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Isolation, characterization and antioxidant capacity assessment of the bioactive compounds derived from *Hypoxis rooperi* corm extract (African potato)

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Abstract

The rhizome of the plant *Hypoxis rooperi* (''African potato'') is known for its traditional and ethnomedical uses in the treatment of benign prostatic hyperplasia and other diseases. We have characterized an extract derived from *H. rooperi*, isolated its major bioactive compound, hypoxoside, and obtained its aglycone, rooperol, by enzymatic digestion. Absorption, fluorescence emission and bidimensional NMR complete spectral data of these compounds were obtained. The antioxidant capacity of both compounds was fully analyzed through the thiobarbituric acid reactive substances (TBARS) and Trolox equivalent antioxidant capacity (TEAC) assays, and it was compared to catechins and olive biophenolics. Both compounds showed a strong antioxidant capacity, although rooperol exhibited a higher antioxidant activity against lipid peroxidation which correlated to its strong affinity for phospholipid membranes as derived from its extremely high lipid/water partition coefficient ($K_p = 3.4 \times 10^4$). The study of the lipophilic (EtOH) and hydrophilic (water) TEAC values revealed that more hydrophobic compounds, had greater lipophilic TEAC values than hydrophilic ones, probably indicating that lipophilic TEAC assay may be more reliable for these compounds. The *H. rooperi* extract also showed higher antioxidant efficacy compared to other strong antioxidant herbal extracts, such as olive leaf or green tea. Moreover, neither evidence of acute oral toxicity nor adverse effects were observed when the *H. rooperi* commercial extract containing 45% hypoxoside was used at a dosage of 2000 mg/kg. The results obtained in this work may contribute to understanding the biological activity described for these dicatechols and the African potato extract for food and cosmetic applications.

Keywords: *Hypoxis rooperi*; Hypoxoside; Rooperol; Antioxidant; TBARS; TEAC; ORAC; Membrane affinity

1. Introduction

The plant *Hypoxis hemerocallidea*, also named as *Hypoxis rooperi* (*Hypoxidaceae*), usually grows in meanders, grassland and mountainous areas of South America, Southern Africa, Australia and coastal regions of Asia. Members of *Hypoxidaceae* are herbaceous perennials with tuberous rhizomes or corms and abundant adventitious roots, which enable them to survive under high-stress conditions, and bright yellow star shaped flowers borne on long (Geerinck, 1968). The corm of the *H. hemerocallidea* has been used in folk medicine to treat a variety of diseases, such as the common cold, flu, hypertension, adult-onset

Abbreviations: AAPH, 2,2′-Azobis (2-methyl-propionamine) dihydrochloride; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate); AUC, area under the fluorescein decay curve; BHT, butylated hydroxytoluene (2-,[6]-di-*tert*-butyl-*p*-cresol); C, (+)-catechin; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGCG, (−)-epigallocatechin gallate; EYPC, egg yolk phosphatidylcholine; FL, disodium fluorescein (3,6-dihydroxy-spiro[isobenzofuran-1[3H],9[9H]-xanthen]-3-one); HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum coherence; LD50, lethal dose 50%; LUV, large unilamellar vesicles; MDA, malondialdehyde; MLV, multilamellar vesicles; NMR, nuclear magnetic resonance; ORAC, oxygen radical absorbance capacity; SUV, small unilamellar vesicles; TBARS, thiobarbituric acid reactive substances; TEAC, Trolox equivalent antioxidant capacity; TEP, 1,1,3,3-tetraethoxypropane; UV–vis, ultraviolet–visible light.

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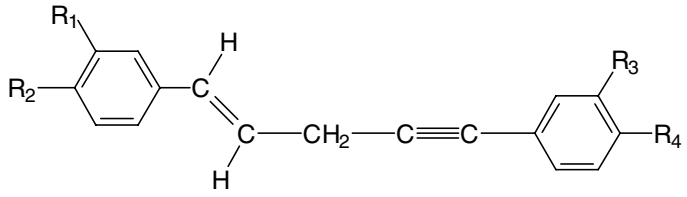
diabetes mellitus, psoriasis, urinary infections, testicular tumours, prostate hypertrophy and internal cancer, HIV/AIDS and some central nervous system disorders (Albrecht, 1995; Dreikorn, 2000; Dreikorn & Schonhofer, 1995; Fagelman & Lowe, 2002; Mills, Foster, et al., 2005; Van Wyk, Van Oudshoorn, & Gericke, 1997; Wilt, Ishani, Rutks, & MacDonald, 2000). Since it became popular, the plant is frequently named as *African potato*, which is an inappropriate name, since the ground portion of the plant does not resemble a potato. In the early 1970s, a hydroalcoholic extract of *H. rooperi* was patented with a long list of beneficial properties: anti-inflammatory, antibiotic, antiarthritic, antiatherosclerotic, diuretic and stimulant of muscular and hormonal activities (Liebenberg, 1969). Dietary supplements and pharmaceutical products based on this plant and focused on the treatment of benign prostatic hyperplasia (BPH) are commercially available in the market (Mills, Cooper, Seely, & Kanfer, 2005; Nicoletti, Galeffi, Messana, & Marini-Bettolo, 1992).

H. hemerocallidea aqueous extracts have demonstrated to possess antinociceptive and anti-inflammatory activities using the albumin-induced rat paw edema model (Liebenberg, 1969; Ojewole, 2002; Ojewole, 2005). In addition, *in vitro* experiments showed that ethanolic extracts from *H. rooperi* exhibited higher inhibitory effects on COX-1 than aqueous extracts, which was also reported to mildly inhibit COX-2 (Jager, Hutchings, & van Staden, 1996; Steenkamp, Gouws, Gulumian, Elgorashi, & van Staden, 2005). In addition, *H. rooperi* aqueous extract showed the capacity to scavenge $HO^{\cdot}$ and caused dose-related hypoglycaemia in normal and diabetic rats (Mahomed & Ojewole, 2003; Ojewole, 2005).

The biological activity of this plant was initially associated with undefined steroid glycosides, whereas later studies related it to the presence of glycosides of unsaturated aglycones (Nicoletti et al., 1992), which can be considered as norlignans. The major constituent found in the rhizome of the *H. hemerocallidea*, is hypoxoside: (*E*)-1,5-bis (3′-hidroxi-4′-*O*-β-D-glucopiranosylphenyl) pent-1-en-4-ino (Fig. 1), a glycosilated norlignan derived from cinamic acid, as it occurs to the lignans, but it is linked to other than β-β′ and with the loss of the chain terminal carbon (Betto, Gabriele, & Galeffi, 1992). Hypoxoside was first isolated and purified from the *Hypoxis obtusa* rhizome (Marini-Bettolo, Patamia, & Nicoletti, 1982). Later, it was discovered that β-glucosidase converted hypoxoside to its aglycone form, rooperol (Fig. 1) (Drewes, Hall, Learmonth, & Upfold, 1984), a bioactive dicatechol bearing powerful antioxidant properties in a human blood system, which may derived from its catechol moieties and unsaturated carbon chain (Van der Merwe, Jenkins, Theron, & van der Walt, 1993).

Hypoxoside is supposed to be pharmacologically inactive, as it is presumed to be transformed into its biologically-active aglycone form, rooperol, in the large intestine of humans and experimentation animals by bacterial β-glucosidase activity (Kruger, Albrecht, Liebenberg, & van Jaarsveld, 1994). Since free catechols are relatively unstable for pharmaceutical formulations, therefore these compounds are used as rooperol acetylated esters, which are quickly metabolized in the gastrointestinal tract to free rooperol (Guzdek, Nizankowska, Allison, Kruger, & Koj, 1996). The anti-inflammatory action of rooperol and its analogues is more likely to take place through the inhibition of the 5-lipoxygenase pathway, rather than cyclooxygenase (Guzdek et al., 1996; Van der Merwe et al., 1993). Rooperol has also been postulated to exert antimetastatic activity through its capacity to stimulate the synthesis of collagen type I, what could impede tumor cell invasions (Dietzsch, Albrecht, & Parker, 1999). Hypoxoside has not exhibited toxicity as an oral prodrug for cancer therapy in a phase I clinical trial (Albrecht, Kruger, et al., 1995, Albrecht, Theron, & Kruger, 1995; Smit et al., 1995).



