African Journal of Biotechnology Vol. 7 (11), pp. 1624-1629, 3 June, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Quantitative and qualitative analysis of sterols/sterolins and hypoxoside contents of three *Hypoxis* (African potato) spp.

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Accepted 25 April, 2008

The glycoside, hypoxoside, identified and isolated from the corms of the African potato (*Hypoxis hemerocallidea*) has shown promising anticancer activities. The African potato is used as an African traditional medicine for its nutritional and medicinal properties. Most research has been carried out on *H. hemerocallidea* (formerly known as *H. rooperi*), with very little or nothing on other *Hypoxis* spp. Thin layer chromatography (TLC) was used to confirm the presence of sterols/sterolins, whereas a GC method was developed to identify and quantify sterols (especially β-sitosterol) in chloroform extracts of *H. hemerocallidea, H. stellipilis* and *H. sobolifera* var. *sobolifera*. High performance liquid chromatography (HPLC) was used to identify and quantify hypoxoside content in these *Hypoxis* spp. TLC results showed that *H. sobolifera* var. *sobolifera* contained the most sterols and sterolins compared to the other two *Hypoxis* spp. Gas chromatography (GC) results show that β-sitosterol and campesterol were the two main phytosterols present in the *Hypoxis* extracts. *H. sobolifera* var. *sobolifera* and *H. hemerocallidea* contained the most β-sitosterol and hypoxoside, respectively. *H. sobolifera* and *H. hemerocallidea* contained 74.69 µg of β-sitosterol and 12.27 µg of hypoxoside per 5 mg of chloroform extracts, respectively. These results show a significant difference in the sterol/sterolin and hypoxoside contents between species of the genus *Hypoxis*, which may influence their degree of biological activities.

Key words: *Hypoxis*, TLC, GC, HPLC, sterol(in)s, hypoxoside.

INTRODUCTION

Hypoxis hemerocallidea Fisch., C.A. Mey. and Avé-Lall., syn. *H. rooperi,* (commonly known as the African potato; belonging to the family *Hypoxidaceae*) topped the list of the 60 most frequently traded plant species in the Eastern Cape, South Africa, when studies were conducted among street traders, traditional healers, store-owners and clinic patients (Dold and Cocks, 2002).

Glycosides, isolated from *Hypoxis* species, have a common pent-1-en-4-yne backbone or a slight modification of it (Sibanda et al., 1990; Messana et al., 1989; Marini-Bettolo et al., 1985; Nicoletti et al., 1992 and Marini-Bettolo et al., 1991). The glycoside, hypoxoside ((E)-1, 5-bis (4'-β-D-glucopyranosyloxy-3'-hydroxyphenyl) pent-4-en-1-yne) (Marini-Bettolo et al., 1982 and Drewes et al., 1984) has shown promising anticancer activities. *In vitro* conversion, catalyzed by β-glucosidase, of non-toxic hypoxoside to cytotoxic rooperol (Drewes and Liebenberg, 1987) has shown growth inhibition of 60 human cancer cell lines tested including breast, colon, uterus, melanoma and non-small cell lung cancer cell lines (Albrecht et al., 1995 and Smit et al., 1995).

Sterols are amphiphilic molecules consisting of hydroxyl groups forming the hydrophilic heads and sterane skeletons with side chains forming the hydrophobic tails (Heldt, 2005). Cholesterol ($C_{27}H_{45}OH$) is the main sterol found in mammals where it plays an important role in the structure and function of cell membranes, production of bile, as precursor of hormones and a role in the immune

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system (De Brabander et al., 2007). Sterols found in plants are known as phytosterols and over 250 phytosterols and their related compounds have been identified (De Brabander et al., 2007) in foods like plant oils, nuts, seeds, cereals, fruits and vegetables (Piironen et al., 2000; Ostlund, 2002). Phytosterols differ from cholesterol in being alkylated at C-24 with C_1 or C_2 substituents (Buchanan et al., 2000). In nature, plants contain sterols with their associated sterolins (glucosides), which are easily destroyed by glycosidic enzymes (Pegel, 1976).

