Isolation and Quantification of β-sitosterol, ergosterol and stigmasterol from *Hypoxis rigidula* Baker var. *rigidula* and *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall (Hypoxidaceae)

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Abstract

Hypoxis rigidula Baker var. *rigidula* and *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall (Hypoxidaceae) are frequently used African medicinal plant that has been used by traditional healers for daily healthcare needs. Phytosterols isolated from *Hypoxis spp*, have been found to be effective in lowering plasma cholesterol concentration by inhibiting the absorption of cholesterol in the small intestine. Phytosterols serve to stabilize phospholipid bilayers in plant cell membranes just as cholesterol does in animal cell membranes. The aim of this study was to isolate phytosterols from *Hypoxis rigidula* and evaluate its cholesterol reduction activity. The objectives were to extract phytosterols from *Hypoxis* using distillation; identify phytosterols using thin layer chromatography (TLC) and quantify phytosterols present in the plant extracts using high performance liquid chromatography (HPLC-UV). TLC gave the following phytosterol standard values: β-sitosterol at Rf 0.63±0, ergosterol at Rf 0.66±0 and stigmasterol at Rf 0.68±0. The chloroform extracts of both *H. hemerocallidea* and *H. rigidula* the presence of β-sitosterol, ergosterol and stigmasterol. HPLC analysis gave the following concentrations of phytosterols; 48.4 and 35.2 µg/ml of stigmasterol were found in *H. rigidula* and *H. hemerocallidea* respectively and 86.7 and 48.4 µg/ml of ergosterol were found in *H. rigidula* and *H. hemerocallidea* respectively. These results show a significant difference in the phytosterol content between the two species of the genus *Hypoxis.*

Keywords: *Hypoxis rigidula*, *Hypoxis hemerocallidea,* phytosterols, secondary metabolites, cholesterol, .

Introduction

Despite the vast technology advancement in medicine, about 80% of the population in Africa still rely on traditional healers & medicinal plants for their daily healthcare needs. Many plants are used as remedies and as ingredients to prepare herbal medicines for many human disorders. *Hypoxis* (Figure 1) is a frequently used African medicinal plant (Ojewole, 2006). Extracts of the corm have been ingested by man for a diversity of ailments (Nair and Kanfer, 2008). There has been an increased interest in the use of phytosterols, their glycosides (sterolins) and steryl esters for reducing cholesterol and increasing immunity and for this reason there are many herbal formulations containing sterols, most of which have been fortified with additional amounts of free sterols, stanols and sterolins (Nair and Kanfer, 2008). The glycoside, hypoxoside, has shown anti-cancer properties (Boukes *et al*., 2008).

Phytosterols are typical constituents of plant cell walls (Marangoni and Poli, 2010), they are members of the family 'terpene' which includes more than 100 different phytosterols and more than 4000 other types of triterpenes plant membranes contain several types of phytosterols that are similar in structure to cholesterol but a methyl or ethyl group at C-24. They have the same basic function as cholesterol does in animals and that is in the structure and function of cell membranes (Nair *et al*., 2006; Moreau *et al*., 2002)

β-sitosterol and stigmasterol are the two major phytosterols generally present in plant cell membranes (Figure 2). They are similar in structure but β-sitosterol has been shown to be a membrane reinforce while stigmasterol is not (De-Eknamkul and Potduang, 2003). β-sitosterol is the most abundant phytosterol (Nair and Kanfer, 2008) and has been used in controlling cholesterol plasma levels (Bouic, 1996). It has been shown to exhibit anti-inflammatory, anti-neoplastic, anti-pyretic and immunomodulating activity (Careri *et al*., 2001).

