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# New phytochemicals from the corms of medicinally important South African *Hypoxis* species☆

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ABSTRACT

*Hypoxis* species (Hypoxidaceae) are amongst the most widely used medicinal plants in southern Africa. Although the phytochemistry of *Hypoxis hemerocallidea* has been extensively investigated, little is known regarding the secondary metabolites of the other indigenous species, including *Hypoxis colchicifolia* and *Hypoxis galpinii*. Two new phenolic glycosides, 3-hydroxy-4-*O*-β-D-glucopyranosyl-benzaldehyde and 1,5-*bis*(3,4-dihydroxyphenyl)-1,2-dihydroxy-4-pentyne-2-*p*-coumaroyl-β-D-glucopyranoside were isolated from corms of *H. colchicifolia* and *H. galpinii*, respectively. The norlignan glycosides (hypoxoside, dehydroxy hypoxoside and *bis*-dehydroxy hypoxoside) were isolated from *H. colchicifolia* for the first time, using high performance countercurrent chromatography and elucidated by 1D and 2D NMR as well as high resolution MS. In addition, geraniol glycoside and β-sitosterol, previously described in Hypoxidaceae, were isolated from the same species. The presence of hypoxoside in all three species investigated indicated that the interchangeable use of *Hypoxis* species by communities has some merit. Establishing the biological properties of the new constituents will provide more insight into the medicinal value of the genus.



## 1. Introduction

The genus *Hypoxis* represents approximately 90 species and is the largest in the family Hypoxidaceae R.Br. (Singh, 2007, 2009). Although the latest checklist of the South African Biodiversity Institute (Germishuizen et al., 2006) records more than 40 species of *Hypoxis* as indigenous to South Africa, Singh (2009) revised these to only 29 distinct species. She indicated that *Hypoxis colchicifolia* Baker is synonymous with *Hypoxis latifolia, Hypoxis gilgiana, Hypoxis oligotricha* and *Hypoxis distachya*, and incorporated *Hypoxis rooperi, Hypoxis elata, Hypoxis patula* and *Hypoxis obconica* into *Hypoxis hemerocallidea* Fisch. & CAMey. Although *H. hemerocallidea* is the most sought after *Hypoxis* species, *H. colchicifolia* is also widely marketed throughout South Africa (Dold and Cocks, 2002). Traditionally, the corms of *H. colchicifolia* are used as a diuretic and for the treatment of psychiatric disturbances. It is also use to kill small vermin and is administered to cattle to cure gall sickness and redwater (bovine babesiosis) (Hutchings, 1996). The plant has cultural significance within the Zulu community and is used to prepare infusions as a cure for barrenness and impotency (Gertsner, 1939; Bryant, 1996). Extracts of *H. hemerocallidea* are used to treat a variety of diseases and disorders, including prostate hypertrophy (Bandeira et al., 2001), urinary and venereal disorders (Louw, 2002), cancer (Koduru et al., 2007) and diabetes (Erasto et al., 2005). Extracts are applied topically to relieve skin disorders such as rashes and wounds (Grierson and Afolayan, 1999) and are used for HIV/AIDS management (Manfredi and Chiodo, 1999). Infusions are taken to treat dizziness, bladder disorders and insanity (Hutchings, 1996). Not only are several of the species, including *Hypoxis rigidula, H. hemerocallidea, Hypoxis obtusa, Hypoxis galpinii, H. colchicifolia* and *Hypoxis acuminata*, morphologically alike (Singh, 2009), but they are all used in traditional medicine and reportedly contain similar chemical constituents (Boukes et al., 2008).

*Hypoxis* species are known to produce a variety of phytoglycosides, including several norlignan glycosides (Marini-Bettolo et al., 1982; Drewes et al., 1984; Galeffi et al., 1989; Sibanda et al., 1991) and phenolic glycosides (Cheng et al., 2009; Matsuo et al., 2011). Hypoxoside, a norlignan diglycoside, is the best known of these

☆ This paper forms part of a special issue of Phytochemistry Letters dedicated to the memory of Andrew Marston (1953–2013), outstanding phytochemist who is much missed by his friends.

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metabolites, since its aglycone, rooperol, is believed to play an important role in the medicinal properties of *Hypoxis* (Albrecht, 1996). A number of sterols, including β-sitosterol, and sterolins have also been detected in *Hypoxis* species (Boukes et al., 2008). However, it is well established that combinations of bioactive compounds, rather than single components, provide an important concurrent effect on multiple pharmacological targets (Pallares et al., 2012). Although it is known that *H. colchicifolia* and *H. galpinii* Baker (synonym *Hypoxis stricta*) produce hypoxoside (Drewes and Liebenberg, 1987), there is a lack of data regarding other secondary metabolites produced by these species. For this reason, structurally and functionally diverse compounds, produced *via* different metabolic pathways in the corms, should be investigated to establish whether *H. colchicifolia* and *H. galpinii* can be substituted for *H. hemerocallidea* in the market place. Many of the *Hypoxis* species are referred to by the same common name and are used interchangeably as traditional medicine by rural communities in South Africa. It is therefore of interest to determine if the chemical constituents of the species overlap.