Compound		R_1	R_2	R_3	R_4
1	Hypoxoside:	-OH	-O-β-D-glucopyranosyl	-OH	-O-β-D-glucopyranoz
2	Dehydroxyhypoxoside:	-H	-O-β-D-glucopyranosyl	-OH	-O-β-D-glucopyranosyl
3	Bis-dehydroxyhypoxoside:	-H	-O-β-D-glucopyranosyl	-H	-O-β-D-glucopyranosyl
4	Rooperol:	-OH	-OH	-OH	-OH
5	Dehydroxyrooperol	-H	-OH	-OH	-OH
6	Bis-dehydroxyrooperol	-H	-OH	-H	-OH

Fig. 1. Chemical structures of the norlignans (**1**–**6**) derived from *Hypoxis rooperi* extract.

In this work the bioactive compounds from a commercial *H. rooperi* extract have been identified and isolated. Their purification by preparative HPLC has been exhaustively described for the first time. Their structures were elucidated by bidimensional NMR and their spectroscopic parameters were determined. The antioxidant activity of hypoxoside and rooperol was fully analyzed, in comparison to other known strong antioxidants, by thiobarbituric acid reactive substances (TBARS) and Trolox equivalent antioxidant capacity (TEAC) assays, performing a correlation of their capacity to inhibit lipid peroxidation with their phospholipid/water partition coefficient. The antioxidant capacity (using ORAC and TBARS assays) and the acute oral toxicity in mice of a *H. rooperi* commercial extract containing 45% hypoxoside was also assessed.

2. Materials and methods

2.1. Chemicals

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and the natural lipid egg yolk phosphatidylcholine (EYPC) were obtained from Avanti Polar Lipids (Birmingham, AL, USA), dissolved in chloroform/methanol (1:1) and stored at −20 °C. 2,2′-Azobis (2-methyl-propionamine) dihydrochloride (AAPH), 1,1,3,3-tetraethoxypropane (TEP), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2-thiobarbituric acid (4,6-dihydroxy-2-mercaptopyrimidine) (TBA), butylated hydroxytoluene (2,[6]-di-*tert*-butyl-*p*-cresol) (BHT), disodium fluorescein (3,6-dihydroxy-spiro[isobenzofuran-1[3H], 9,[9H]-xanthen]-3-one) (FL), (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG) and β-glucosidase were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Oleuropein and hydroxytyrosol were obtained from Extrasynthese (Lyon, France). Stock solutions of the bioactive compounds **1** and **4** were prepared in methanol and stored at −20 °C. *H. rooperi* (45% hypoxoside), green tea (70% catechins) and olive leaf (25% oleuropein) extracts were kindly provided by Monteloeder, S. L. (Elche, Spain). All other compounds were of analytical, spectroscopic or chromatographic reagent grade and were obtained from Merck KGaA (Darmstadt, Germany). Double-distilled and deionized water was used throughout this work.

2.2. Analytical HPLC

Ten milligrams of the rhizome extract from *H. rooperi* were dissolved in 2 ml of distilled water and centrifuged for 20 min at 11,000*g*. Five μl of the supernatant were injected in an analytical reverse phase column LiChrospher® 100 RP-18 (5 μm, 250 × 4 mm i.d.) from Merck and subjected to HPLC analysis. The separation of norlignans compounds was carried out in a high performance liquid chromatograph LaChrom (Merck-Hitachi) series 7000, equipped with a pump, autosampler, column oven and UV–vis diode array detector (wavelength selected at 260 nm to detect hypoxoside and rooperol). The chromatographic separation was performed at 25 °C and a flow rate of 0.8 ml/min with a mobile phase composed of (A) 0.05 M KH_2PO_4 and (B) acetonitrile. The multigradient solvent system was as follows: 0–40 min, from 20% B to 70% B; 40–42 min, from 70% B to 20% B; 42–50 min, fixed at 20% B.

2.3. Semipreparative HPLC

For the purification of hypoxoside, 250 mg of *H. rooperi* commercial extract were dissolved in 10 ml of distilled water, centrifuged at 1600*g* at room temperature and filtered through a 0.45 μm nylon filter. The filtered supernatant of the extract was injected into a preparative reverse phase column LiChrospher® 100 RP-18 (15 μm, 250 × 25 mm i.d.) from Merck KGaA (Darmstadt, Germany) and subjected to a semipreparative elution at the same chromatographic conditions as the analytical method but at a flow rate of 31.25 ml/min. In the case of the purification of rooperol, this was first obtained by β-glucosidase digestion performed on the whole extract. Briefly, 250 mg of extract were dissolved in 10 ml of 0.1 M phosphate buffer pH 5.5 and 15 mg of β-glucosidase (60 U/mg) were added. Digestion was performed during 6 hr at 37 °C with gently shaking. Then, the mixture was centrifuged at 1600*g* to eliminate insoluble particles and extracted with 5 ml of diethyl ether (Kruger et al., 1994). Rooperol was purified by semipreparative HPLC as described for hypoxoside elsewhere within this section.

Semipreparative purifications were carried out using a WellChrom (Merck-Knauer, Berlin, Germany) preparative high performance liquid chromatograph equipped with two K-1800 pumps, dynamic mixing chamber, manual injector, UV–vis detector and fraction collector. EuroChrom® software version 3.01 (Merck-Knauer) was used for data acquisition and analysis. Major peaks appearing in their respective chromatograms, identified as hypoxoside and rooperol, respectively, were collected and analyzed by analytical HPLC in order to check its purity. Each isolated molecule was identified by comparing the UV spectra with previously published data (Kruger et al., 1994). However, the identity of the compounds was corroborated by bidimensional HSQC ^{1}H and ^{13}C NMR and compared to previously published data (Drewes et al., 1984; Drewes, Scogins, & Wenteler, 1989; Marini-Bettolo et al., 1982). Each eluted fraction containing only pure compound was then solvent exchanged to pure methanol through the use of preparative C18 cartridges in order to eliminate salts and evaporated to dryness in rotavapor. Last traces of solvent were removed by keeping the samples under high vacuum overnight and after that, each purified compound was weighted and subjected to HPLC analysis to check for possible degradation during the purification process.

2.4. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectra were recorded on a Bruker AVANCE 500 spectrometer and probe HD 5 mm TXI 13C Z, at a resonance frequency of 500.0 MHz for ^{1}H and 125.0 MHz for ^{13}C. The spectra were collected at 296 K in methanol-d_3 or acetone-d_6 as deuterated solvents.

2.5. Determination of the phospholipid/water partition coefficient (K_p)

Fluorescence measurements of the dicatechols were recorded with an SLM-8000C spectrofluorimeter fitted with Glan-Thompson polarisers. Their respective K_p values were determined from its intrinsic fluorescence intensity increase upon the incorporation to large unilamellar vesicles (LUVs) composed of DMPC at 30 °C, in comparison to that in the aqueous phase, from experiments where compounds concentration was kept constant and phospholipid concentration was varied (Caturla, Perez-Fons, Estepa, & Micol, 2005; Caturla, Vera-Samper, Villalain, Mateo, & Micol, 2003). The phospholipid/water partition coefficient, K_p, was defined as

$$K_p = \frac{n_L/V_L}{n_w/V_w}, \quad (1)$$

where n_i stands for moles of compound in phase i and V_i for volume of phase i; the phase was either aqueous (i = w) or lipidic (i = L). The quantitation of K_p was done according to:

$$\Delta I = \frac{\Delta I_{max}[L]}{1/(K_p\gamma) + [L]}, \quad (2)$$

where ΔI ($\Delta I = I - I_0$) stands for the difference between the fluorescence intensity of each of the norlignans measured in the presence (I) and in the absence of the phospholipid vesicles (I_0); $\Delta I_{max} = I_\infty - I_0$ is the maximum value of this difference once the limiting value is reached (I_∞) upon increasing the phospholipid concentration [L], and γ is the molar volume of the phospholipid (0.737 M^{-1} for DMPC in the fluid phase) (Marsh, 1990). The phospholipid content was determined as previously reported (Böttcher, Van Gent, & Priest, 1961). Samples were excited at 298 nm for both compounds, hypoxoside and rooperol, and fluorescence emission was recorded at 332 and 342 nm for hypoxoside and rooperol, respectively.