β-sitosterol is understood to decrease plasma cholesterol concentrations extrinsically by displacing cholesterol from bile salt micelles, increasing bile salt excretion by competitively blocking cholesterol absorption from the intestinal lumen, or hindering the cholesterol esterification rate in the intestinal mucosa. Additional intrinsic actions of phytosterols may include modification of hepatic acetyl-CoA carboxylase and cholesterol 7-a hydroxylase enzyme activities in animals and humans (Jones et al., 1999). Phytosterols have been added to margarines as functional foods with the ability to reduce both total cholesterol and LDL cholesterol levels (Weber *et al*., 2002). Phytosterols have been recognized as cancer

preventative biological-active substances together with other secondary plant products such as carotenoids, flavonoids and phytoestrogens (Careri *et al*., 2001).

This study focuses on the isolation and quantification of phytosterols with possible cholesterol reducing properties from *Hypoxis rigidula and Hypoxis hemerocallidea*.

Materials and Methods

Collection of plant material

Plant material of the *Hypoxis rigidula* and *Hypoxis hemerocallidea* plant species were collected in Durban, Province of Kwazulu Natal, South Africa, and identified by Professor H. Baijnath, a botanist of the University of Kwazulu Natal (Westville).

Isolation of phytosterols

Corms of *Hypoxis rigidula* and *H. hemerocallidea* were washed, peeled and chopped. Chloroform was added to the plant material in a 1:1 (v: w) ratio, placed in a shaker at 150rpm, 37 ˚C overnight. The supernatant was removed and the extracting method repeated with the same plant material until the supernatant was clear. The chloroform was evaporated in vacuo and mass of extracts determined. After mass determination the extracts were re-dissolved in chloroform until further use (Boukes *et al*., 2008)

Thin Layer Chromatography

Ergosterol, β-sitosterol and stigmasterol standards and *Hypoxis* extracts were dissolved in chloroform and spotted onto 10 x 10 cm silica coated aluminum TLC plate with toluene – diethyl ether (40:40, v/v) as mobile phase for sterol identification. The TLC plate was developed for ±10 minutes, dried at room temperature and developed by dipping into a solution containing vanillin (15 g), ethanol (250 ml) and conc. sulfuric acid (2.5 ml) for 15 s and dried at room temperature. Once dried, plate was heated at 100˚C for five min (Boukes *et al.*, 2008)

HPLC-UV

HPLC analysis was performed using D-7000 HPLC System Manager equipped with UV-Vis detector. Chromatographic separation was carried out using a C_8 narrow-bore column (150×2.1 mm, 5 µm) under isocratic conditions. The mobile phase was a mixture of methanol; acetonitrile; isopropyl alcohol (40:40;20, v/v/v) at the flow-rate of 1 ml/min. The operative wavelength was set at 216 nm. Injection volume, 1 µl (Phillips and Blythe, 2003)

Results

Identification by Thin layer chromatography

The presence of phytosterols in the chloroform extracts of *Hypoxis spp* was confirmed by TLC. The mobile phase was toluene diethyl ether [40:40:]. This mobile phase is a modification of the mobile phase, toluene – diethyl ether – 1.75 M acetic acid (1:1:1, v/v/v), which was modified because it resulted in better phytosterol separation (Boukes et al., 2008).

Quantitative analysis and identification by High Performance Liquid Chromatography

HPLC was used to separate, identify and quantify phytosterols in the *Hypoxis* spp extracts because it seems appropriate for the analysis of thermally unstable compounds such as phytosterols. Compared to Gas Chromatography (GC), HPLC offers an advantage of working under milder column temperatures and under non destructive detection conditions (Lagarda *et al*., 2006).

Figure 4 shows the two phytosterols which were identified in *H. hemerocallidea*, with reference to their retention times we were able to deduce which compound eluted at which time. Ergosterol eluted at 4.13 minutes while Stigmaterol eluted at 4.92 minutes. The chromatogram (Fig. 5) shows the two phytosterols which were identified in *H. rigidula*, with reference to their retention times we were able to deduce which compound eluted at which time. Ergosterol eluted at 4.20 minutes while Stigmaterol eluted at 4.92 minutes.

