, and *Serratia marcescens* among many others. *E. coli* is the most common etiological agent of urinary tract infections (UTIs) in both hospital and community acquired infections. According to Bitew, et al. [10], uropathogens associated infections are implicated to greater than or equal to 6 million outpatient visits and 479,000 hospitalizations annually in the United States alone. Whereas the resultant treatment costs are estimated to be greater than 2.47 billion USD. Consequently, the UTIs are associated with the rise in antibiotic resistance in the hospital setting [11]. The profile of bacterial uropathogens have changed drastically over the years due to their evolving adaptive strategies contributing to antibiotic resistance [10,12,13]. For this reason, it becomes imperative to search and screen for more antimicrobial potentials against resistant pathogens. The present study thus explored the potential antibacterial activities of the *H. hemerocallidea* extracts and compounds 1 - 5 isolated from it or taxonomically related *H. colchicifolia* and *H. galpinii* against the mentioned bacterial uropathogens.

Materials and methods

Reagents and solvents

All solvents were of analytical (AR) grade. Methanol (MeOH), ethanol (EtOH), acetone, chloroform (CHCl₃), sulphuric acid, ascorbic acid, 2,2-dipicryl-diphenyl hydrazyl (DPPH), Amoxicillin, ciprofloxin, Luria Bertani (LB) soft agar or Mueller Hinton (MH) agar were purchased from either Rochelle chemicals and Lab equipment CC, South Hills or Sigma-Aldrich, Johannesburg South Africa.

Plant materials and standard compounds

Crude water and methanol extracts of *H. hemerocallidea* as well as five pure compounds hypoxoside 4, galpinoside 5, hemerocalloside 3, curculigoside C 2 and geraniol glycoside 1 with UPLC-MS purity of 95% were obtain from a previous study. Possible degradation of each standard compound was confirmed using TLC analysis of each compound developed in chloroform: methanol: water (CHCl₃: MeOH: H₂O 70:30:2 v/v/v). The retardation factor (R_f) value for each compound was compared with literature for the same compounds analyzed using the same mobile phase [9].

Qualitative free radical scavenging (Antioxidant) DPPH Dot blot staining assay

Visual limit of detection determination: The qualitative free radical scavenging activity of the test samples and controls were investigated using the method described by Mon and co-workers [14] with minor modifications. Using

a glass Pasture pipette, 5 drops of the water and methanol extracts of *H. hemerocallidea* as well as standard compounds (hypoxoside 4, galpinoside 5, hemerocalloside 3, curculigoside C 2 and geraniol glycoside 1) were separately spotted on a 5 x 5 cm silica gel pre-coated glass plates (Silica gel 60 F254, Merck, Germany) and dried using a creek of Table fan air. The modifications included the sample concentrations spotted as follows: 1.0, 0.5, 0.1, 0.09, 0.08, 0.07, 0.06, 0.05 and 0.01 mg/mL of extracts, standards and vitamin C (positive control). A 0.4 mM (1.57 g DPPH/10 mL MeOH) solution was prepared immediately and smeared on the Dot blot TLC plate using a cotton wool.

Quantitative determination of antioxidant activity (*in vitro* DPPH, Hydrogen peroxide free radical scavenging assay and Ferric reducing antioxidant power): The quantitative anti-oxidant activity (AOXA or %DPPH) were examine using spectrophotometry measurement of 1,1-diphenyl-dipicryl hydrazyl (DPPH) free radical scavenging assay. 1.0 mL of 1.0, 0.5, 0.1, 0.08, and 0.05 mg/mL of water, methanol extracts of *H. hemerocallidea*, and the 5 standards (1-5) were mix with 1 mL of 0.4 mM DPPH solution in methanol. The mixtures were allow to react at room temperature in the dark for 30 minutes. Blank solutions of 1.0 mL DPPH + 1.0 mL methanol were use as the negative control while L-ascorbic acid (Vitamin C) and 0.4 Mm of the DPPH was the positive control. The tests were carried in triplicate and mean values were recorded. Hydrogen peroxide free radical scavenging assay was done with the same protocol using 20 mM of 30% w/v H₂O₂ mixed with PBS (pH = 7.67). Two milliliters (2.0 ml) of the hydrogen peroxide mixed with 1.0 ml each of the serially diluted (1.0, 0.5, 0.1, 0.08, and 0.05 mg/mL) test samples solution were separately allowed to react in the dark before reading the absorbance. The negative control was H₂O₂-PBS solution with no test samples while vitamin C was the positive control. The decrease in absorbance was measured at 518 nm or 230 nm for DPPH and the H₂O₂ assays respectively using spectrophotometer (spectrophotometers Nanocolor® uv/vis, Macherey-Nagel GmbH & Co. KG, Germany). The ferric reducing power antioxidant (FRAP) assay was conducted using test extracts, standards and control. The working plant extracts, standards and control solutions were prepared by diluting the 1 mg/ml stock solution to 1.0, 0.5, 0.1, 0.08, and 0.05 mg/mL. A milliliter (1.0 ml) of the working solution of the plant extracts, standards and control was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.60) and 2.5 ml of a 1% (w/v) solution of potassium ferricyanide (K₃[Fe(CN)₆]). The mixture was vortexed, and then incubated in water bath at 50 °C for 20 min. Thereafter, 2.5 ml of a 10% (w/v) trichloroacetic acid was added, and then centrifuged at 3000 rpm for 10 min. A volume of 2.5 mL of the supernatant was transferred into a test tube and mixed with and 0.5 mL of a 0.1% (w/v) solution of ferric chloride solution. The mixture was well vortexed and the increasing absorbance measured