2.6. Measurement of the Trolox equivalent antioxidant capacity (TEAC)

The Trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the radical cation of ABTS by antioxidants, was performed as previously described (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993; Re et al., 1999). Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–24 h before use. For the study of phenolic compounds the $ABTS^{\bullet+}$ solution was diluted with ethanol or water (lipophilic and hydrophilic assay, respectively) to an absorbance of 0.70 (±0.02) at 734 nm. For the photometric assay 1 ml of the $ABTS^{\bullet+}$ solution and 100 μL antioxidant solution were mixed for 45 s and measured immediately after 5 min at 734 nm (absorbance did not change significantly up to 10 min). Compounds were assayed at five different concentrations determined within the linear range of the dose–response curve. A calibration curve was prepared with different concentrations of Trolox (0–20 μM). Results were expressed in mM of Trolox.

2.7. Radical-scavenging capacity by thiobarbituric acid-reactive substances assay (TBARS)

The quantitative evaluation of the antioxidant capacity of the compounds against lipid peroxidation was determined through TBARS assay. Small unilamellar vesicles (SUVs) were prepared as previously described (Caturla et al., 2003) by sonication of multilamellar vesicles (MLVs) of egg yolk phosphatidylcholine (EYPC) during 6 cycles of 30 s and diluted to a final concentration of 0.76 mM with Tris buffer (140 mM NaCl, 20 mM Tris, pH 7.4). One milliliter of SUVs dispersion was incubated for 10 min at 37 °C with the antioxidants and after that the free radical generator AAPH was added (10 mM final concentration) to the mixture in order to induce peroxidation of unsaturated fatty acids. The reaction was incubated at 37 °C with occasional vortexing and stopped after 60 min by adding 200 μl of BHT (4% p/v in ethanol) and frozen at −80 °C until needed. The colorimetric reaction with thiobarbituric acid was then carried out by adding 250 μl of sodium dodecyl sulfate (3% p/v), 500 μl of TBA (1% p/v) and 500 μl of HCl 7 mM to the samples and incubating them at 95 °C for 15 min. Then, TBA–MDA chromogen was determined using the same HPLC system and column as previously described within this section. The analysis were conducted by injecting 40μl of sample in an isocratic mode with methanol-50 mM potassium phosphate buffer, pH 6.8 (40:60, v/v), using a flow rate of 0.8 ml/mim. The TBA–MDA product was monitored by fluorescence detection with excitation at 515 nm and emission at 553 nm. A calibration curve of MDA (0–3.2 nmol), obtained by acidic hydrolysis of 1,1′,3,3′-tetraethoxypropane (TEP), was used. This was obtained preparing a solution of 10 mM TEP in 1% H_2SO_4 and allowing the mixture to stand at room temperature for 24 h before use (ε_{244} of MDA = 13,700 M^{-1} cm^{-1}).

2.8. Assay of the oxygen radical absorbance capacity (ORAC)

To assay the capacity of the extracts to scavenge peroxyl radicals a validated ORAC method, which uses fluorescein (FL) as the fluorescent probe ($ORAC_{FL}$), was utilized (Ou,

Hampsch-Woodill, & Prior, 2001). Promptly, 0.5 g of the extract were extracted with 20 mL of acetone/water (50:50, v/v) and the mixture was shaken at 400 × rpm at room temperature on an orbital shaker for 1 h. The extract was centrifuged at 100*g* for 15 min, and the supernatant was ready for the ORAC analysis after the appropriate dilution with 75 mM phosphate buffer solution at pH 7.4. The automated ORAC assay was carried out on a Fluostar Galaxy spectrofluorometric analyzer (BMG Labtechnologies GmbH; Offenburg, Germany). In the final assay mixture (200 μL total volume), FL and AAPH were used at 90 nM and 12.8 mM, respectively. Several dilutions of Trolox (1–40 μM) were used to construct the calibration curve. A freshly prepared AAPH solution was used for each experiment. The temperature of the incubator was set at 37 °C and the FL fluorescence was recorded every minute after the addition of AAPH.

The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area of the FL decay curve (area under curve, AUC) as previously described (Ou et al., 2001). ORAC values were expressed as Trolox equivalents as millimole or micromole per gram of antioxidant substance.

2.9. Acute oral toxicity assessment

Ten-week-old male and female ICR strain mice were used for the study. The animals were housed in cages in a temperature-controlled animal room (23 ± 1 °C) with a relative humidity of 55 ± 5% and were fed a standard diet. The animal care and handling was done according to the regulations of Council Directive 86/609/EEC about Good Laboratory Practice (GLP) on animal experimentation. The animals were allowed to fast by withdrawing the food and water for 18 h and divided into four groups of eight individuals (two groups for the control and two groups for the test substance) before the administration. Briefly, the powder (*H. rooperi* extract, 45% hypoxoside) was dissolved in 0.9% NaCl sterile solution, pH 7.4 at a concentration of 80 mg/ml and centrifuged to eliminate insoluble particles. The test substance was administered by oral gavage in a total volume of 0.5 mL at a single dose of 2000 mg/kg. Weight control and observations were continued for 14 days. On day 14, the mice were anesthetized, killed by exsanguinations, and examined by necropsy. For reasons of animal welfare concern and as regulations recommend (OECD Test Guideline 420; fixed dose procedure), testing on animals in GHS Category 5 ranges (2000–5000 mg/Kg) was discouraged.

3. Results and discussion

3.1. Semipreparative HPLC purification of bioactive compounds from H. rooperi extract

H. rooperi extract is being increasingly utilized in pharmacological preparations focused on prostate health. Nevertheless, due to its strong antioxidant capacity and its potential anti-inflammatory activity it might become a promising ingredient for functional food or dietary supplements. Therefore, its main bioactive compounds have been purified and their antioxidant activity and toxicity assessed.

A commercial hydroalcoholic powered extract of *H. rooperi* rhizomes standardized in 45% hypoxoside was analyzed by analytical HPLC. Fig. 2A shows the chromatogram at 260 nm of the extract in which the major compound as judged by the retention time and UV–vis spectrum data published from other authors (Kruger et al., 1994) was the norlignan glucoside hypoxoside (compound **1**). Compounds **2** and **3** were identified as its derivatives dehydroxyhypoxoside and bis-dehydroxyhypoxoside, respectively (Fig. 1). The complete β-glucosidase digestion of the extract yielded the chromatographic separation showed in Fig. 2B, in which the compounds observed were identified as rooperol (compound **4**), the aglycone form of hypoxoside, and dehydroxyrooperol and bis-dehydroxyrooperol, i.e. the respective aglycone forms of compounds **2** and **3** (Fig. 1) (Kruger et al., 1994).

A semipreparative scale purification from 250 mg of *H. rooperi* extract (Fig. 3) was performed in order to obtain enough amount of the pure compounds to assay their antioxidant activity and to confirm their identity by HSQC-NMR. Retention times, capacity factor and yield data for the analytical and preparative chromatographic separations of the different compounds are provided in Table 1. The purified compounds were used for detailed spectroscopic and structure elucidation as described below.