In this investigation, secondary metabolites present in corms of *H. colchicifolia* and *H. galpinii* were isolated using silica gel column chromatography and high performance countercurrent chromatography (HPCCC). Silica gel separations are plagued by low yields resulting from irreversible adsorption of compounds to the stationary phase and extensive tailing (Yang and Ito, 2005). Although currently largely obsolete, countercurrent distribution (CCD) techniques were originally designed to eliminate losses associated with stationary phase effects (Akerlund, 1984). Marini-Bettolo et al. (1982) isolated 100 mg of hypoxoside from *H. obtusa* using CCD. However, the development of high performance countercurrent chromatography (HPCCC) led to more efficient separations, particularly for polar and water-soluble compounds. This technique involves liquid–liquid equilibrium between immiscible solvents, aided by centrifugation (Hatti-Kaul, 2000). The compounds isolated from the two *Hypoxis* species were identified using nuclear magnetic resonance (NMR) spectroscopy and ultra-performance liquid chromatography–mass spectrometry (UPLC-MS). These compounds will be used as reference standards to establish chemotypical variations within the genus and to develop methods for quality control purposes.

## 2. Results and discussion

### 2.1. Identification of isolated compounds

Seven compounds (six from *H. colchicifolia* and one from *H. galpinii*), including two new compounds, were isolated.

β-Sitosterol (**1**) from the $CHCl_3$ extract of *H. colchicifolia.* The spectroscopic data of **1** are in agreement with data reported by Kamboj and Saluja (2011) for β-sitosterol. This sterol has been identified in extracts of *H. hemerocallidea, Hypoxis stellipidis* and *Hypoxis sobolifera* (Boukes et al., 2008). The immune-boosting and cholesterol lowering properties of sterols, such as **1** and their use as functional food supplements are well documented (Bouic, 2001).

Geraniol glycoside (**2**) from *H. colchicifolia* (Table 1). An $R_f$ value of 0.71 was obtained by TLC using $CHCl_3$–MeOH–$H_2O$ (70:30:2) as developing solvent. Compound **2** was previously reported from *Hypoxis acuminata* by Bredenkamp et al. (1989).

3-Hydroxy-4-*O*-β-D-glucopyranosylbenzaldehyde (orcinal glycoside) (**3**) from *H. colchicifolia* (Fig. 1; Table 1).

Chemical shifts obtained from the $^1H$ and $^{13}C$ NMR spectrum of **3** indicated the presence of an aldehyde proton ($\delta$ 9.80), three aromatic protons ($\delta$ 7.21, 7.39 and 7.28) and one anomeric carbon at $\delta$ 101.2 ppm. The $^{13}C$ NMR of **3** resonated at $\delta$ 192.1 (C=O), 151.2 (*ortho*-oxygenated carbon), 147.6 (phenoxy-C) and at 101.2 ppm (anomeric carbon). Also evident from the $^{13}C$-ATP experiment were three quaternary carbons at $\delta$ 151.2, 148.0, 131.5, one methylene at $\delta$ 61.1 and nine methines at $\delta$ 192.1, 123.5, 115.8, 115.1, 101.2, 77.7, 76.4, 73.6 and 70.2 ppm, respectively. The linkages between the aldehyde, phenol and sugar moieties at C-1 and C-4 of the phenol were determined for **3** by heteronuclear multiple-bond correlation (HMBC) (Fig. 1). This spectrum showed that the aldehyde proton ($\delta$ 9.87 ppm) was correlated to the phenyl C-1 ($\delta$ 131.49) and the glucose H-1 ($\delta$ 4.94) correlated to phenyl C-4 ($\delta$ 151.2 ppm). The attachment of the glucose to the C-4 of the ring was further proven from the HMBC correlation at H-C ($\delta$ 7.24–147.40) and the COSY phenyl H–H correlation at $\delta$ 7.24–7.36. This compound is chemically related to orcinol glycoside, reported by Gupta et al. (2005) from *Curculigo orchioides* (Hypoxidaceae). However, the methyl group in orcinol glycoside was absent in **3**; instead, a carbonyl carbon signal that resonated at $\delta$ 192.1 was evident. These presences of the hydrogen on the carbonyl carbon was deduced from the heteronuclear single quantum correlation (HSQC) experiment that indicates a short-range J-coupling between H–C at $\delta$ 192.1 and its proton at $\delta$ 9.80. On the basis of these diagnostic features **3** was identified as 3-hydroxy-4-*O*-β-D-glucopyranosylbenzaldehyde. As far as could be ascertained, this compound has not been reported before.

The presence of orcinal glycoside in *H. colchicifolia* is likely to be the result of the biosynthetic transformation of orcinol glycoside in the plant. Orcinol glycoside was found to exhibit moderate antioxidant activity against the hydroxyl radical ($IC_{50}$ = 1.39 mM) and the superoxide anion ($IC_{50}$ = 2.49 mM), when compared to the positive control, epigallocatechin gallate (EGCG; hydroxyl radical: $IC_{50}$ = 0.43, superoxide anion: $IC_{50}$ = 0.53) (Wu et al., 2005). It is a reasonable assumption that orcinal glycoside would also display good antioxidant activity.