Phytosterols cannot be synthesized by humans and are thus consumed from the diet. The most commonly found phytosterols are sitosterol (C_{29}), campesterol (C_{28}) and stigmasterol (C_{29}) (Pegel, 1980; Ostlund, 2002). Phytosterols are incorporated in a variety of food products (functional foods (Vorster et al., 2003)) due to their cholesterol-lowering effect, hence providing protection against cardiovascular disease (Tapiero et al., 2003). Studies with phytosterols, especially β-sitosterol, have shown inhibition of several cancer cell lines including colon (Raicht et al., 1980; Choi et al., 2003 and Awad et al., 1998), prostate (von Holtz et al., 1998) and breast (Steenkamp and Gouws, 2006; Ju et al., 2004; Awad et al., 2003 and Awad et al., 2001). The role of plant sterols as immune modulators (Bouic et al., 2001; Bouic and Lamprecht, 1999; Bouic 2002 and Breytenbach et al., 2001) and anti-inflammatory agents (Quilez et al., 2003; Pegel, 1979) has also been described.

Some of the compounds (hypoxoside, β-sitosterol and other sterols/sterolins) that may be responsible for the medicinal properties of *Hypoxis* have been identified in *H. hemerocallidea* but the quantity of these in different *Hypoxis* spp. is unknown. Various species of *Hypoxis* are sold and used indiscriminately without any evidence that they contain equal quantities of sterols/sterolins and hypoxoside. The main aims of this study were to identify and quantify the sterol/sterolin and hypoxoside contents of *H. hemerocallidea*, *H. stellipilis* Ker Gawl. and *H. sobolifera* var *sobolifera* (Jacq.) Nel.

MATERIALS AND METHODS

Reagents and chemicals

Sterol standards (β-sitosterols [purity > 90%], campesterol [purity > 65%], cholesterol [purity > 99%], desmosterol [purity ≥ 85%], ergosterol [purity ≥ 95%], fucosterol [purity ~ 95%], stigmasterol [purity ~ 95%] and stigmastenol [purity > 95%]) were purchased from Sigma Chemical Co. (MO, USA). HPLC grade acetonitrile and methanol were purchased from Romil Ltd. (Cambridge, UK). Water was obtained from a Milli-Q Compact System (Millipore, Bedford, MA).

Plant material

Corms of *H. hemerocallidea* (PEU 14798) and *H. stellipilis* (PEU 14841) were purchased in Port St Johns and Port Elizabeth (Xhosa traditional medicine shop), respectively, in the Eastern Cape, South Africa. Corms of *H. sobolifera* var *sobolifera* (PEU 14840) were collected near Plettenberg Bay in the Southern Cape, South Africa. Corms of the three *Hypoxis* spp. were planted in the same soil type and exposed to equal amounts of sunlight, humidity and water for at least six months before they were harvested and used fresh. The plants were identified by Y. Singh from the South African National Biodiversity Institute (SANBI) and voucher specimens were deposited in the Nelson Mandela Metropolitan University herbarium.

Isolation of hypoxoside and sterols/sterolins

Corms of *H. hemerocallidea*, *H. stellipilis* and *H. sabolifera* were washed, peeled, grated and crushed using a mortar and pestle. Chloroform was added to the plant material in a 1:1 (v:w) ratio, vortexed (5 min), extracted (15 min) and centrifuged (3645 x g for 5 min) at room temperature. The supernatant was removed and the extracting method repeated with the same plant material. The chloroform was evaporated *in vacuo* and mass of extracts determined. After mass determination the extracts were redissolved in chloroform until further use.

TLC

β-Sitosterol (2 µg) and stigmasterol (2 µg) standards (Supelco, USA) and *Hypoxis* extracts (500 µg) dissolved in chloroform were spotted onto 20 x 20 cm silica coated aluminum plates (Merck, Germany) and air dried. Chromatogram tanks were equilibrated for one hour using toluene – diethyl ether (40:40, v/v) and chloroform – ethyl acetate – formic acid (5:4:1, v/v/v) as mobile phases for sterol and sterolin identification, respectively. TLC plates were developed for ±20 min or until the solvent front was ±1 cm from the top of the plate. Detection of sterols/sterolins was performed as described by Scott and Springfield (2004). In brief, TLC plates were dried at room temperature and developed by firstly dipping into a solution containing 5% sulfuric acid in 96% ethanol for 15 s followed by a solution containing 1% vanillin in 96% ethanol for 15 s and dried at room temperature. Once dried, plates were heated at 80 – 100°C for five min. Photos of the developed TLC plates were taken with the AlphaImager™ 3400 (Alpha Innotech.).