at 700 nm using the afore mentioned spectrophotometer. Values obtained were converted to percentage antioxidant activity (%DPPH) using the formula:

$$\% \text{ DPPH scavenging capacity} = (A_0 - A_s / A_0) \times 100 \quad (1)$$

Where A_0 indicates absorbance of the negative control 1.0 mL of DPPH solution + 1.0 ml of methanol, 2.0 ml of H_2O_2 PBS or 2.5 mL of the supernatant + 0.5 mL ferric chloride solutions and A_s represents the absorbance of the positive control - 1.0 ml of DPPH solution + 1.0 ml, 2.0 ml H_2O_2 -PBS solution or 2.5 mL supernatant + 0.5 mL of ferric chloride mixed with 1.0 ml of extracts, standards or vitamin C solution.

Dot Blot stain antimicrobial bio-autography: The Dot Blot stain for antimicrobial bio-autography was conducted as described by Mon and co-workers [14] with modifications. The 5 x 5 cm silica gel pre-coated glass plates (Silica gel 60 F254, Merck, Germany) was diagonally divided into two equal halves. Five glass Pasture pipette drops of 1.0 mg/mL of the water, methanol extracts or the standards compounds (hypoxoside 4, galpinoside 5, hemerocallside 3, curculigoside C 2 and geraniol glycoside 1) were spotted at the bottom corner of the plate. Ciprofloxacin and amoxicillin (0.1 mg/mL) positive control were spotted at the top corner of the TLC plate. The plates were dried using a creek of Table fan air. About 5 mL of the Luria Bertani (LB) soft agar or Mueller Hinton (MH) agar solution seeded with 0.5 mL of *S. aureus* (ATCC 25923), *S. marcescens* (ATCC 14041), *P. aeruginosa* (ATCC 9721), *P. mirabillis* (ATCC 33583) and *E. coli* (ATCC 10536) prepared overnight and standardized at 0.05 McFarland standard, poured on the prepared Dot blot TLC plate. The plates were incubated at 37 °C for 24 hours, thereafter zones of inhibition appearing as creamy white blots or clear on a red (*S. marcescens*) or creamy white (*S. aureus*, *P. mirabillis* and *E. coli*) or green-yellowish (*P. aeruginosa*) pigment backgrounds were observed.

Antibacterial minimum inhibition (MIC) by 96-well assay: The bacterial cultures of *S. aureus* (ATCC 25923), *S. marcescens* (ATCC 14041), *P. aeruginosa* (ATCC 9721), *P. mirabillis* (ATCC 33583) and *E. coli* (ATCC 10536) were grown for 18-24 hours at 37°C and thereafter adjusted to $OD_{600 \text{ nm}}$ 0.08-0.1. The 96-well polystyrene microtiter plate assay was used to determine the lowest concentration of extracts that inhibits bacterial growth [15]. The two-fold serial dilution microplate method was used to determine the MIC values. Briefly, aliquots (100 μ l) of 25 mg/mL crude extracts and 1 mg/mL isolated pure standard compounds were serially diluted with Mueller Hinton (MH) broth in each well. A 100- μ l aliquot of standardized bacterial suspension were added to each well. Mueller Hinton broth and 1% DMSO were use as negative and solvent control, respectively. Ciprofloxacin (0.01 mg/mL) and amoxicillin (0.01 mg/mL) were use as positive controls. *Para*-iodonitrotetrazolium (INT, 0.2 mg/ml) 40 μ L was added after 24 hours of incubation, thereafter incubated for further 30 min to 1 hour until optimal colour development was observed. Tests were carried out in triplicate. The MIC was recorded as the lowest concentration of the extract/isolated standard compound that inhibited bacterial growth as described by Pauw and Eloff [15].

Results

Standard compounds purification and identification

The stability of each standard compound used in this study was confirmed using simple TLC developed in $CHCl_3$:MeOH: H_2O (70:30 2 v/v/v). Single compact spot for each compound was obtained with Rf (0.76, 0.50, 0.47, 0.45, and 0.43 for geraniol glycoside 1, curculigoside C 2, hemerocallside 3, hypoxoside 4 and galpinoside 5, - Figure 1. The Rf values matched those previously reported using the same mobile phase [9].