3.2. Characterization of the bioactive compounds

UV spectra in methanol solution of purified hypoxoside (**1**) and rooperol (**4**) herein studied (Fig. 4A) were consistent with those published by Kruger et al. obtained from the diode-array detection (Kruger et al., 1994). The absorption spectrum showed three maxima at 212, 258 and 298 nm for hypoxoside (**1**) and 212, 260 and 302 nm for rooperol (**4**). The spectrum showed strong absorptions characteristic of alkene or alkine groups conjugated with phenol aromatic ring. The spectrum of hypoxoside was almost identical to that one of rooperol but showing an ipsochromic shift due to the presence of the glucoside moiety. The molar absorptivities for hypoxoside (**1**) and rooperol (**4**) were close to 25,000 ± 100 $M^{-1}\,cm^{-1}$ at 260 nm in methanol (Table 1), in both cases excellent correlation coefficients were obtained for the isolated compounds as standards. No previous data on the molar absorptivity of rooperol (**4**) are available, however, the value obtained in this work for hypoxoside (**1**) increased one order of magnitude that one previously reported (Marini-Bettolo et al., 1982), likely due to the higher purity of the standard herein purified. In addition, fluorescence excitation spectrum (data not shown) was

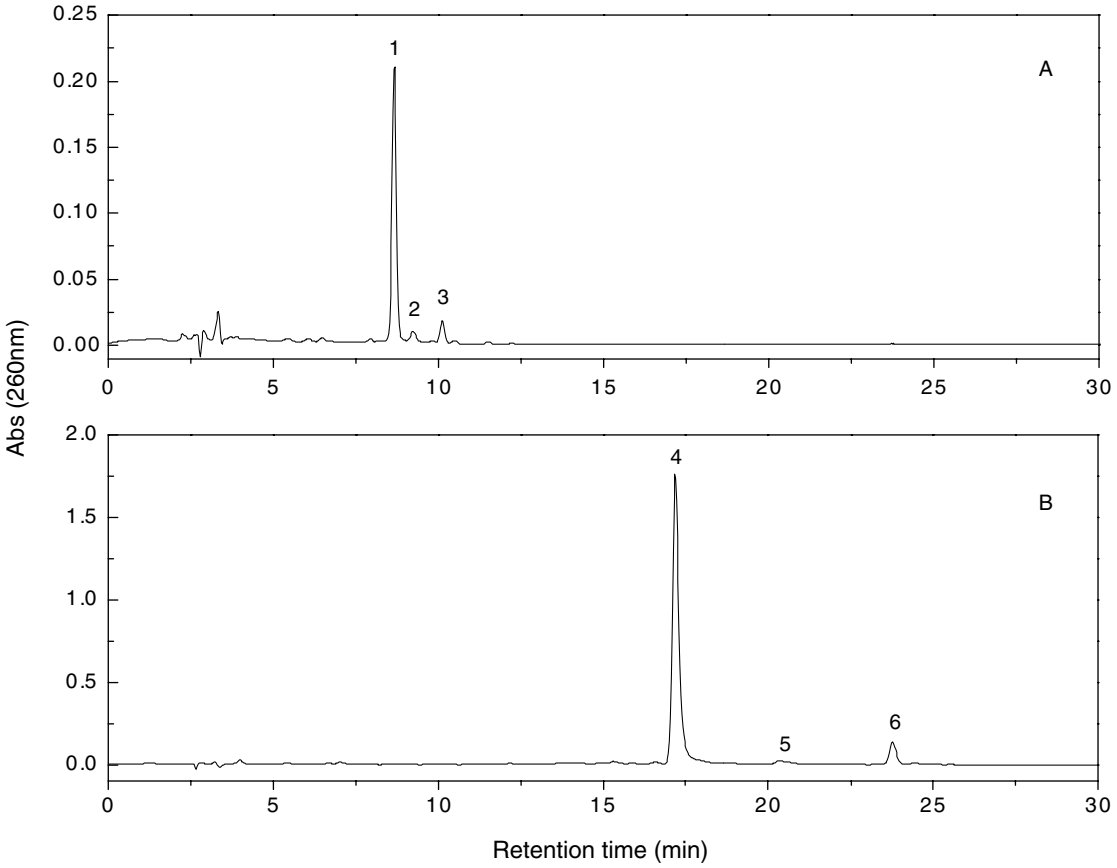


Fig. 2. High-performance liquid chromatography profiles of the *Hypoxis rooperi* extract (A) and its β-glucosidase (B) at 260 nm (1, hypoxoside; 2, dehydroxyhypoxoside; 3, bis-dehydroxyhypoxoside; 4, rooperol; 5, dehydroxyrooperol; 6, bis-dehydroxyrooperol).

identical to UV absorption spectrum of the compounds (Fig. 4), which corroborates the higher purity of the standard here purified. Fig. 4B shows the fluorescence emission spectra of hypoxoside (**1**) and rooperol (**4**) in dilute solutions (less than 0.1 a.u.) at 298 and 305 nm of wavelength excitation, respectively. These spectra were much more intense in ethanol or methanol than in aqueous solutions likely due to their higher quantum yield in these solvents, as it has been reported for phenol (Parker, 1968) and phenol derivatives (Caturla et al., 2003; Gomez-Fernandez et al., 1989).

Analysis of the structures of hypoxoside or (*E*)-1,5-bis-(4′β-D-glucopyranosyloxy-3′-hydroxyphenyl)-pent-4-en-1-yne (**1**) and its aglycone rooperol or (*E*)-1,5-bis-(3′, 4′-dihydroxyphenyl)-pent-4-en-1-yne (**4**) were elucidated by mono and bidimensional spectra in deuterated solvents as it has been previously reported by other authors (Drewes et al., 1989; Marini-Bettolo et al., 1982). The ^{1}H and ^{13}C NMR data of hypoxoside (**1**) and rooperol (**4**) are shown in Table 2. Assignation of NMR signals of rooperol (**4**) by HSQC experiment in acetone-d_6 is shown in Fig. 5, and this was used to determine the unequivocal chemical shift between nuclei. Moreover, all the signals of hypoxoside (**1**) spectrum were assigned by HSQC for the first time. The ^{1}H NMR spectra of **1** showed the characteristic signals of the sugar substitution labelled with H-Gluc, i.e. at δ4.74 and 4.72 (2 × d, $J = 7.2$ Hz, 2 × H-1 of each glucoside moiety), 3.85 (H-6, $J_{gem} = 12$ Hz) and 3.69 (H-6, $J_{gem} = 12$ Hz), 3.48–3.42 (H-3 and H-5) and 3.40–3.37 ppm (H-2 and H-4). The rest of the signals of compound **1** matched to those of its aglycone **4** with almost identical shifts.

Signals corresponding to the protons on C-1, C-2 and C-3 in the backbone showed identical shifts and constants for compounds **1** and **4** (see Table 2). These carbon signals were additionally correlated to the presence of a double triplet ($J_1 = 15.7$ Hz and $J_2 = 5.5$ Hz) in vinyl proton H-2, which was correlated to C2 (see inset, Fig. 5). This fact confirmed the *trans* configuration for the two compounds analyzed.

For the ^{13}C NMR spectrum of rooperol (**4**), the assignments were performed on the bibliography basis (Drewes et al., 1989; Marini-Bettolo et al., 1982), but we found some discrepancies regarding C-2 in this compound, which we established at δ121.6 ppm (see Fig. 5, inset), contrary to δ117.7 ppm, previously reported by other authors (Marini-Bettolo et al., 1982). In addition, the complete correlation of the NMR data has been obtained for the assignment of C2 and C5 carbons of the aromatic rings through HSQC for the first time.