GC

GC analysis of sterols was performed using a Thermo Finnigan Focus gas chromatograph equipped with a FID and an Autoinjector AI3000, with Delta Chromatography 5.0 software. The column used for GC separation was a SAC™-5 capillary column (Supelco, 30 m × 0.25 mm i.d. × 0.25 µm film thickness). The thermal conditions were: 80°C for 2 min; 10°C.min^{-1} to 300°C; 300°C for 14 min. The carrier gas was He (1 ml.min^{-1} constant flow) and the injection volume was 2 µl (splitless). A sterol mixture (containing 100 µg/ml of each sterol standard) was spiked individually to identify the peaks. An increase in peak area was used as criteria to identify each peak.

HPLC

High performance liquid chromatography (HPLC) analysis of hypoxoside was performed using a Beckman System Gold high performance liquid chromatograph equipped with Solvent Module 128, Diode Array Detector Module 169. The column used for HPLC separation was a Nucleosil C_{18} column (Supelco, 5 µm, 150 × 4.6 mm i.d.). Detection of hypoxoside was performed as described by Nair and Kanfer (2006). In brief, acetonitrile – water (20:80, v/v) was used as mobile phase in isocratic mode at a flow rate of 1 ml/min and the injection volume was 10 µl. Detection was achieved in the

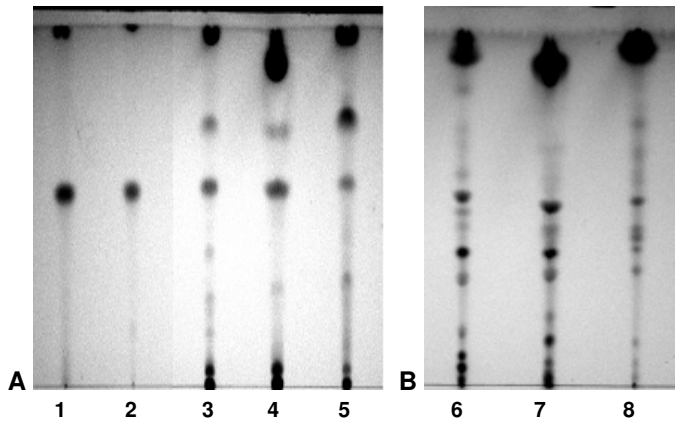

Figure 1. TLC plate of the (A) sterols and (B) sterolins found in chloroform *Hypoxis* spp. extracts (1) β-sitosterol, (2) stigmasterol, (3/6) *H. hemerocallidea*, (4/7) *H. sobolifera* and (5/8) *H. stellipilis*.

range of 200 - 400 nm and hypoxoside was detected at a wavelength of 260 nm. Stock solutions of hypoxoside (1 mg/ml), *H. hemerocallidea* (5 mg/ml), *H. stellipilis* (10 mg/ml) and *H. sobolifera* (10 mg/ml) were prepared in methanol and filtered through 0.2 µm syringe filters (Corning Incorporated, New York, USA).

RESULTS AND DISCUSSION

Chloroform has been shown to be very effective in dissolving sterols (Toivo et al., 2000) due to its non-polar nature. The presence of sterols and sterolins in the chloroform extracts of *H. hemerocallidea*, *H. stellipilis* and *H. sobolifera* var *sobolifera* was confirmed via TLC. Modification of the mobile phase, toluene – diethyl ether – 1.75 M acetic acid (1:1:1, v/v/v) used by Scott and Springfield (2004), to toluene – diethyl ether (40:40, v/v) resulted in better sterol separation (Figure 1A). The spots of the sterol standards (stigmasterol and β-sitosterol) had the same Rf value of 0.53. Cholesterol, campesterol, desmosterol, ergosterol, fucosterol and stigmastenol migrated the same distance on the TLC plates (data not shown), with the spots differing only in colour ranging from pink to blue. This made it impossible to identify and quantify individual sterols in the *Hypoxis* extracts, using TLC.

The mobile phase used for sterolin identification consisted of chloroform – ethyl acetate – formic acid (5:4:1, v/v/v). The sterolins in the *Hypoxis* extracts could not be identified or quantified due to the unavailability of sterolin standards (Figure 1B). From the results obtained, different sterolins (based on Rf values) were detected and differences in sterolin composition could clearly be seen between the three *Hypoxis* species (Figure 1).