Compounds **4**, **5** and **6** were identified as hypoxoside, dehydroxy hypoxoside and *bis*-dehydroxy hypoxoside, respectively, using NMR, UPLC-MS data and literature values (Table 1). Although these analogues are difficult to resolve due to their structural similarities and highly polar nature, they were

**Table 1**
Compounds isolated from *Hypoxis* species with their corresponding retention times and fragmentation data as established by UPLC-MS.

| Comp ID | Compound name | Retention time (min) | Mass to charge ratio (*m/z*) | MS–MS fragmentation | Reference |
|---|---|---|---|---|---|
| 1 | β-Sitosterol | N/A | 414.01 | 396, 354, 329, 303, 255, 231, 213, 199, 173, 159, 145, 133, 119, 105, 81. | Kamboj and Saluja (2011) |
| 2 | Geraniol glycoside | 7.60 | 447.16 | 447 (M−H), 295 (M−$C_{10}H_{19}O$), 163 (M−$C_{10}H_{19}O$–$C_{11}H_{19}O_9$–$C_5H_9O_4$), 155 (M−$C_5H_9O_4$) | Bredenkamp et al. (1989) |
| 3 | Orcinal glycoside | 1.64 | 299.11 | 299 (M−$H^+$); 137 ($C_7H_5O_3$) | New |
| 4 | Hypoxoside | 4.58 | 605.11 | 641 (M+$Cl^-$); 605 (M−$H^+$); 443 ($C_{23}H_{23}O_9$); 281 ($C_{17}H_{12}O_4$) | Drewes et al. (1984) |
| 5 | Dehydroxy hypoxoside | 4.89 | 589.11 | 625 (M+$Cl^-$); 589 (M); 427 ($C_{23}H_{23}O_8$); 264 ($C_{17}H_{12}O_3$) | Laporta et al. (2007) |
| 6 | *bis*-Dehydroxy hypoxoside | 5.20 | 609.09 | 609 (M+$Cl^-$); 573 (M); 411 ($C_{23}H_{23}O_7$); 248 ($C_{17}H_{12}O_2$) | Laporta et al. (2007) |
| 7 | Galpinoside | 4.27 | 623.09 | 623 ([M−H); 461 (M−$C_9H_7O_3$); 325 (M−$C_{17}H_{15}O$); 299 ($C_{15}H_{17}O_8$); 163 (M−$C_{23}H_{25}O_{10}$); 160 (M−$C_{17}H_{15}O_8$–$C_9H_7O_3$) | New |

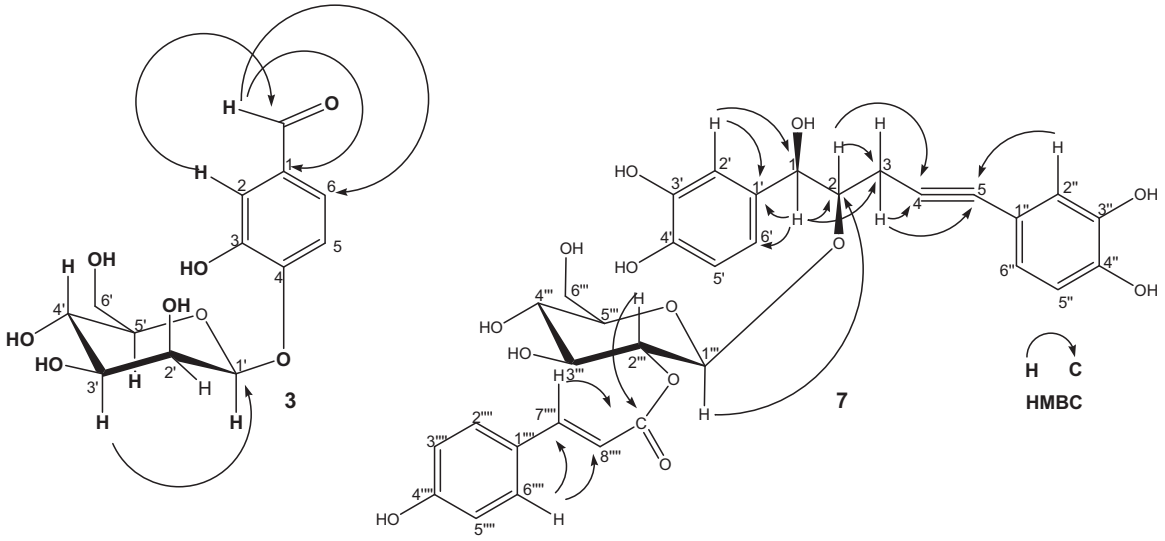


**Fig. 1.** Chemical structures of new compounds **3** (orcinal glycoside) and **7** (galpinoside), indicating their HMBC correlations, isolated from *Hypoxis colchicifolia* and *H. galpinii*, respectively.

successfully separated using HPCCC (Fig. 2). This technique is popular for the separation of polar extracts that are often difficult to separate using classical methods such as column chromatography (Marston and Hostettmann, 2006). The high performance countercurrent chromatograph (HPCCC) used in this study can achieve a g-level (the ratio of the column radius, *r*, to rotor radius, *R*) of 240, as opposed to the 55–80 g generated in high speed countercurrent chromatography (HSCCC), implying short cycle times and faster flow rates, comparable to those of preparative scale high performance liquid chromatography (www.dynamicextractions.com/technology/countercurrent-chromatography-history.html).