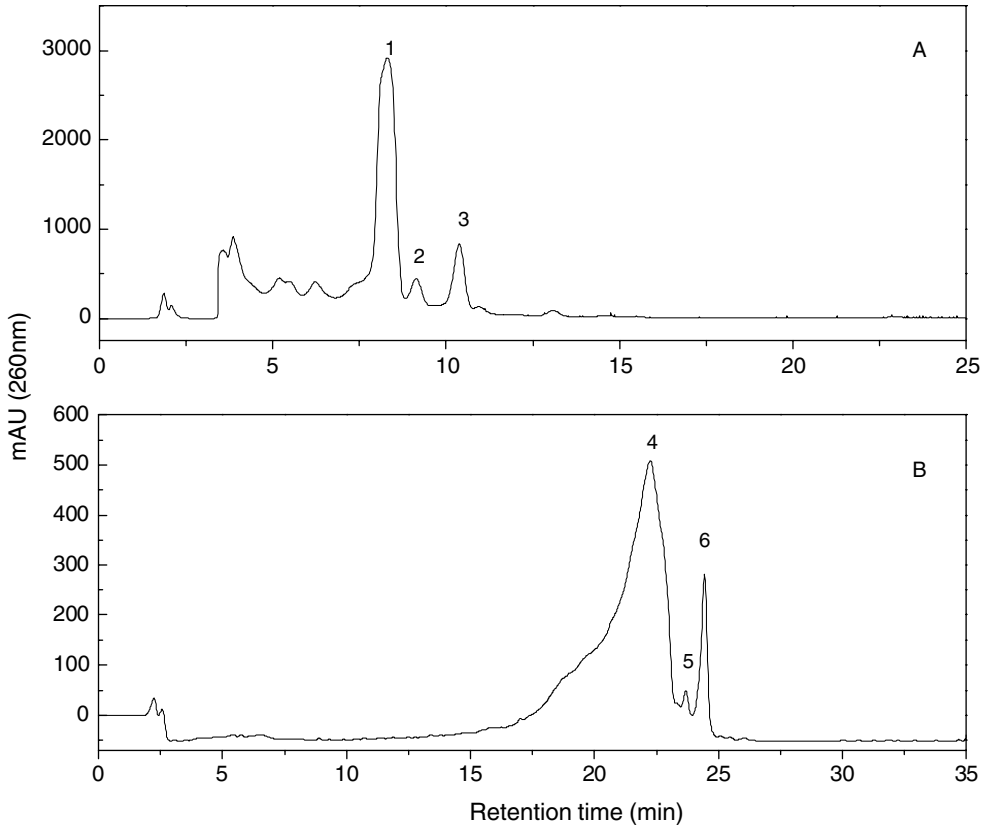


Fig. 3. Semipreparative HPLC profiles of the *Hypoxis rooperi* extract (A) and its β-glucosidase digestion (B) at 260 nm. Compounds are the same as in Fig. 2.

Table 1
Capacity factors, yield, and molar absorptivity of the compounds isolated from *Hypoxis rooperi* extract and its β-glucosidase digestion

Compound	Capacity factors		Yield (%w)[a]	ε^{260} (M^{-1} cm^{-1})[b]
	Analytical HPLC	Preparative HPLC		
1	2.32 ± 0.04	3.12 ± 0.05	20.1	25,100 ± 100
2	2.57 ± 0.02	3.58 ± 0.02	n.d.	–
3	2.91 ± 0.03	4.20 ± 0.02	3.1	–
4	4.79 ± 0.13	8.79 ± 0.01	2.55	24,900 ± 100
5	5.85 ± 0.15	10.77 ± 0.08	n.d.	–
6	7.02 ± 0.19	11.10 ± 0.11	1.36	–

[a] Relative error <10%.
[b] Determined in methanol.

3.3. Antioxidant capacity of the compounds from H. rooperi extract

The antioxidant capacity of the *H. rooperi* extract and the main compounds derived from it, hypoxoside and rooperol has been extensively studied in this work for the first time by using several methods for assessing in vitro antioxidant capacity. TBARS and TEAC assays were used to establish the antioxidant capacity of these norlignans. Fig. 6A shows the antioxidant capacity of hypoxoside and rooperol against lipid peroxidation by using TBARS assay. Both compounds showed exponential decay curves for malondialdehyde formation as their concentration was increased. Nevertheless rooperol exhibited a higher capacity to inhibit lipid peroxidation showing an IC_{50} value as low as 2.6 μM, in comparison to hypoxoside, i.e. 12.6 μM. Rooperol exhibited higher antioxidant activity than hypoxoside, as measured by TEAC assay (Fig. 6B), especially in the lipophilic medium (ethanol), 2.40 μM TE versus 1.48 μM TE. This fact occurred in both solvents, EtOH and water. However, the difference in the antioxidant potency between these two compounds was less dramatic through the TEAC assay than that one observed by the TBARS assay. These results may reveal that the difference in their respective redox potential values is not as big as that one related to their capacity to scavenge free radicals within a hydrophobic environment such as a phospholipid membrane, system in which rooperol seems to be specially efficient.

In addition, the capacity of the norlignans, hypoxoside and rooperol, to inhibit lipid peroxidation was compared to that one of other potent antioxidants from green tea or olive such as (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, oleuropein and hydroxytyrosol. Table 3 shows the concentration of the compounds at which 50% of the inhibition of total lipid peroxidation was achieved as measured by TBARS-HPLC assay. Among catechins, ECG was the strongest antioxidant as it has been previously reported (Caturla et al.,

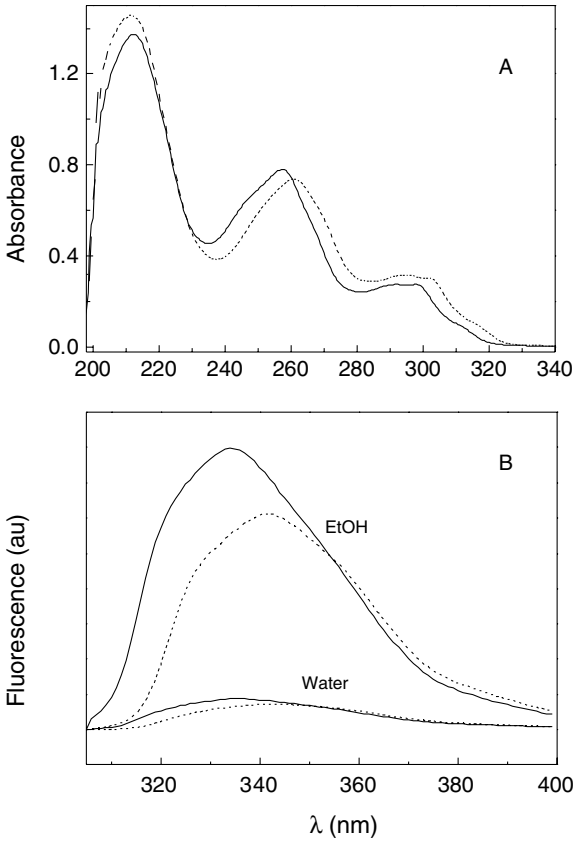
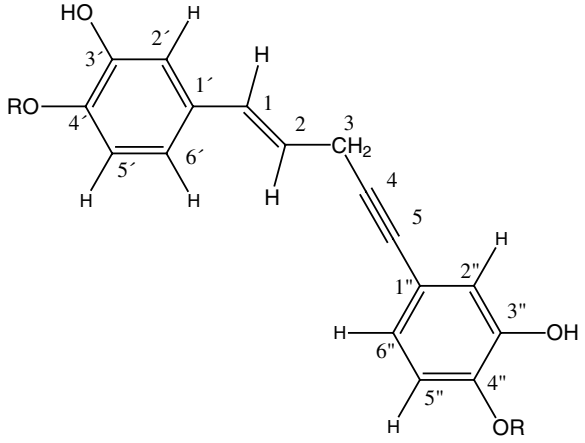


Fig. 4. (A) UV spectra of hypoxoside (**1**; solid line) and rooperol (**4**; dashed line) in methanol. (B) Corrected fluorescence emission spectra of **1** (solid line) and **4** (dashed line) in methanol.