Table 2 shows the concentration of the phytosterols present in the Hypoxis species extracts as calculated from the standard curves of the two phytosterol standards.

Discussion

TLC is a separation technique which is adequate for sample clean up, purification, qualitative assays and preliminary phytosterol estimation studies. Chloroform was used as a solvent due to its non polar nature and has been shown to be very efficient in dissolving extracts (Boukes *et al*., 2008). The presence of phytosterols in the hypoxis extracts were was confirmed by TLC. The two extracts *H. hemerocallidea* and *H. rigidula* had the same Rf value as β-sitosterol, this confirms the presence of this phytosterol in the extracts, however it does not mean that this is the only phytosterol present in these two *Hypoxis* spp extracts. Figure 3 depicts that phytosterols are very similar in structure and because of these similarities; β-sitosterol was the only phytosterol observed in the extracts and also it may be difficult for TLC to separate them efficiently. In addition to the similarity in structures, the molecular weight of phytosterols is very close together with β-sitosterol having a molecular weight of 414.72 g/mol which is higher than that of ergosterol (396.65g/mol) and stigmasterol (412.69 g/mol). With these results we can conclude that B-sitosterol is also more polar phytosterol which is the reason it was identifiable in the extracts more so than the other phytosterols.

HPLC was the technique used to separate, quantify and identify the phytosterols present. A standard curve of the phytosterols concentration; ergosterol and stigmastrol, was used to quantify the content of each of the phytosterols in the extracts. Standard curves of ergosterol concentration (ranging between 10 – 500 µg/ml) as a function of peak height (R^2 = 0.998, R_T= 4.15) and stigmasterol concentration (ranging between 10 – 600 µg/ml) as a function of peak height (R^2 = 0.996, R_T= 5.10) were used to quantify ergosterol and stigmasterol content. *H. rigidula* contained more Ergosterol and stigmasterol than *H. hemerocallidea* and these results are shown on table 2.

It was found that *H. rigidula* had a higher concentration of ergosterol and stigmasterol than the more commonly used species *H. hemerocallidea*. An investigation by Boukes *et al* (2008) of three hypoxis species found that another hypoxis speci, *H. sobolifera*, had a higher concentration of phytosterols than *H. hemerocallidea.*

Since this investigation and the one by Boukes *et al* (2008) has found other hypoxis that contain higher concentrations of phytosterols than the more commonly used *H. hemerocallidea*; whose survival is threatened because of extensive use by locals, the use of it should be limited to ensure the species survival

From this investigation we can conclude that as a good source of phytosterols, *H. rigidula* may possess cholesterol reducing properties which are better than *H. hemerocallidea.* Further investigation may be conducted on the cholesterol reducing properties of phytosterols found in Hypoxis species.

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Figure 1 A: Family Hypoxidaceae. B: Hypoxis Corm. Published on the Internet http://ww2.bgbm.org/herbarium/ (Barcode: B 10 0003343 / ImageId: 206455) [accessed 07-Apr-10]