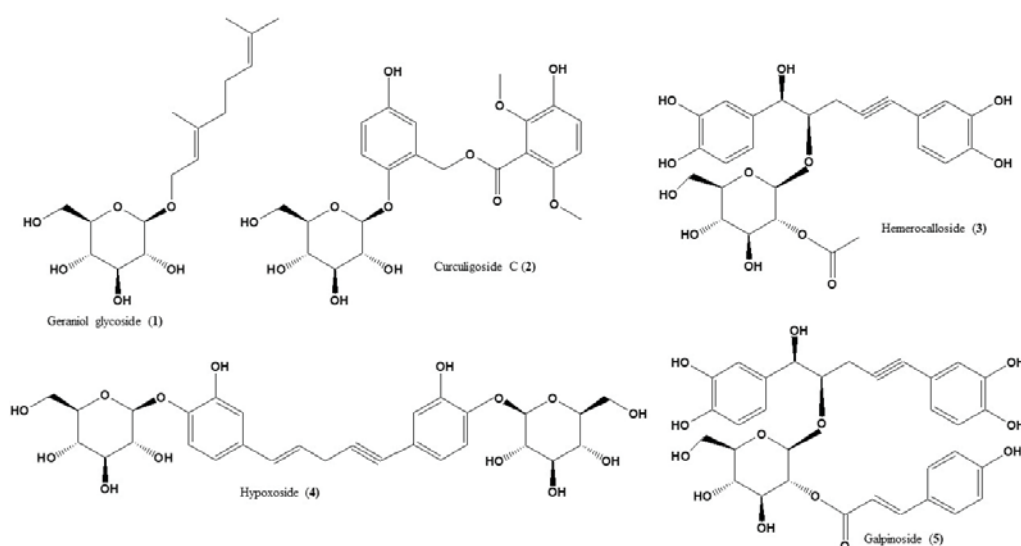


Figure 1: Structures of geraniol glycoside 1, curculigoside C 2, hemerocallside 3, hypoxoside 4 and galpinoside 5, standard compounds used in this study.

Visual limit of detection by qualitative free radical scavenging (Antioxidant) DPPH

Dot Blot staining assay: A dot-blot staining test was preferred over the classical TLC bio-autography to evaluate the qualitative anti-oxidant activity of the *Hypoxis* extracts, using standards (hypoxoside 4, galpinoside 5, hemerocalloside 3, curculigoside C 2, geraniol glycoside 1) and L-ascorbic acid (vitamin C) as a positive control. Result obtained (Figure 2 top) revealed that galpinoside 5 and hemerocalloside 3 exhibit free radical scavenging properties that is comparable to that of ascorbic acid judging by the white-yellow bleaching of the purple DPPH background.

Established by the thickness of the white-yellow coloration, the *Hypoxis hemerocallidea* methanol and water extracts also indicated relatively moderate anti-oxidant activity than hypoxoside 4, the commercial molecules from most *Hypoxis* species (Figure 2 top). The limit of detection, LOD of any analytical instrument or method is often determined by injecting very low concentration, (usually ≤ 1 mg/mL) of an analyte into the instrument and using area under the curve (AUC) to determine the lowest concentration that can be detected. This study, implemented this idea by spotting 1.0 - 0.001 mg/mL of *Hypoxis hemerocallidea* water and methanol extracts, standard galpinoside 5, hemerocalloside 3,

curculigoside C 2 and geraniol glycoside 1 (Figure 2 bottom) on a TLC glass plate to visualize their anti-oxidant activities limit of detection. Hypoxoside 4, indicated moderate radical scavenging activity with a visual limit of detection value of 0.1 mg/mL, a value equal to that of the *Hypoxis hemerocallidea* water extract (Figure 2 bottom). The anti-oxidant activity of galpinoside 5, hemerocalloside 3 standards and methanol extract has never been reported. Our findings revealed DPPH free radical scavenging visual LOD values of 0.06 mg/mL comparable to the positive control vitamin C. Whereas traces of curculigoside C 2 were observed with visual LOD at 1 mg/mL with the cream discoloration of the purple DPPH fading off in a matter of seconds, geraniol glycoside 1 was not visually detected in the concentration range studied.

Quantitative determination of antioxidant activity (in vitro DPPH and H₂O₂ free radical scavenging assay)

The *in vitro* quantitative anti-oxidant activity was determined by analyzing the same extracts, standards and positive control used for the qualitative DPPH visual LOD assay. This was important to further confirm the exact antioxidant activity of *H. hemerocallidea* methanol extract, galpinoside 5, hemerocalloside 3 and vitamin C with the same visual anti-oxidant LOD of 0.06 mg/mL.