Table 2
^{1}H and ^{13}C NMR chemical shifts comparison of hypoxoside (**1**; R = glucose) and its aglycone rooperol (**4**; R = H)[a]



	^{1}H NMR			^{13}C NMR	
	1[b]	**4**[c]		**1**[b]	**4**[c]
H1	6.56	6.51	C1	130.9	131.6
H2	6.07	6.08	C2	121.6	124.3
H3	3.26	3.23	C3	22.2	23.2
			C4	84.2	86.2
			C5	82.7	83.2
			C1′	115.1	114.4
H2′	6.94	7.08	C2′	118.5	118.2
			C3′	145.6; 145.1[d]	148.3; 148.0[d]
			C4′	144.8; 144.7[d]	146.8; 146.2[d]
H5′	6.76	6.85	C5′	115.3	120.0
H6′	6.81	6.82	C6′	123.8	124.5
			C1″	129.6	134.3
H2″	6.91	7.06	C2″	112.7	118.5
			C3″	[d]	[d]
			C4″	[d]	[d]
H5″	6.77	6.87	C5″	115.2	114.3
H6″	6.77	6.77	C6″	118.3	119.3
H1-Gluc.		4.74; 4.72	C1-Gluc.		104.1; 103.7
H2-Gluc.		3.40–3.37[e]	C2-Gluc.		78.2
H3-Gluc.		3.48–3.42[f]	C3-Gluc.		74.8
H4-Gluc.		[e]	C4-Gluc.		71.2
H5-Gluc.		[f]	C5-Gluc.		77.6
H6-Gluc.		3.85; 3.69	C6-Gluc.		62.3

[a] Chemical shifts in ppm, methyl group of the deuterated solvents was used as reference for shift tabulated values (Gottlieb, Kotlyar, & Nudelman, 1997).
[b] CD_6CO, deuterated solvent.
[c] CD_3OH, deuterated solvent.
[d] Signals may be interchanged.
[e] Multiples corresponding to H2-Gluc and H4-Gluc.
[f] Multiples corresponding to H3-Gluc and H5-Gluc.

2003; Ou et al., 2002), i.e. 2.3 μM, followed by EGCG and EC, which showed similar antioxidant capacity. Catechin evidenced the lowest antioxidant capacity of all the catechins studied, around 4× less potent than EGCG. The IC_{50} value obtained for rooperol, i.e. 2.6 μM, was close to that one found for the very strong antioxidant catechin, ECG. Rooperol also showed an IC_{50} value lower than those obtained for other compounds claimed to be powerful antioxidants such as oleuropein or hydroxytyrosol from olive leaf and fruit (Benavente-García, Castillo, Lorente, Ortuño, & Del Río, 2000; Gordon, Paiva-Martins, & Almeida, 2001; Saija et al., 1998), when measured at the same assay conditions. In agreement to previously reported, we found oleuropein to be a stronger antioxidant than hydroxytyrosol to inhibit lipid peroxidation (Saija et al., 1998), which showed an IC_{50} value almost nine times bigger than ECG.

Regarding TEAC values obtained for the analyzed compounds, differences were smaller than those observed through TBARS assay. Previously reported comparative studies on TEAC (Miller & Ruiz-Larrea, 2002; Rice-Evans, Miller, & Paganga, 1996) showed values around 4 only for very powerful antioxidants phenols such as quercetin, galloylated catechins or some anthocyanins. The strongest antioxidant molecules in EtOH through TEAC assay in our study were found to be EGCG, with almost four times the antioxidant capacity of Trolox (3.87 mM), followed by ECG (3.44 mM). In contrast, EC and C showed lower TEAC values, i.e. 2.70 and 2.02 mM, respectively. As previously mentioned, rooperol and hypoxoside showed lower lipophilic TEAC values than galloylated catechins. For the majority of the compounds studied, their lipophilic TEAC values were higher than their respective hydrophilic TEAC values, especially for those compounds being more hydrophobic such as rooperol, ECG or EGCG (Caturla et al., 2003). Oleuropein and hydroxytyrosol did not show

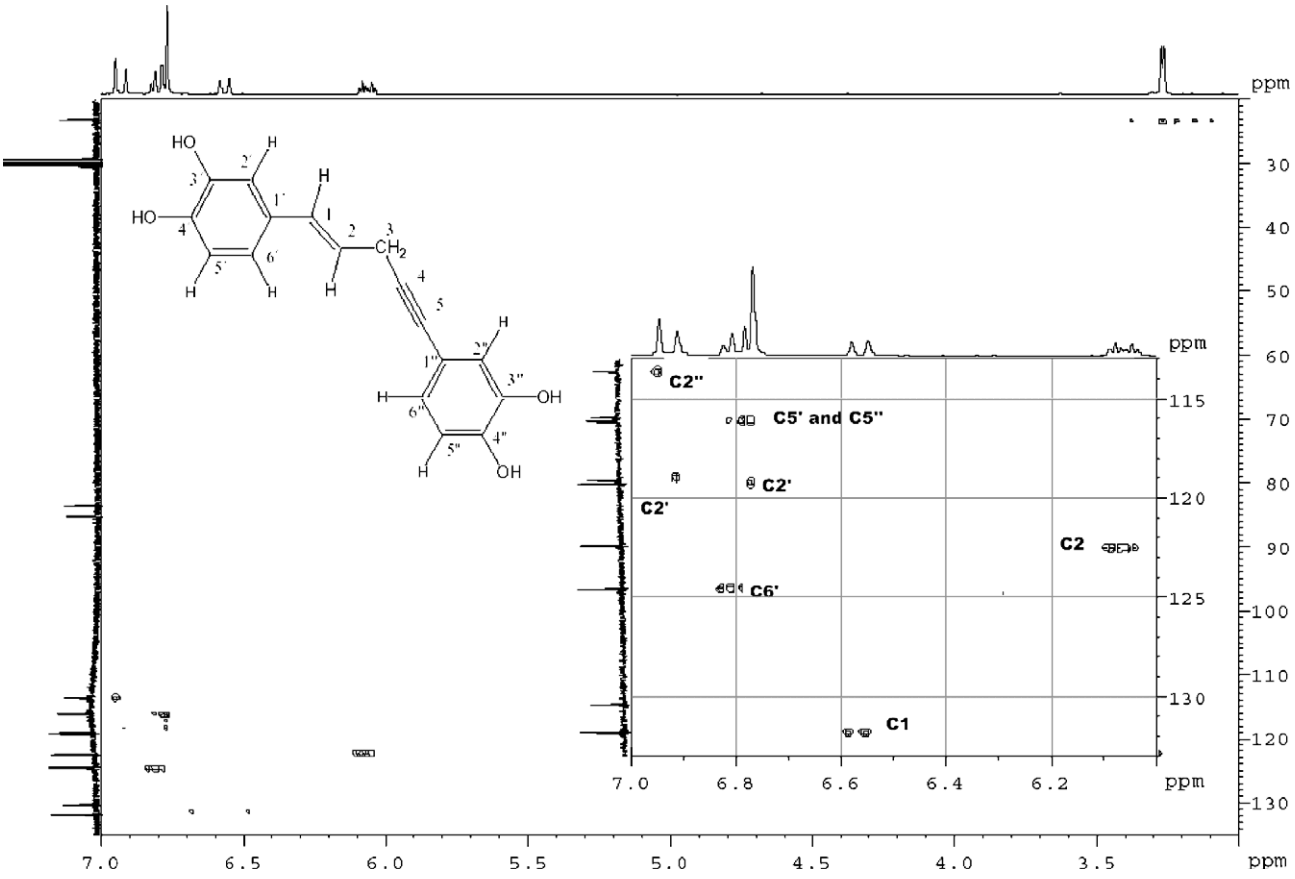


Fig. 5. {^{1}H, ^{13}C} HSQC NMR spectrum of rooperol (**4**) in acetone-d_6. Inset: magnification corresponded to downfielded spectrum.