The chromatographic (Table 1) and spectroscopic data obtained for the norlignan glycosides, hypoxoside (**4**), dehydroxy hypoxoside (**5**) and *bis*-dehydroxy hypoxoside (**6**) corresponded to those reported in the literature (Marini-Bettolo et al., 1982; Drewes et al., 1984; Laporta et al., 2007). Hypoxoside was first isolated from *H. obtusa* by Marini-Bettolo et al. (1982) and later by Drewes et al. (1984) from *H. rooperi* (synonym *H. hemerocallidea*). Rooperol, the aglycone of hypoxoside, is known to have valuable anticancer and

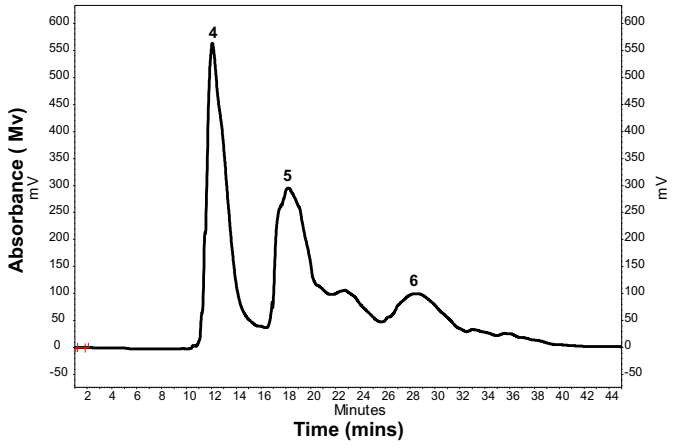


**Fig. 2.** High performance countercurrent chromatogram obtained by normal phase mode for a mixture containing hypoxoside (**4**; $R_t$ 11.5–14.5 min), dehydroxy hypoxoside (**5**; $R_t$ 18.3–21.8 min) and *bis*-dehydroxy hypoxoside (**6**; $R_t$ 25.5–33.0 min). Solvent: $CHCl_3$–*n*-BuOH–MeOH–$H_2O$ (7.5:10:7.5:25). UV λ = 260 nm.

antimutagenic properties (Albrecht et al., 1995). Whereas the biological activities of hypoxoside are well documented, those of the derivatives (**5** and **6**) are unknown.

Compound **7**, a dark brown amorphous solid was isolated from the acetone extract of the aerial parts of *H. galpinii*. M.p. 165–167 °C; $\alpha_D^{20}$ +0.025 (*c* 0.1 MeOH). $UV_{MeOH}$ $\lambda_{max}$ 256 and 299 nm. The IR absorptions at 3523, 3010, 2550–2108, 2263 and 1660 $cm^{-1}$ indicated the presence of hydroxyl, olefinic or phenyl, ketone, alkyne functional groups. The negative HRESIMS of **7** displayed a molecular adduct ion peak $[M-H]^+$ at *m/z* 623.1837, suggesting the molecular formula $C_{32}H_{31}O_{13}$ (Table 1). The $^1H$ NMR spectrum of **7** displayed signals for six aromatic protons in two ABX systems, which might result from catechol-like moieties, both being correlated by the $^1H$–$^1H$-COSY spectrum, and two aliphatic signals (Table 2). Both sets of catechol-moieties, one at δ 6.84 (1H, br s) and δ 6.72 (2H, br d, *J* = 1.2 Hz), and the other at δ 6.79 (1H, d, *J* = 2.0 Hz), δ 6.68 (1H, dd, *J* = 8.5, 2.0 Hz) and δ 6.59 (1H, d, *J* = 8.5 Hz) indicate a nyasicol-type norlignan, together with four aliphatic protons at δ 4.52 (1H, d, *J* = 8.5 Hz), δ 3.93 (1H, m), δ 2.40 (1H, dd, *J* = 17.5, 2.6 Hz) and δ 2.10 (1H, dd, *J* = 17.5, 6.1 Hz), while the acetylene moiety was confirmed by bands in the IR spectrum at 2550–2108 $cm^{-1}$ (Marini-Bettolo et al., 1982). One of the sets of catechol moieties was conjugated, with an acetylene moiety confirmed by HMBC correlations of H-2″ at δ 6.79 (1H, d, *J* = 2.0 Hz) and H-6″ at δ 6.68 (1H, dd, *J* = 8.5, 2.0 Hz) to C-5 at δ 83.6. H-2 at δ 3.93 (1H, m). In addition, H-3 at δ 2.40 (1H, dd, *J* = 17.5, 2.6 Hz) and δ 2.10 (1H, dd, *J* = 17.5, 6.1 Hz) were also correlated to C-4 at δ 84.1 in the HMBC spectrum (Fig. 1).