GC is the technique of choice to analyze the presence of sterols in food (Cunha et al., 2006; Contarini et al., 2002; Toivo et al., 2000; Lagarda et al., 2006 and Goudjil et al., 2003) due to shorter analysis times, less peak interference, improved resolution, greater detection sensitivity (low nanogram range) and thermal stability of the capillary columns (Abidi, 2001). The SAC-5^{TM} capillary column, consisting of 95% dimethylpolysiloxane and 5% phenyl, is specially packed for the analysis of plant and animal sterols. The sterol/stanol standard mixture (100 µg of each sterol/mL), consisting of β-sitosterol (30.6 min), campesterol (29.3 min), cholesterol (27.8 min), desmosterol/ergosterol (28.9 min), fucosterol (28.3 min), stigmasterol (29.7 min) and stigmastenol (30.9 min) was well separated, except for desmosterol and ergosterol, which eluted at the same retention time, with good resolution on the SAC-5^{TM} column within a period of 32 min (Figure 2).

From GC analysis, it was clear that β-sitosterol was the main phytosterol found in the chloroform extracts of *H. hemerocallidea*, *H. stellipilis* and *H. sobolifera*. Trace amounts (<10 µg per 5 mg of *Hypoxis* extract) of campesterol were also found in all three *Hypoxis* spp. extracts, whereas trace amounts of desmosterol/ ergosterol, stigmasterol, stigmastenol were found only in certain *Hypoxis* spp. A standard curve of β-sitosterol concentration (ranging between 10 - 100 µg/mL) as a function of peak height (R^2 = 0.9531, R_T = 30.6 min) was used to quantify β-sitosterol content. *H. sobolifera* contained the most β-sitosterol with ±2.5 and 7.5 times more β-sitosterol compared to *H. hemerocallidea* and *H. stellipilis*, respectively (Table 1).

The presence of β-sitosterol and campesterol as the two major phytosterols in *Hypoxis* correspond to published data (Moghadasian, 2000; Pegel, 1976). According to a minireview by Moghadasian (2000), 95% of dietary phytosterols consist of sitosterol and campesterol (approximately 65 and 30%, respectively), whereas the other phytosterols (mainly stigmasterol) and stanols make up the other 5%.

This is the first time that GC was used to identify and quantify the presence of sterols in *Hypoxis* extracts. Nair et al. (2006) have used high performance liquid chromatography to determine the presence of β-sitosterol, stigmasterol and stigmastenol in commercially available oral dosage forms reported to contain material or extracts of *Hypoxis*. Using the SAC-5^{TM} capillary column eliminated time-consuming preparation steps, for example extraction of lipid fraction from sample material, saponification (alkaline hydrolysis), extraction of non-saponifiables and derivatization of the sterol standards and *Hypoxis* extracts (Toivo et al., 2000).

The presence of hypoxoside has been identified in several South African species of *Hypoxis* (Nicoletti et al., 1992) including *H. hemerocallidea* (Nair and Kanfer, 2006), but not in *H. stellipilis* or *H. sobolifera*. The HPLC method described by Nair and Kanfer (2006) was used to quantify hypoxoside content in the three *Hypoxis spp* (Figure 3). Hypoxoside was detected after 12.5 minutes and quantified from a standard curve of hypoxoside concentration (ranging between 5 - 100 µg/mL) as a function of peak area (R^2 = 0.9971, R_T = 30.6).