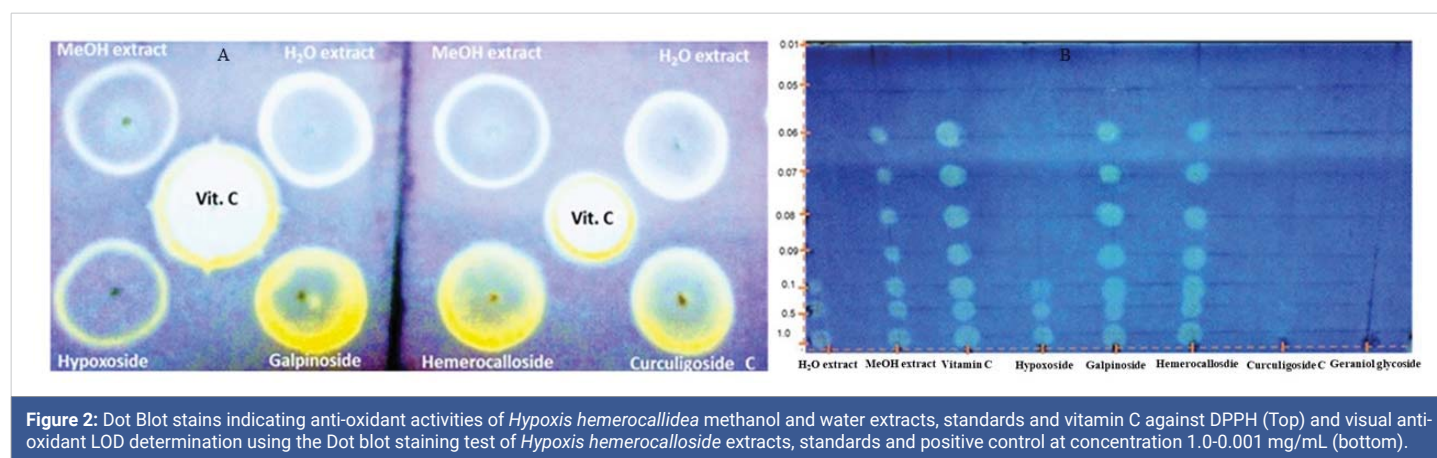


Figure 2: Dot Blot stains indicating anti-oxidant activities of *Hypoxis hemerocallidea* methanol and water extracts, standards and vitamin C against DPPH (Top) and visual anti-oxidant LOD determination using the Dot blot staining test of *Hypoxis hemerocalloside* extracts, standards and positive control at concentration 1.0-0.001 mg/mL (bottom).

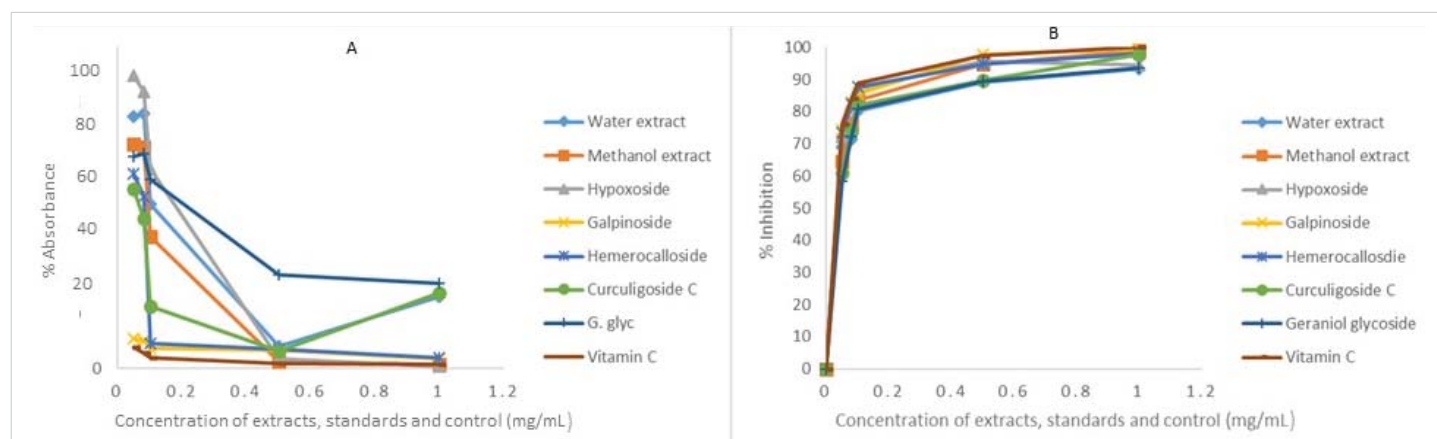


Figure 3: DPPH absorbance-concentration function (A) percentage inhibition of DPPH at 518 nm of *H. hemerocallidea* extracts (water, methanol), standards and vitamin C. Each value is expressed as Mean \pm standard deviation ($n = 3$)

The same reason also necessitated the *in vitro* anti-oxidant activity evaluation of *H. hemerocallidea* water extract and hypoxoside 4 that exhibited identical visual anti-oxidant LOD of 0.1 mg/mL. Figure 3 depicts the absorbance versus concentration function for the *Hypoxis* extracts, standards and vitamin C. The reduction in the absorbance of DPPH (Figure 3A) and the H_2O_2 (Figure 4A) is an indication of the degree to which the extract and standard compounds can inhibit the DPPH and H_2O_2 radical generation activity [14]. This is associated with the free radical scavenging activities of tested samples. Conversely, the increasing absorbance for the FRAP assay (Figure 5A) further validated the antioxidant potentials of test samples. The results from the three antioxidant assays concurs with results obtained for the visual LOD of the qualitative anti-oxidant activity obtained using the Dot Blot assay as galpinoside 5, displays a free radical scavenging power that is comparable with ascorbic acid.