A sugar moiety was detected by the presence of an anomeric proton at δ 4.91 (1H, d, *J* = 8.5 Hz) and H-2‴ at δ 4.95 (1H, dd, *J* = 9.9 Hz, 8.5 Hz). This coupling constant for an anomeric proton and the downfield shift of H-2 of the β-glucose indicated that *p*-coumaric acid is conjugated to the C-2 position (Marini-Bettolo, 1991). Also, the chemical shift of C-1‴ at δ 101.1 confirmed the β-configuration of the *O*-glycosidic linkage. Typical protons of *p*-coumaric acid were detected in the $^1H$ NMR spectrum at δ 7.14 (H-2‴ and H-6‴, seemingly d, *J* = 8.2 Hz), δ 6.70 (H-3‴ and H-5‴, seemingly d, *J* = 8.2 Hz), δ 7.53 (H-7‴ d, *J* = 16.0 Hz), and δ 6.21 (H-7‴ d, *J* = 16.0 Hz). In the HMBC spectrum (Fig. 1), there were correlations between H-1‴ at δ 4.92 (1H, d, *J* = 8.0 Hz) and C-2 at δ 83.5, and H-2‴ δ 4.95 (1H, dd, *J* = 8.0 Hz, 8.0 Hz) and C=O of *p*-coumaric acid at δ 168.8. On the basis of these MS and NMR data

**Table 2**
$^{1}H$ and $^{13}C$ NMR data ($\delta$/ppm)[a] for compound **7** (galpinoside) in CH3OH-$d_4$.

| Position | Compound **7** | |
|---|---|---|
| | $\delta_H$ (J/Hz) | $\delta_C$ |
| 1 | 4.52 (d, 8.5) | 76.6 |
| 2 | 3.93 (m) | 83.5 |
| 3 | 2.40 (dd, 17.5, 2.6) | 22.9 |
| | 2.10 (dd, 17.5, 6.1) | |
| 4 | – | 84.1 |
| 5 | – | 83.6 |
| 1′ | – | 133.0 |
| 2′ | 6.84 (br s) | 115.4 |
| 3′ | – | 146.4[b] |
| 4′ | – | 146.3[b] |
| 5′ | 6.72 (br d, 2.0) | 119.5 |
| 6′ | 6.72 (br d, 2.0) | 120.3 |
| 1″ | – | 116.3 |
| 2″ | 6.79 (d, 2.0) | 116.6 |
| 3″ | – | 146.8 |
| 4″ | – | 146.1 |
| 5″ | 6.59 (d, 8.5) | 119.4 |
| 6″ | 6.68 (dd, 8.5, 2.0) | 125.0 |
| 1‴ | 4.91 (d, 8.5) | 101.1 |
| 2‴ | 4.95 (dd, 9.9, 8.5 | 76.7 |
| 3‴ | 3.64 (m) | 74.3 |
| 4‴ | 3.46 (m) | 75.5 |
| 5‴ | 3.49 (m) | 76.3 |
| 6‴ | 3.74 (m) | 62.0 |
| 1⁗ | – | 131.1 |
| 2⁗ | 7.14 (d, 8.2) | 127.4 |
| 3⁗ | 6.70 (d, 8.2) | 116.8 |
| 4⁗ | – | 161.2 |
| 5⁗ | 6.70 (d, 8.2) | 116.8 |
| 6⁗ | 7.14 (d, 8.2) | 127.4 |
| 7⁗ | 7.53 (d, 16.0) | 146.6 |
| 8⁗ | 6.21 (d, 16.0) | 115.3 |
| C=O | – | 168.8 |

the chemical structure of **7** was elucidated to be 1,5-*bis*(3,4-dihydroxyphenyl)-1,2-dihydroxy-4-pentyne-2-*p*-coumaroyl-β-D-glucopyranoside, referred to as galpinoside.

This compound has been reported by Marini-Bettolo (1991) from *Hypoxis interjecta* and *Hypoxis multiceps*. However, the compound was an enzyme hydrolyzed product obtained from interjectin, which has one additional glucose attached to the C-4″ position of **7**. There are two chiral centres in the compound, C-1 and C-2. In the case of 1,5-*bis*(3,4-dihydroxyphenyl)-1,2-dihydroxy-4-pentyne-2-β-D-glucopyranoside (nyasicoside), the absolute configuration of C-1 and C-2 were assigned as the *R* and *S* configuration, respectively (Chifudera et al., 1994). However, later, Chang et al. (1999) revised the configuration of C-2 as *R* by CD experiments. In order to confirm the configurations in this study, the relative configuration of C-1 and C-2, *threo* (1*R*,2*R*) or *erythro* (1*R*,2*S*) was determined by $^{1}H$ NMR spectroscopy. Although enantiomers cannot be distinguished by NMR spectroscopy, diastereomers would be expected to be differentiated once one of the absolute configurations is known, for example *R* for C-1.

Coupling constants of H-1 of (1*R*,2*R*)-(−)-pseudoephedrine and (1*R*,2*S*)-(−)-ephedrine were compared to each other. The coupling constant of H-1 of pseudoephedrine is 9.1 Hz and for ephedrine was determined as 3.2 Hz. Thus, the relative configuration of **7** was thought to be the *threo* form, probably the 1*R*,2*R* configuration.

All the $^{1}H$ and $^{13}C$ NMR assignments are listed in Table 2. Some doubtful assignments of phenolic protons in previous research (Chifudera et al., 1994), for example B-ring protons were clearly resolved with the help of $^{1}H$–$^{1}H$-COSY, HSQC and HMBC.