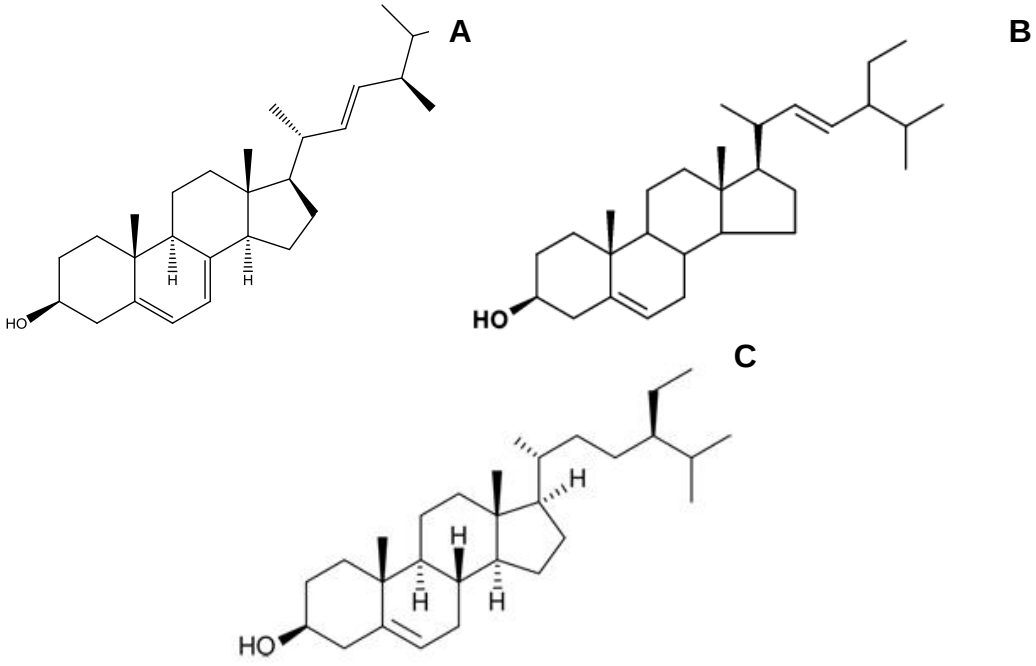

Figure 2 Structure of phytosterols A – Ergosterol, B- Stigmasterol and C- β –Sitoterol

Figure 3 TLC plate of the phytosterols found in chloroform extracts of Hypoxis spp. Lane 1 - *H. rigidula*, Lane 2 - *H. hemerocallidea* (corm), Lane 3 - β-Sitosterol (standard), Lane 4 - Stigmasterol (standard), Lane 5 - Ergosterol (standard) and Lane 6 - *H. hemerocallidea* (leaves)