The percentage free radical scavenging activity of the test *H. hemerocallidea* extracts, standards and vitamin C were evaluated to further validate the degree to which the extract and standard compounds can inhibit the DPPH and H_2O_2 radical generation activity as well as reduce Fe^{3+} to Fe^{2+} . As

indicated (Figure 3B-5B and Table 1), galpinoside 5, exhibited potent anti-oxidant activity with $IC_{50} = 0.030$ (DPPH), 0.04 (H_2O_2) mg/mL with a reducing power ($IC_{50} = 0.17$) comparable to that of vitamin C, with $IC_{50} = 0.028$ (DPPH), 0.038 (H_2O_2) (0.11) mg/mL respectively. This was followed by hemerocalloside 3, and methanol extracts with average $IC_{50} = 0.041$ mg/mL and $IC_{50} = 0.056$ mg/mL respectively for both free radical test methods. Whereas curculigoside C 2 has DPPH $IC_{50} = 0.045$ mg/mL and hypoxoside 4 ($IC_{50} = 0.037$) one-to-one, geraniol glycoside 1, exhibited the lowest percentage antioxidant activity with ($IC_{50} = 0.048$ mg/mL).

Dot blot stain antimicrobial bio-autography

The Dot Blot staining assay (Figure 6) revealed that only the water extract indicated positive change for inhibition of *S. marcescens* as represented by the zone of inhibition in the area of water extract. However, both methanol and water extracts of *H. hemerocallidea* showed inhibitory activity against *S. aureus*, *S. marcescens* and *P. aeruginosa*. From the standards compounds, only curculigoside C 2, inhibited *E. coli* with an MIC of 3.13 mg/mL. Hypoxoside 4, galpinoside 5, hemerocalloside 3 and geraniol glycoside 1, exhibited no antimicrobial activities.

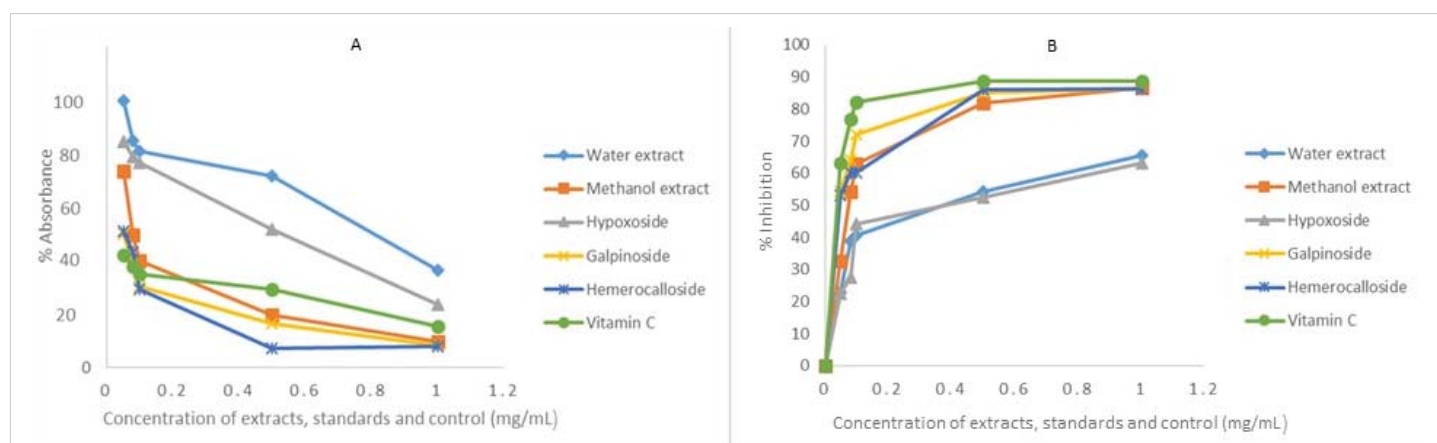


Figure 4: H_2O_2 absorbance-concentration function (A) percentage inhibition of H_2O_2 at 230 nm of *H. hemerocallidea* extracts (water, methanol), standards and vitamin C. Each value is expressed as Mean \pm standard deviation ($n = 3$).