### 2.2. Ultra performance liquid chromatography analysis

The purities of the isolated compounds were established to be in excess of 95% using UPLC–MS. These compounds then served as reference standards for their identification in extracts using this technique. The traditional use of *Hypoxis* mainly involves the preparation of aqueous extracts of the fresh corms. However, in this study, methanol (MeOH) was used to prepare polar extracts from the corms of the three *Hypoxis* species, since TLC indicated similar fingerprints for aqueous and MeOH extracts. Comparison of the UPLC–MS chromatograms obtained (Fig. 3) revealed corresponding peaks at retention times 4.58 min (*m/z* 605.11) and 4.89 min (*m/z* 589.11), which were identified as hypoxoside and dehydroxy hypoxoside, respectively. The prominent peak at 5.20 min (*m/z* 609.09) in the chromatogram of *H. colchicifolia* corresponded to *bis*-dehydroxy hypoxoside. The corresponding peaks were also evident in the chromatograms of the other two species. Laporta et al. (2007) previously reported the presence of these three compounds in *H. rooperi* (synonym *H. hemerocallidea*). Peak areas for hypoxoside using the PDA detector were similar for *H. hemerocallidea* (47919 AU), *H. colchicifolia* (45621 AU) and *H. galpinii* (44732 AU). Significantly higher levels of dehydroxy hypoxoside (34198 AU) and *bis*-dehydroxy hypoxoside (18671 AU) were present in the corms of *H. colchocifolia* than in the other two species. However, *H. hemerocallidea* contained similar amounts of these metabolites (dehydroxy hypoxoside: 15014 AU, *bis*-dehydroxy hypoxoside: 4558 AU) to *H. galpinii* (dehydroxy hypoxoside: 14990 AU, *bis*-dehydroxy hypoxoside: 4467 AU).

A peak in the chromatogram of *H. colchicifolia* (Fig. 3) at 1.14 min (*m/z* 377.02) was identified as sucrose, while that occurring at 5.67 (*m/z* 611.10) in all three species was not identified. However, the peaks at 4.27 min (*m/z* 623.09) and 7.60 min (*m/z* 447.16), common to all three species, represented galpinoside and geraniol glycoside, respectively. The peak, specific to *H. colchicifolia*, with retention time 4.03 min (*m/z* 643.11), could not be identified.

In conclusion, two new compounds, orcinal glycoside and galpinoside, were isolated from South African *Hypoxis* species, adding to the list of secondary metabolites from the genus. In addition, dehydroxy hypoxoside, *bis*-dehydroxy hypoxoside, geraniol glycoside and β-sitosterol are herein reported for the first time as metabolites of *H. colchicifolia*. The presence of the phenolic glycosides in *Hypoxis* is of clinical importance, because such compounds comprising C6-linkers-C6 have value in mitigating osteoporosis (Wang et al., 2012) and may act as efficient inhibitors of fibrils that are responsible for conditions such as Alzheimer's disease (Riviere et al., 2007). Further investigations of the biological properties of the new compounds isolated in this study will shed light on the medicinal value of the genus. Chemical profiling of other *Hypoxis* species, using the isolated compounds as standards, will reveal interchangeable species for medicinal use.

## 3. Experimental

### 3.1. Plant material

Specimens of *H. colchicifolia* and *H. galpinii* were available from previous research and voucher specimens (HC024 and HG008, respectively) were subsequently identified by Prof. Braam van Wyk from the University of Pretoria, prior to their deposition at the Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria, South Africa. The tap roots were removed from the fresh corms, which were separated from the plant. After washing, the corms were chopped into small pieces and oven dried at 30 °C for 36 h prior to extraction. The resulting dried plant material was then pulverized using a Retsch® MM 400 ball milling

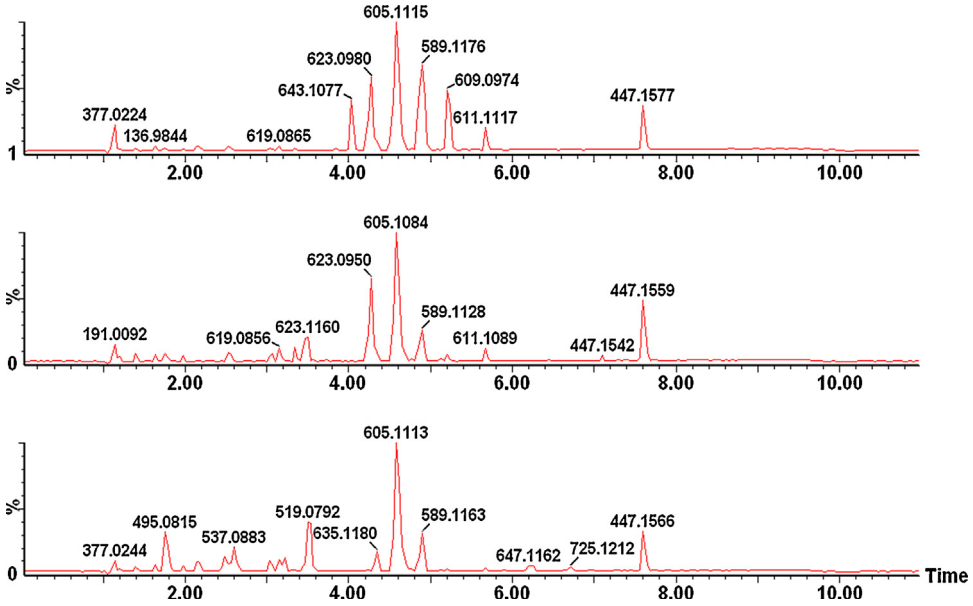


**Fig. 3.** Chromatograms (UPLC-MS) of the methanol extracts of corms of (A) *Hypoxis colchicifolia*, (B) *Hypoxis galpinii* and (C) *Hypoxis hemerocallidea*.

machine (Monitoring and Control Pty Ltd., Haans, Germany) at a frequency of 30.0 Hz for 120 s to yield fine brown powders. These powders were sieved using a 500 μm mesh size (Endcotts Filters Ltd., London, UK) to ensure consistency of particle size.