Of the three *Hypoxis* species tested for hypoxoside

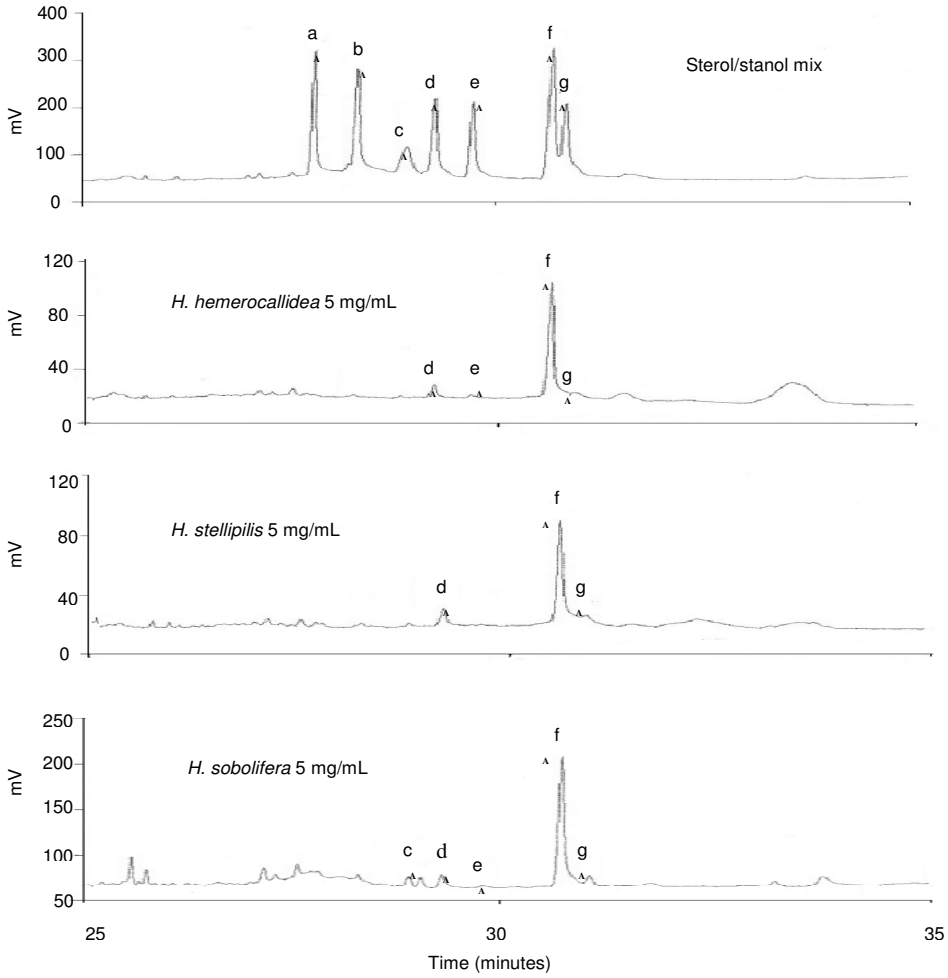

Figure 2. GC chromatograms of standards and chloroform *Hypoxis* extracts: (a) cholesterol, (b) fucosterol, (c) desmosterol/ergosterol, (d) campsterol, (e) stigmasterol, (f) β-sitosterol and (g) stigmastenol.

Table 1. Content and total percentage of β-sitosterol per 5 mg of chloroform *Hypoxis* extracts.

***Hypoxis* spp.**	**Content (µg)**	**Yield (%)***
H. hemerocallidea	29.38	0.59
H. stellipilis	10.05	0.2
H. sobolifera	74.69	1.49

*w/w

Table 2. Content and total percentage of hypoxoside per 5 mg of chloroform *Hypoxis* extracts

Hypoxis spp.	**Content (µg)**	**Yield (%)***
H. hemerocallidea	12.27	0.12
H.stellipilis	7.93	0.08
H. sobolifera	Undetectable	-

* w/w

content, only *H. hemerocallidea* and *H. stellipilis* contained hypoxoside. *H. sobolifera*, which showed the highest anticancer activity (unpublished data), had undetectable levels of hypoxoside (Table 2).

Since chloroform, the solvent used in this study, is not the best solvent for hypoxoside extraction, the presence of this glycoside in *H. sobolifera* was investigated using more polar solvents. A water extract of *H. sobolifera* has shown no hypoxoside content, whereas ethanol, methanol and acetone extracts of *H. sobolifera* yielded 60.66, 49.13 and 60.35 µg per 5 mg of extract, respectively. Hypoxoside is therefore present in *H. sobolifera* but in much smaller amounts than in *H. hemerocallidea* and *H. stellipilis*. Previous studies have used 30 - 75% ethanol (Pegel, 1979) and methanol (Nair and Kanfer, 2006) to extract hypoxoside from *H. hemerocallidea*. Traditional healers/herbalists use water and boiling to make *Hypoxis* extracts, which may yield hypoxoside and sterolins