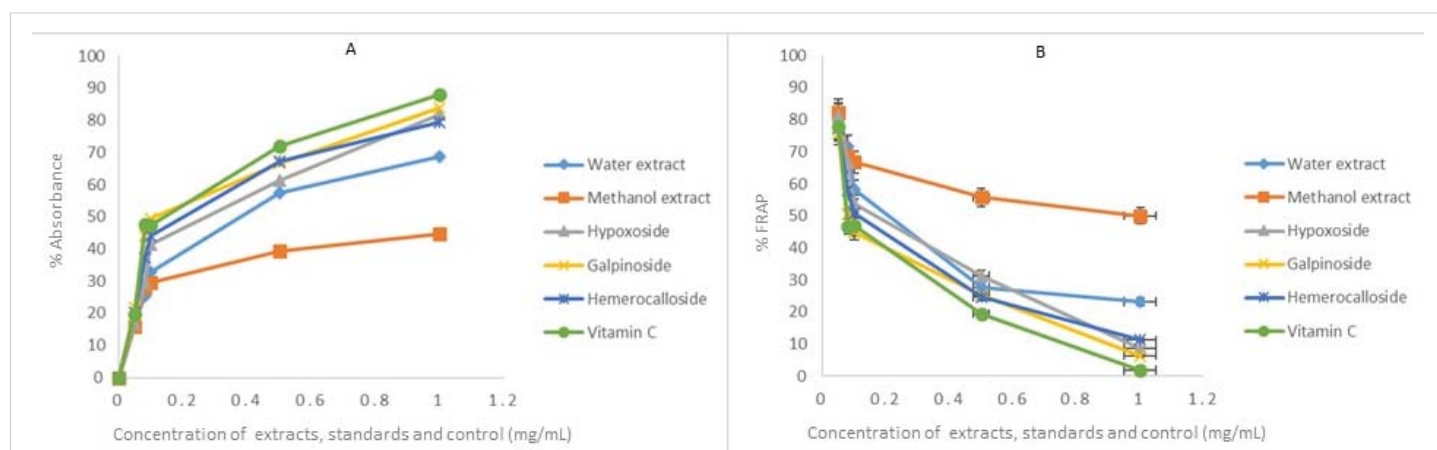


Figure 5: Fe^{3+} to Fe^{2+} reducing power of test *H. hemerocallidea* extracts, standards and vitamin C. Each value is expressed as Mean \pm standard deviation ($n = 3$).

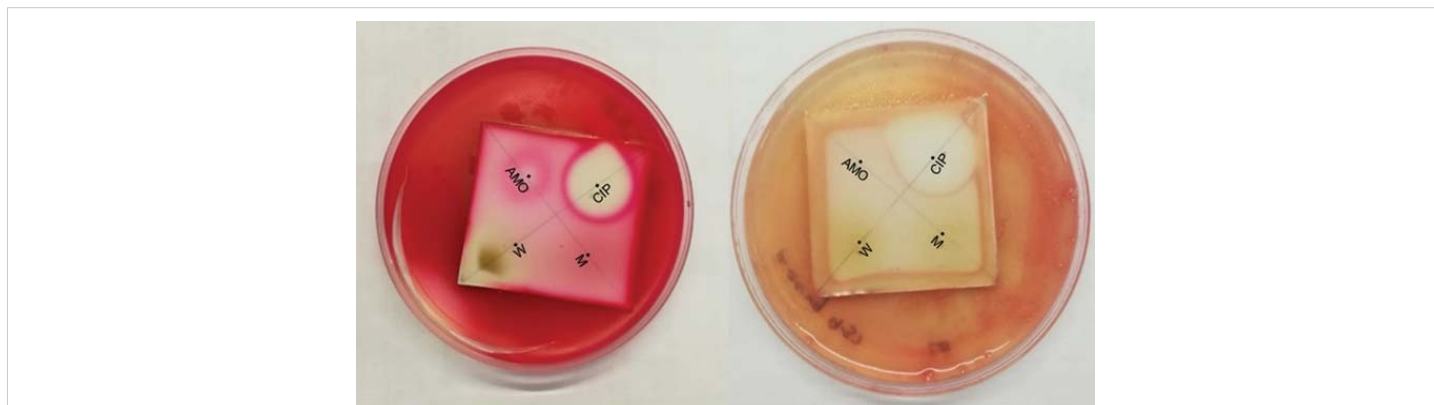


Figure 6: Selected anti-microbial Dot Blot staining bioautography. Left: *S. marcescens* and right: *P. aeruginosa*. CIP (ciprofloxacin 0.01 mg/ml), AMO (amoxicillin 0.01 mg/ml), W (water extract of *H. hemerocallidea*) and M (methanol extract of *H. hemerocallidea*).

Table 1: Calculated IC_{50} indicating the percentage free radical activity of *Hypoxis* water, Methanol extracts, standards galpinoside 5, hemerocalloside 3, curculigoside C 2, geraniol glycoside 1 and vitamin C positive control.

Analyte	Free radical scavenging Assay		
	DPPH IC_{50} (mg/mL)	H_2O_2 IC_{50} (mg/mL)	FRAP IC_{50} (mg/mL)
<i>H. hemerocallidea</i> water extract	0.037 ± 0.10	0.08 ± 1.29	0.23 ± 2.58
<i>H. hemerocallidea</i> methanol extract	0.039 ± 1.40	0.073 ± 2.04	0.15 ± 1.25
Hypoxoside (4)	0.037 ± 0.97	0.09 ± 1.40	0.35 ± 2.87
Galpinoside (5)	0.030 ± 1.40	0.04 ± 1.30	0.17 ± 2.62
Hemerocalloside (3)	0.032 ± 0.98	0.05 ± 1.44	0.20 ± 2.64
Curculigoside C (2)	0.045 ± 1.42	Nd	Nd
Geraniol glycoside (1)	0.48 ± 1.41	Nd	Nd
Vitamin C	0.028 ± 0.97	0.038 ± 1.08	0.11 ± 2.91

Nd: Not determined due to limited test standards.