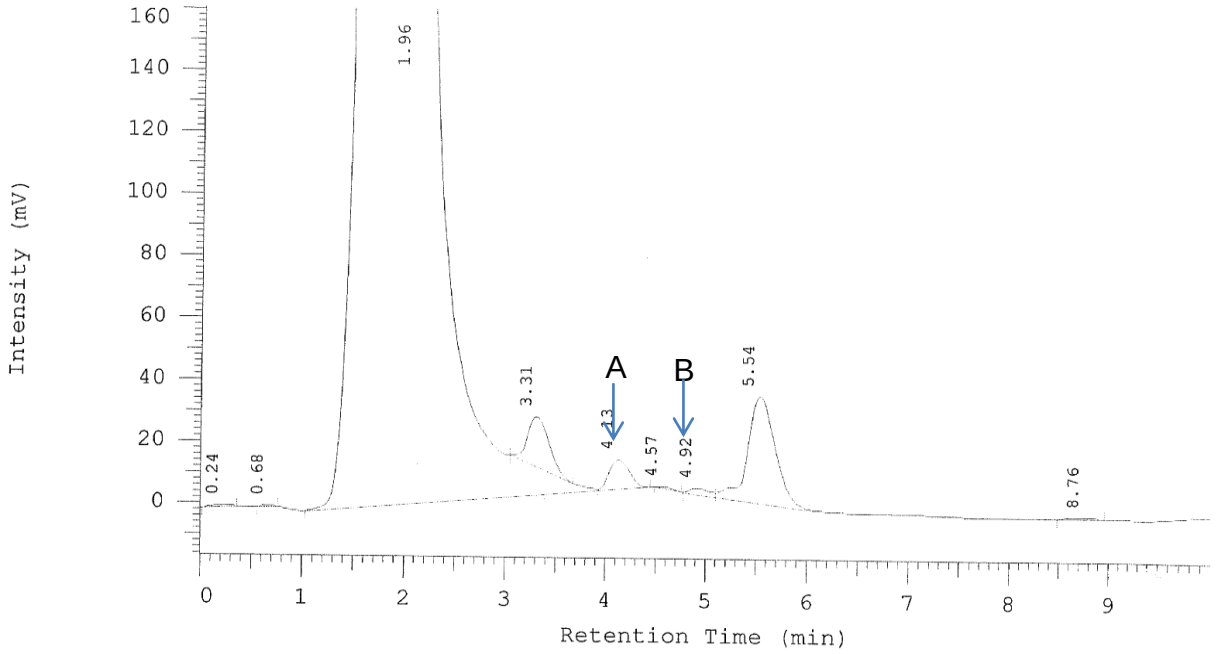

Figure 4 HPLC chromatogram of *H. hemerocallidea.* R_T A- 4.13 (ergosterol), R_T B- 4.92 (stigmasterol),

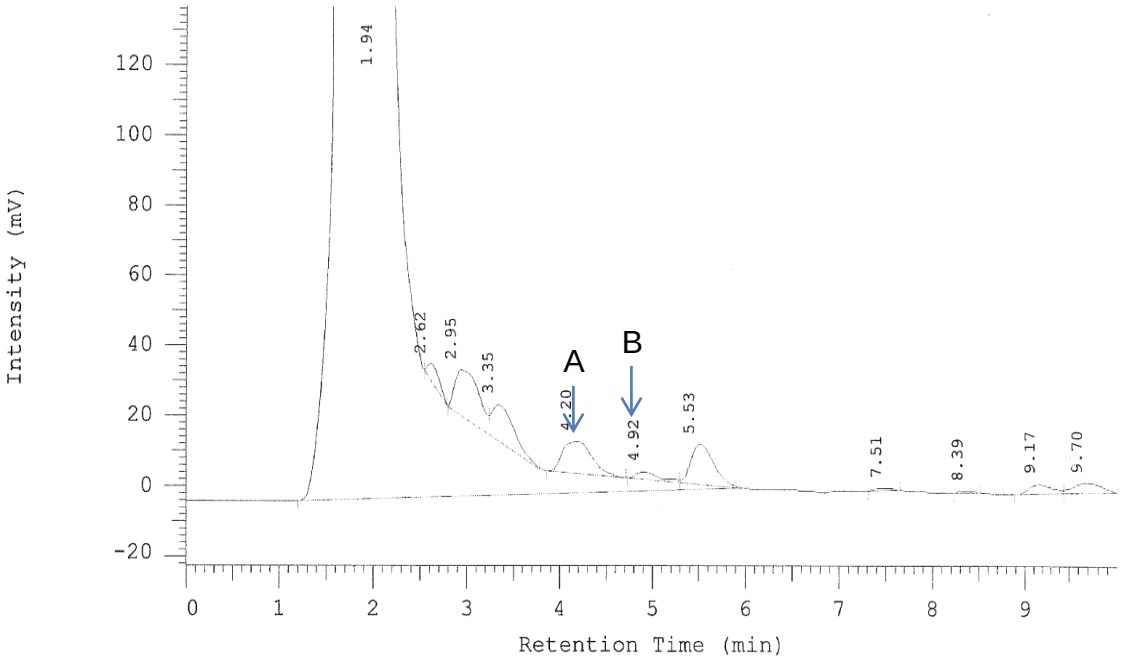

Figure 5 HPLC chromatogram of *H. rigidula.* R_T A- 4.20 (ergosterol), R_T B- 4.92 (stigmasterol),

Table 1 Resolution factor (Rf) values of phytosterol standards

Standards	**Rf value(x ± sd)**	
β- sitosterol	0.63±0	
ergosterol	0.66±0	
stigmasterol	0.68±0	
H. rigidula		β- sitosterol
H. hemerocallidea (corm)		β- sitosterol
H. hemerocallidea (leaves)		stigmasterol

Table 2: Concentration of phytosterols in the Hypoxis spp. extracts

Plant	Stigmasterol	Ergosterol
H. rigidula	48.4 µg/ml	86.7 µg/ml
H. hemerocallidea	35.2 µg/ml	48.4 µg/ml