### 3.2. Extraction of secondary metabolites

All solvents, purchased from Merck (Pty) Ltd. (Johannesburg, Gauteng, South Africa), were of analytical reagent grade. Powdered *H. colchicifolia* (427 g) was divided into three equal portions, which were individually treated until the mass became manageable for combination. Each portion of the plant material was extracted with 3 ml × 250 ml $CHCl_3$ by sonication at 30 °C for 30 min and filtered (No. 4, Whatman, Buckinghamshire, UK). The resulting filtrates were combined and evaporated under reduced pressure at 40 °C using a rotary evaporator (Büchi Labortechnik R200, Flawil, Switzerland). Thereafter, the remaining portions of plant material were each extracted with methanol (MeOH; 3 ml × 250 ml), which after combination and evaporation of the solvent, resulted in a residue with a mass of 116 g.

### 3.3. Isolation of compounds

Extracts were sonicated (Bandelin Electronic, Berlin, Germany) in an appropriate solvent before application to columns for separation. For vacuum liquid chromatography (VLC), a vacuum pump (Sartorius Stedim, Goettingen, Germany) was attached to a glass column (Labotec (Pty) Ltd., Midrand, South Africa), packed with silica gel (Kieselgel 60, Machery-Nagel, Neumann-Neander, Düren, Germany), to increase the flow rate of the mobile phase through the column.

*H. colchicifolia*: The $CHCl_3$ extract (3.08 g) was batch eluted by VLC using $CHCl_3$ and MeOH. The $CHCl_3$ eluate was further purified by CC; petroleum ether as eluent yielded **1** (190 mg). A small amount of **2** (16.5 mg) was isolated from the MeOH batch using $CHCl_3$–acetone (90:10) for elution.

The initial MeOH extract was passed through a VLC column, which was eluted sequentially with EtOAc and EtOH. Since the EtOH fraction contained all the compounds of interest, this fraction (35.0 g) was further purified on a second VLC column by sequential batch elution using $CHCl_3$ and EtOH. The EtOH fraction (9.35 g), containing the target metabolites, was subsequently purified by conventional column chromatography (CC) using $CHCl_3$–MeOH–$H_2O$ (70:30:2) as eluent. Following TLC analysis, the fractions were combined as follows: A (0.57 g; $R_f$ 0.72–0.70), B (1.50 g; $R_f$ 0.70–0.65), C (2.34 g; $R_f$ 0.50–0.48) and D (5.12 g; $R_f$ 0.45–0.20). Compound **2** (167 mg) was obtained from Fraction A upon re-chromatography on silica gel using $CHCl_3$–acetone (90:10) as eluent, thus bringing the total amount of **2** to 184 mg. Fraction B was further chromatographed on a Sephadex® LH-20 (Sigma–Aldrich, Johannesburg, Gauteng, South Africa) column with $CHCl_3$–MeOH–$H_2O$ (70:30:2) as mobile phase to yield **3** (12.5 mg). Fraction D applied to silica gel, using EtOAc–MeOH (90:10) as the mobile phase, afforded pure **4** (800 mg) and a mixture of **4**, **5** and **6** (126 mg). The mixture of **4**, **5** and **6** was later purified by a single HPCCC separation.

*H. galpinii:* The plant was extracted with EtOAc, followed by acetone. The residue (5.70 g) resulting from the acetone extract, containing the target compounds, was further purified by silica gel CC using $CHCl_3$–MeOH–$H_2O$ (70:30:2). The resulting fractions, containing the target compound, were combined (1.02 g) and applied to a second silica gel column using the same mobile phase to yield **7** (310 mg).

### 3.4. High performance countercurrent chromatography

Preparative HPCCC was achieved using a hydrodynamic multilayer coil-planet g-type centrifuge (Spectrum, Dynamic Extractions Ltd., Slough, UK), fitted with two semi-preparative columns (175 ml total volume, 1.6 mm i.d.) connected in series. Solvent was pumped with an HPLC quaternary gradient pump (Model Q-grad, Scientific System Inc., State College, PA 16803, USA). The HPCCC was equipped with a reticulating chiller to maintain a constant column temperature of 30–32 °C. Compounds in the eluent, collected by a fraction collector (Model FC203B, Gilson, Middleton, USA), were detected using a UV–VIS detector (Sapphire 600, EACOM, Prague, Czech Republic), fitted with a preparative flow cell, at 260 nm. Data acquisition was accomplished by EZChrom software data and HPCCC runs were monitored by Agilent Interface 35900E.