Minimum Inhibitory Concentrations (MICs)

The MIC values for test samples (*Hypoxis* water, methanol extracts, standards galpinoside 5, hemerocalloside 3, curculigoside C 2, geraniol glycoside 1) that exhibited activities against the test pathogens (*P. aeruginosa*, *S. aureus*, *S. marcescens*, *P. mirabilis* and *E. coli*) are presented in table 2. From these findings, the *H. hemerocallidea* water extract showed potent and noteworthy activities of ≤ 1 mg/mL [17] against all test pathogens except for *P. aeruginosa*. *H. hemerocallidea* water extract (MIC value of 0.78 mg/mL) inhibited *S. aureus* and *S. marcescens*, while 0.39 mg/mL inhibited *P. mirabilis* and the best MIC of 0.20 mg/mL against *E. coli* was also observed. The MIC value of 1.56 mg/mL of

the same extract at least inhibited *P. aeruginosa*. In contrast, the MIC values for methanol extract ranged between 1.56 and 3.13 mg/mL were not as “noteworthy” because the extracts exhibit MIC > 1 mg/mL [17]. The methanol extract (MIC = 1.56 mg/mL) was noted against *S. aureus*, while *S. marcescens*, *P. aeruginosa*, *P. mirabilis* and *E. coli* were inhibited at MIC = 3.13 mg/mL. All the isolated test pure compounds inhibited the test pathogens by a concentration of 0.25 mg/mL except for curculigoside C 2, which exhibited MIC = 0.13 mg/mL against *E. coli*. This observation further confirms that *E. coli* was better susceptible to extracts and compounds from *H. hemerocallidea*. When compared to the ciprofloxacin and amoxicillin (MIC range of 0.003 - 0.25 mg/mL and 0.01 - 0.25 mg/mL respectively); *H. hemerocallidea*

Table 2: Minimum inhibitory concentrations (mg/mL) for *Hypoxis hemerocallidea* water, methanol extracts pure isolated compounds and positive control against selected uropathogens.

Test samples	MIC (mg/mL)				
	<i>S. aureus</i>	<i>S. marcescens</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>	<i>E. coli</i>
Plant extracts					
Water	0.78	0.78	1.56	0.39	0.20
Methanol	1.56	3.13	3.13	3.13	3.13
Compounds					
Galpinoside	0.25	0.25	0.25	0.25	0.25
Hemerocalloside	0.25	0.25	0.25	0.25	0.25
Hypoxoside	0.25	0.25	0.25	0.25	0.25
Curculigoside C	0.25	0.25	0.25	0.25	0.13
Controls					
Ciprofloxacin	0.003	0.003	0.003	0.003	0.25
Amoxicillin	0.01	0.25	0.25	0.25	0.25
1% DMSO	6.25	6.25	6.25	6.25	6.25



water extract (MIC 0.20 mg/mL against *E. coli*), curculigoside C 2, (MIC range of 0.13 - 0.25 mg/mL) were recorded. Similar results were observed for galpinoside 5, hemerocalloside 3, and hypoxoside 4 (all three with MIC of 0.25 mg/mL). These results suggest ≤ 0.25 mg/mL as the threshold MIC value for the *Hypoxis* plant polar extracts, isolated standards *vis-a-vis* the commercial antibiotics like ciprofloxacin and amoxicillin.

Discussion

The isolated standards compounds were identified using literature references as hypoxoside 4, galpinoside 5, hemerocalloside 3 [9], curculigoside C 2 and geraniol glycoside 1 [16]. Hypoxoside 4 is a pentyne derived natural pro-drug present in every *Hypoxis* species. However, hypoxoside 4, could be hydrolyzed to rooperol by the action of glucosidase enzyme. Although the compound hypoxoside 4 is now commonly isolated in *Hypoxis hemerocallidea* as its major compound, it was initially isolated from other *Hypoxis* species including *H. obtusa*. Hypoxoside 5 is available on the market as vital medicinal compound [18]. Galpinoside 5, and others were previously isolated from South African *Hypoxis* species, adding to the list of secondary metabolites from the genus. Although these compounds were identified, no evaluation of their antibacterial and antioxidant activities were conducted.

This study hereby identifies galpinoside 5, hemerocalloside 3 and curculigoside C 2 as polar phenolic compounds isolated from polar extracts of *Hypoxis* plant as the compounds that possess the anti-oxidant activity. The good antioxidant activity (LOD of 0.01 mg/mL) of galpinoside 5, and hemerocalloside 3, standard compounds whose anti-oxidant activity has never been reported alongside the methanol extract of the plant, is comparable to the positive control vitamin C. Whereas curculigoside C 2, has a moderate visual antioxidant activity at a concentration of 1 mg/mL and geraniol glycoside 1, had none. This result mirrors that reported by Mannathoko and co-workers [19] for similar antioxidant activity of 0.05 mg/mL for methanol extract of *H. hemerocallidea* corm and vitamin C (ascorbic acid). The mechanism of the anti-oxidant activity of galpinoside 5, and hemerocalloside 3, could be *via* the diacatechol-type (rooperol or nordihydroguaiaretic acid) pathway previously reported by other groups [20 - 21]. Most compounds with good antioxidant activities usually have 3,4-dihydroxy groups that is responsible for the reduction of the free radicals from the DPPH or H_2O_2 solution. The same reason is also responsible for the relatively poor antioxidant activity of curculigoside C 2, observed at 1 mg/mL with a 3-hydroxy group in its structure and the zero activity of geraniol glycoside 1, being a consequence of the lack of phenyl and hydroxyl group in its structure. The synergistic presence of galpinoside 5, hemerocalloside 3 and curculigoside C 2, with catechol-type or rooperol-type structure in the methanolic extract of *H. hemerocallidea* should be responsible for its

relatively better anti-oxidant activity over the water extract and hypoxoside 4. The two sugar units in hypoxoside 4, would reduce the anti-oxidant activity. Work is currently underway in our laboratory to compare the activity of the aglycones of the active antioxidant compounds to that of rooperol, an aglycone with better antioxidant than the parent hypoxoside 4, prodrug.