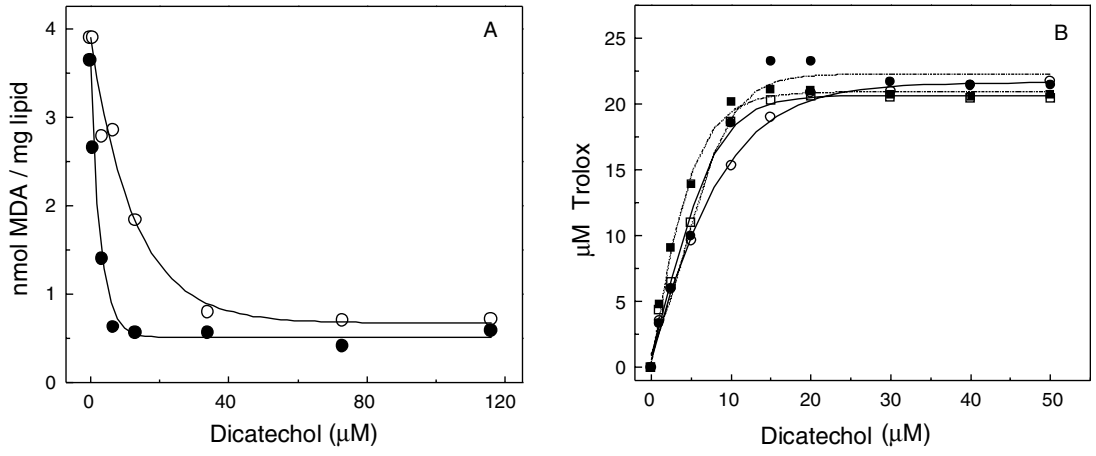


Fig. 6. (A) Quantitative evaluation of the antioxidant capacity of hypoxoside (open symbols) and rooperol (bold symbols) against lipid peroxidation as determined by TBARS assay. (B) Capacity of hypoxoside (open symbols, solid line) and rooperol (closed symbols, dashed line) to scavenge radicals ($ABTS^{\cdot+}$) studied by the TEAC assay and expressed as the Trolox equivalent antioxidant capacity (circles, water; squares, EtOH). Curves are representative of three independent experiments. The relative error of average values shown in the curves was less than 5%.

significant differences in their hydrophilic TEAC values, as observed previously (Paiva-Martins, Gordon, & Gameiro, 2003), although oleuropein was again a stronger antioxidant than hydroxytyrosol in the lipophilic medium through TEAC assay (0.99 versus 0.62) in agreement with the results observed in the TBARS assay. All these results may reflect the capability of the lipophilic TEAC assay to determine the capacity of hydrophobic antioxidants in a more reliable way than the hydrophilic TEAC assay. This variability for TEAC values depending on the solvent has also been previously observed by other authors (Van den Berg, Haenen, Van den Berg, & Bast, 1999).

Table 3
Lipid/water partition coefficients, lipophilic and hydrophilic TEAC values and IC_{50} TBARS values of several antioxidant molecules from green tea, African potato and olive leaf

Compound	K_p[a] (lipid/H_2O)	Antioxidant capacity			Source
		TEAC mM (EtOH)[b]	TEAC mM (H_2O)[c]	TBARS[d] IC_{50} (μM)	
(+)-Catechin, C	2000	2.02	2.12	10.0	*Camellia sinensis*
(−)-Epicatechin, EC	2200	2.70	2.14	4.64	*Camellia sinensis*
(−)-Epicatechin gallate, ECG	29,200	3.44	2.51	2.30	*Camellia sinensis*
(−)-Epigallocatechin gallate, EGCG	19,200	3.87	2.50	4.26	*Camellia sinensis*
Hypoxoside	1138	1.48	1.23	12.60	*Hypoxis rooperi*
Rooperol	33,906	2.40	2.27	2.60	*Hypoxis rooperi*
Oleuropein	920	0.99	0.91	9.14	*Olea europaea*
Hydroxytyrosol	250	0.62	0.91	19.20	*Olea europaea*

The relative error for average values (three independent determinations) was less than 5%.
[a] Lipid/water partition coefficient calculated from the intrinsic fluorescence of the compound.
[b] mM Trolox equivalent antioxidant capacity determined in EtOH.
[c] mM Trolox equivalent antioxidant capacity determined in H_2O.
[d] Concentration corresponding to the 50% inhibition of total lipid peroxidation determined by TBARS-HPLC assay.

The highest difference found in Table 3 among hydrophilic TEAC values was smaller than 3× whereas the largest difference among lipophilic TEAC values was close to 6×. In contrast, IC_{50} TBARS values revealed differences greater than 8×, i.e. between hydroxytyrosol and ECG. These results corroborate that hydrophilic TEAC assay may reveal the antioxidant capacity of a compound based on its redox potential but it does not show the capability of the compound to scavenge free radicals under a specific environment such as a biological membrane. Nevertheless, lipophilic TEAC assay seems to monitorize the antioxidant capacity of a hydrophobic compound in a better way. In addition, TBARS assay may be more reliable to determine the capability of a compound to inhibit lipid peroxidation and may expose bigger differences than expected in the antioxidant capacity.

Table 3 also shows the lipid/water partition coefficients of the same compounds for which the antioxidant capacity has been determined, compared to the norlignans hypoxoside and rooperol. The partition coefficients of the norlignans, K_p, were determined from the intrinsic fluorescence intensity increase upon the incorporation to large unilamellar vesicles (LUVs) composed of DMPC, in comparison to that in the aqueous phase. Rooperol showed an extremely high phospholipid/water partition coefficient ($K_p = 3.4 \times 10^4$), although hypoxoside exhibited also a significant membrane affinity ($K_p = 0.11 \times 10^4$), values which represent 92% and 75%, respectively, of the dicatechol molecules being associated to phospholipid membranes, both determined at lipid-saturating conditions. Considering this fact, the interaction of rooperol with membranes may play a crucial role in the mode of action of this molecule which underlies its anti-inflammatory capacity (Guzdek et al., 1996; Ojewole, 2002; Steenkamp et al., 2005; Van der Merwe et al., 1993). As shown in Table 3, those antioxidants exhibiting extremely high lipid/water partition coefficients and therefore having strong membrane affinity such as ECG, EGCG (Caturla et al., 2003) or rooperol showed the lowest IC_{50} TBARS values, therefore strongly inhibiting lipid peroxidation, and also correlated

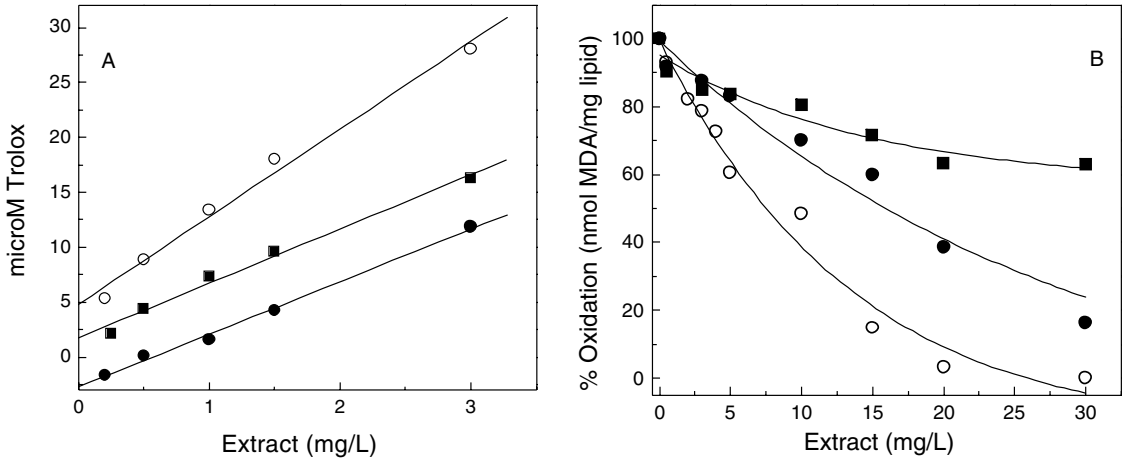


Fig. 7. (A) Antioxidant dose–response curves of *Hypoxis rooperi* extract (open circles) compared to green tea (closed circles) and olive leaf (closed squares) extracts as chain-breaking antioxidants against peroxyl radicals using the $ORAC_{FL}$ assay. (B) Antioxidant capacity of the same extracts using the TBARS assay. Curves are representative of three independent experiments. The relative error of average values shown in the curves was less than 5%.

with the highest lipophilic TEAC values. In addition, oleuropein, which had a higher K_p than hydroxytyrosol (Caturla et al., 2005; Gordon et al., 2001; Paiva-Martins et al., 2003), demonstrated to be a stronger antioxidant than hydroxytyrosol as measured by TBARS and lipophilic TEAC assays, although these compounds do not show big differences in their hydrophilic TEAC values, as previously reported (Paiva-Martins et al., 2003).