After evaluating a variety of solvent mixtures using the method described by Marston and Hostettmann (2006), the solvent

combination of $CHCl_3$–*n*-BuOH–MeOH–$H_2O$ (7.5:10:7.5:25) was selected for HPCCC separation of the compounds. The mixture of **4**, **5** and **6** (126 mg) was dissolved in 3 ml of upper phase (UP) and 3 ml of lower phase (LP) and filtered through a 0.20 μm filter (Bonna-Agela Technologies; Stargate Scientific, Wilgeheuwel, South Africa). Normal phase semi-preparative mode was used by filling the column with 65 ml of the stationary phase (LP). The coils were rotated at 1600 rpm, where after the mobile phase (UP) was pumped at 6.0 ml/min, until dynamic equilibrium was reached at 50 ml. At this stage, the pre-loaded sample was injected into the column and a run time of 60 min, comprising 45 min for elution and 15 min for extrusion, was permitted. Compounds **4** (40 mg), **5** (15 mg) and **6** (10 mg) were isolated as cream coloured fluffy powders at retention times of 11.5–14.5 min, 18.3–21.8 min and 25.5–33.0 min, respectively (Fig. 2). A total mass of 840 mg of **4** was derived from the polar extracts of all three species.

### 3.5. Nuclear magnetic resonance spectroscopy

Spectra were recorded on a Bruker 600 Avance II NMR (Bruker, Bellerica, MA, USA) at 600 MHz for $^1$H NMR and 150 MHz for $^{13}$C NMR. Two dimensional (2D) NMR experiments were performed using standard Bruker microprograms. Measurements were made in $CD_3OD$ and the solvent signals were used for calibration. The purities and identities of isolated compounds were confirmed by comparing $^1$H and $^{13}$C NMR signals, UPLC–MS data, IR signals and UV absorption wavelengths with literature values (Table 1).

3-Hydroxy-4-*O*-β-D-glucopyranosylbenzaldehyde (orcinal glycoside) (**3**) A new compound isolated from *H. colchicifolia* (Fig. 1; Table 1). Colourless semi-solid oil. $UV_{MeOH}$ $\lambda_{max}$ 225, 270 and 309 nm. IR (KBr) $\nu$ 3525 (–OH), 1725 (C=O) and 3012 (Ph-H) $cm^{-1}$. $^1$H NMR (600 MHz, $CD_3OD$) $\delta$ 9.80 (H, s, CH=O), 7.21 (H, s, Ph-2), 7.39 (H, d, $J$ = 8 Hz, Ph-5), 7.28 (H, d, $J$ = 8 Hz, Ph-6), 4.94 (H, d, $J$ = 8 Hz, glc-1), 3.50 (H, t, $J$ = 8.5 Hz, glc-3), 3.48 (H, t, $J$ = 7.5 Hz, glc-2), 3.45 (H, t, $J$ = 8.5 Hz, glc-4), 3.47 (H, m, glc-5), 3.42 (2H, m, glc-6). $^{13}$C NMR ($CD_3OD$) $\delta$ 192.14, 151.16, 147.99, 131.49, 123.49, 115.76, 115.13, 101.17, 77.71, 76.40, 73.62, 70.17 and 61.08 ppm. HRESIMS (negative ion mode) *m/z* 300.24 (Calculated for $C_{13}H_{16}O_8$ 300.08 [M+1]). HRESIMS fragmentation pattern reported in Table 1. An $R_f$ value of 0.65 was obtained by TLC using $CHCl_3$–MeOH–$H_2O$ (70:30:2) as developing solvent.

### 3.6. Ultra high performance liquid chromatography analysis

Methanol extracts of *H. colchicifolia*, *H. hemerocallidea* and *H. galpinii* were introduced by full-loop injection (1.0 μl) into a UPLC (Waters Acquity chromatographic system; Waters, Milford, MA, USA), equipped with a photo diode array (PDA) detector. This detector was used to optimize the separations during the initial analyses. Extracts and pure compounds were separated on an Aquity UPLC BEH $C_{18}$ column (150 mm × 2.1 mm, i.d., 1.7 μm particle size; Waters) maintained at 40 °C. The mobile phase consisted of 0.1% aqueous formic acid (Solvent A) and HPLC grade (Merck™, Germany) acetonitrile (Solvent B), at a flow rate of 0.3 ml/min. Gradient elution was applied as follow: 85% A:15% B–65% A:35% B in 7 min, changed to 50% A:50% B in 1 min (held for 2.5 min), before returning to the initial ratio in 0.5 min (a total run time of 11 min). Data were managed by Markerlynx 4.1 chromatographic software.

The UPLC system was interfaced with a combination time-of-flight/quadrupole Xevo $G_2$QT mass spectrometer (Waters, USA). For the UPLC–MS analyses, the same column, elution gradient and flow rate were used as before. Although both positive and negative ion modes were applied, the results obtained indicated that higher sensitivities and more information were obtained in the negative mode. The mass spectrometer was therefore operated in negative ion electrospray mode using nitrogen as the desolvation gas at a flow rate of 600 L/h. A desolvation temperature of 350 °C and a source temperature of 100 °C were used. The capillary and cone voltages were set to 2500 and 40 V, respectively. Data were collected in the range *m/z* 100–1200.

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