When quantitative determination of antioxidant activity (*in vitro* DPPH, H_2O_2 free radical scavenging assay and ferric reducing power) and percentage antioxidant activities were performed, results (Figure 3B - 5B) underscores 0.5 mg/mL as the optimal concentration for best free radical scavenging properties for the methanol extract, galpinoside 5, hemerocalloside 3 and vitamin C on the one hand and water extract and hypoxoside 4 on the other. This is due to the absorbance of the DPPH, H_2O_2 , and ferric chloride reducing power at this concentration is lowest. This would imply that commercial products and *muti* that contain *Hypoxis* extracts and pure antioxidant compounds like galpinoside 5 and hemerocalloside 3 must maintain this optimal concentration for optimal anti-oxidant activity *ceteris Paribus*. The methanol extract, galpinoside 5 and hemerocalloside 3 has a quantitative free radical scavenging activity that is comparable to that of the positive control vitamin C. However, galpinoside 5 would be the compound with the best comparable anti-oxidant activity to vitamin C. The other analytes and vitamin C has linear absorbance after this concentration to indicate a possible similarity in their free radical scavenging potential compared to vitamin C to agree with results previously published [19-20]. The results obtained for the percentage free radical scavenging properties were 87.98% ($IC_{50} = 0.030$ mg/mL) for galpinoside 5, compared with 89.93% ($IC_{50} = 0.028$ mg/mL) of vitamin C and 87.20% ($IC_{50} = 0.037$ mg/mL) for hemerocalloside 3, supports those previously reported [19, 22] which highlighted that polar extracts of the fresh leaf and corms has high anti-oxidant activity associated with highly polar constituents. This inference is justified from the perspective of galpinoside 5 and hemerocalloside 3 being highly polar compounds isolated from *Hypoxis* plant with solubility in methanol and water. As mentioned by Katerere and Ellof [22], polar phenolic compounds are responsible for the anti-oxidant activity of *Hypoxis hemerocallidea* extract. From Figure 2 (bottom), the moderate antioxidant activity of hypoxoside 4 and the *H. hemerocallidea* water extract with a visual limit of detection value of 0.1 mg/mL is due to compounds lacking dicatechol-type structure.

The poor to moderate antioxidant activity of the water extract and hypoxoside 4, were complimented by the noteworthy MIC of 0.39 and 0.20 mg/mL against *P. mirabilis* and *E. coli* respectively, and ≤ 0.25 mg/mL for hypoxoside 4 and water extract. Bacterial growth inhibition of *E. coli* by *H. hemerocallidea* water extract was marginally greater than the activity of the commercial ciprofloxacin with an MIC value of 0.25 mg/mL. This could be a function of improved synergy



between the compounds in the water extract as compared to the methanol extract. Although the extraction solvent polarity decreases, the inhibition of the organisms by the polar extract of South African *H. hemerocallidea* increases. As example, the acetone < ethanol demonstrate MIC 2.5 mg/mL > 0.31 mg/mL [23] and MeOH < H₂O with 3.13 mg/mL > 0.20 mg/mL (present study). Matotoka and Masoko [23] also documented *H. hemerocallidea* acetone extract with 2.5 mg/mL, 0.63 mg/mL, and 0.03 mg/mL MIC values against *P. aeruginosa*, *S. aureus* and *E. faecalis*, respectively.

Galpinoside 5, hemerocalloside 3 and hypoxoside 4 with MIC value of 0.25 mg/mL for all the test pathogens performed equally as ciprofloxacin against *E. coli* and amoxicillin against *S. marcescens*, *P. aeruginosa*, *P. mirabilis* and *E. coli* (MIC = 0.25 mg/mL). Overall, curculigoside C 2, exhibited the prominent noteworthy antibacterial property against *E. coli* (MIC = 0.13 mg/mL). This activity was significantly improved than ciprofloxacin and amoxicillin (MIC = 0.25 mg/mL). The rationalization for potent results obtained with curculigoside C against all the test pathogens and the possible mechanism of action includes the structure of curculigoside C; where the presence of hydroxyl groups OHs at C-3, C-3' positions and the two vicinal oxygen-bearing groups at the benzene ring [24] when compared to ciprofloxacin and amoxicillin would attest to this. This concentration (0.25 mg/mL) is clinically important for manufacturers and users of herbal medicines formulated from *H. hemerocallidea* polar extract and standards compounds isolated from the plant extracts. The superfluity of health benefits available for consumers of *H. hemerocallidea* products in particular and other *Hypoxis* species in general should not be merely associated to hypoxoside 4, (or rooperol) and β -sitosterol. Justifiably, additional compounds like galpinoside 5, hemerocalloside 3 and curculigoside C 2 present in the polar extract particularly possess the biological activities for which *Hypoxis* plant is indicated for in the present study.

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