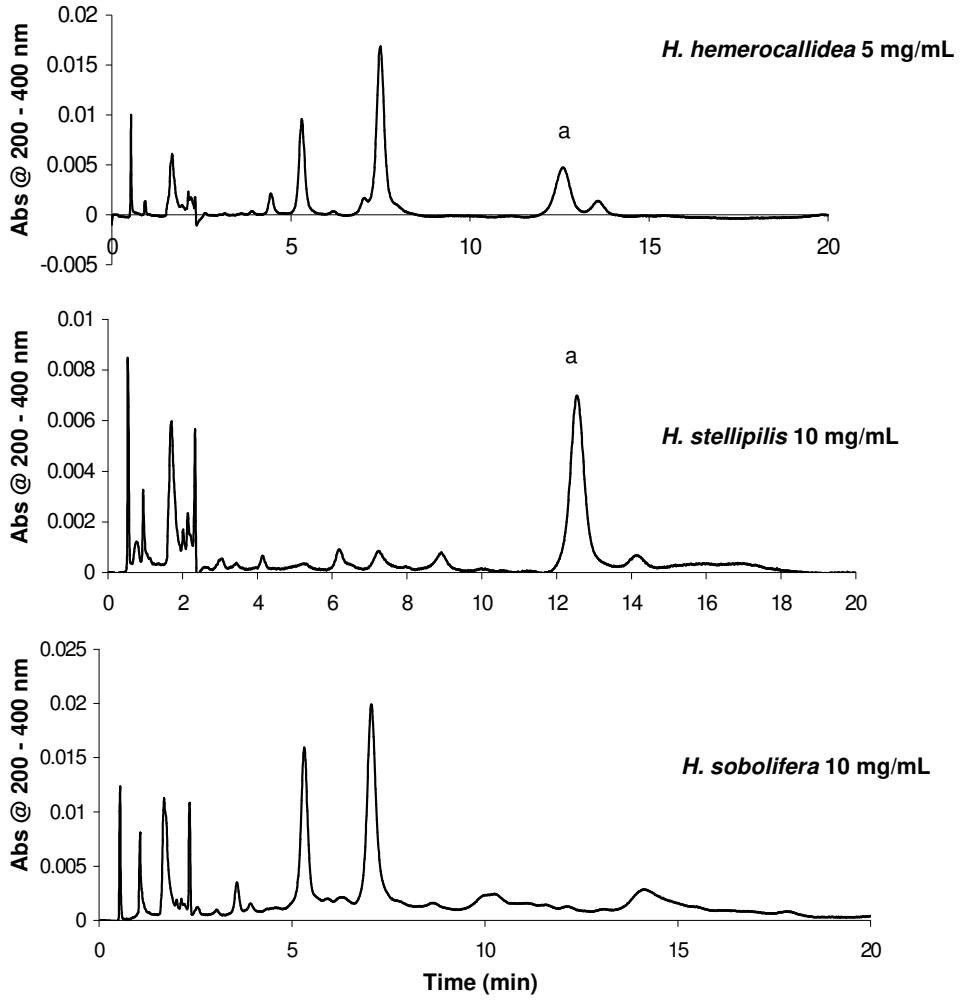

Figure 3. HPLC chromatograms of the hypoxoside content of chloroform *Hypoxis* extracts: (a) hypoxoside detected at 260 nm.

(Pegel, 1976). Due to the polar nature of hypoxoside it would be better to use more polar solvents for extractions in the future, if more emphasis is placed on the effect of hypoxoside in the *Hypoxis* extracts.

This is the first time that hypoxoside and sterol contents were quantified in *H. stellipilis* and *H. sobolifera*, and as far as we know in *H. hemerocallidea*. Both the sterol and hypoxoside contents were shown to vary between the three species. Differences in the hypoxoside and sterol/sterolin contents of the three *Hypoxis* spp. investigated may explain the differences in anticancer activity (unpublished data) obtained against certain cancer cell lines. *Hypoxis* species are used indiscriminately in traditional medicine and are sold under the common name 'African potato' in herbal shops. Further investigation is required to determine the implications of these findings in the uses of *Hypoxis* as a traditional remedy. Consumption of the three different *Hypoxis* species, which have different sterols/sterolins and hypoxoside content, may have adverse or favorable effects depending on the concentration of extract consumed. Sterols/sterolins and hypoxoside may have synergistic effects, which need to be investigated.

ACKNOWLEDGMENTS

The authors want to thank the NRF (GUN 2069228) and the MRC for funding this study, and Dr. C. Albrecht for donating purified hypoxoside.

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