The antioxidant capacity of the *H. rooperi* extract was also compared to other potent antioxidant vegetable extracts containing the compounds studied in Table 3, i.e. green tea and olive leaf, by ORAC and TBARS assays. Antioxidant dose–response curves of these extracts are shown in Fig. 7A, which reveal the antioxidant activity against peroxyl radicals through the measurement of the area under the fluorescence decay curve (AUC) of the sample as compared to that in the absence of antioxidant in the $ORAC_{FL}$ assay. African potato extract showed an $ORAC_{FL}$ value of 7960 ± 500 TE/g dw, a strong value compared to other antioxidant powdered extracts measured in the same conditions (Ou et al., 2001). In contrast, green tea extract (70% catechins) and olive leaf extract (25% oleuropein) exhibited lower antioxidant potency, i.e. 4780 ± 200 and 4950 ± 300 TE/g dw, respectively. The capability of *H. rooperi* extract to inhibit lipid peroxidation was also determined by TBARS assay (Fig. 7B) and compared to the same extracts used in Fig. 7A. *H. rooperi* extract showed again the highest antioxidant capacity, i.e. IC_{50} 7.5 mg/L, compared to green tea and olive leaf extracts, IC_{50} values of 16 and >30 mg/L, respectively. Therefore, *H. rooperi* extract exhibited the highest efficacy to scavenge peroxyl radicals and to inhibit lipid peroxidation from the three extracts studied.

3.4. Oral acute toxicity of H. rooperi extract

The administration of a daily dosage up to 3.2 g of a standardized *H. rooperi* extract showed absence of toxicity in a phase I clinical trial in human patients suffering lung cancer (Albrecht, Kruger, et al., 1995; Smit et al., 1995). Nevertheless, we performed an acute toxicity test at higher concentrations in mice to establish the approximate oral LD_{50}. A single dose of 2000 mg/kg b.wt. of *H. rooperi* extract (45% hypoxoside) was utilized and mice were observed through a 2-week period. No deaths occurred in either the control or the *H. rooperi* groups during treatment. No significant alterations in the body weight of the *H. rooperi*-treated groups (male and female) compared with the control were observed (Fig. 8).

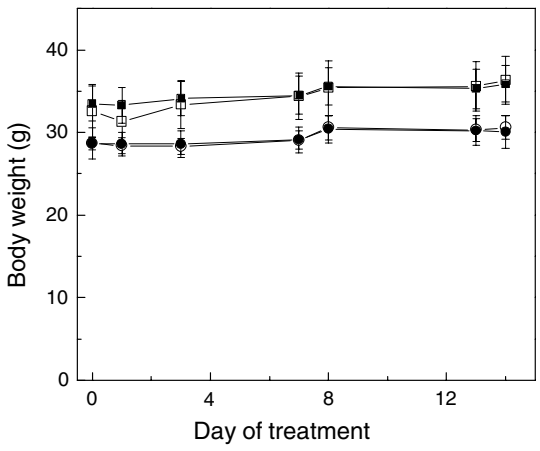


Fig. 8. Body weight changes in male (squares) or female (circles) mice treated orally with extract of *Hypoxis rooperi*. 2000 mg/kg dose (closed symbols) and control group (open symbols).

Once the assay was completed, two individuals (one from each sex) treated with extract were sacrificed and compared with their respective controls through postmortem and pathological examinations. Observation comprised examination of the external surface of the body (skin), all orifices, mucous membranes and the cranial, thoracic and abdominal cavities and their contents. The postmortem analysis of either male or female individuals subjected to treatment did not show abnormalities on vital organs such as brain, heart, lungs, liver, spleen, kidneys or intestines. Digestive system (stomach, duodenum, ileum, etc.) was also completely normal compared to controls. In addition, no particular gender-related effects were observed since no toxicity was noticed in male or female groups. The complete absence of toxicity at a concentration as high as 2000 mg/kg b.wt. means that the substance may be classified either as a very low toxicity substance, i.e. GHS Category 5 (globally harmonized system) or unclassified. Considering that the *H. rooperi* extract used contained 45% hypoxoside, an LD_{50} value for the pure compound, hypoxoside, of $\geqslant$900 mg/kg b.wt. might be extrapolated.

4. Conclusions

The results shown in this work demonstrate that extracts derived from *H. rooperi* rhizome bearing a high content of hypoxoside might have a potential use as a strong antioxidant in nutraceuticals or dietary supplements, besides its pharmacological application. The compounds derived from the extract, especially rooperol, have an outstanding capacity to inhibit lipid peroxidation, as well as galloylated catechins. Rooperol also exhibited an extremely high affinity for phospholipids membranes. Moreover, those compounds having very strong capacity to inhibit free-radical-induced membrane lipoperoxidation, and therefore bearing strong antioxidant capacity through TBARS or lipophilic TEAC assays, such as rooperol and galloylated catechins, correlated with a high lipid/water partition coefficient. These facts reinforce the statement that the antioxidant capacity of a phenolic compound is highly related to its ability to interact with and penetrate the lipid bilayers, as reported by other authors (Erlejman, Verstraeten, Fraga, & Oteiza, 2004; Rice-Evans et al., 1996; Saija et al., 1995). Then, features further than the number of hydroxyl groups or the polymerization degree must account for the total antioxidant activity of a phenolic

compound. In addition, TBARS assay and, in a less extent, lipophilic TEAC assay, seem to provide more reliable values of the antioxidant capacity against lipid peroxidation for hydrophobic compounds, whereas hydrophilic TEAC assay underestimate it.

Acknowledgments

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References

Albrecht, C. F. (1995). Hypoxoside: a putative, non-toxic prodrug for the possible treatment of certain malignancies, HIV-infections and inflammatory conditions. *South African Medical Journal, 85*, 302–307.

Albrecht, C. F., Kruger, P. B., Smit, B. J., Freestone, M., Gouws, L., Miller, R., et al. (1995). The pharmacokinetic behaviour of hypoxoside taken orally by patients with lung cancer in a phase I trial. *South African Medical Journal, 85*, 861–865.

Albrecht, C. F., Theron, E. J., & Kruger, P. B. (1995). Morphological characterisation of the cell-growth inhibitory activity of rooperol and pharmacokinetic aspects of hypoxoside as an oral prodrug for cancer therapy. *South African Medical Journal, 85*, 853–860.

Benavente-García, O., Castillo, J., Lorente, J., Ortuño, A., & Del Río, J. A. (2000). Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chemistry, 68*, 457–462.

Betto, P., Gabriele, R., & Galeffi, C. (1992). Determination of the norlignan glucosides of hypoxidaceae by high-performance liquid chromatography. *Journal of Chromatography, 594*, 131–135.

Böttcher, C. S. J., Van Gent, C. M., & Priest, C. (1961). A rapid and sensitive submicro phosphorus determination. *Analytical Chimica Acta, 24*, 203–204.

Caturla, N., Perez-Fons, L., Estepa, A., & Micol, V. (2005). Differential effects of oleuropein, a biophenol from *Olea europaea*, on anionic and zwiterionic phospholipid model membranes. *Chemistry and Physics of Lipids*.

Caturla, N., Vera-Samper, E., Villalain, J., Mateo, C. R., & Micol, V. (2003). The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biology and Medicine, 34*, 648–662.

Dietzsch, E., Albrecht, C. F., & Parker, M. I. (1999). Effect of rooperol on collagen synthesis and cell growth. *IUBMB Life, 48*, 321–325.

Dreikorn, K. (2000). Phytotherapeutic agents in the treatment of benign prostatic hyperplasia. *Current Urology Reports, 1*, 103–109.

Dreikorn, K., & Schonhofer, P. S. (1995). Status of phytotherapeutic drugs in treatment of benign prostatic hyperplasia. *Urologe A, 34*, 119–129.

Drewes, S. E., Hall, A. J., Learmonth, R. A., & Upfold, U. J. (1984). Isolation of hypoxoside from *Hypoxis rooperi* and synthesis of (*E*)-1,5-bis(3,4-dimethoxyphenyl)pent-4-en-1-yne. *Phytochemistry, 23*, 1313–1316.

Drewes, S. E., Scogins, U. J., & Wenteler, G. L. (1989). Structure determination of a phenolic pent-1-en-4-yne derivative from *Hypoxis rooperi. Phytochemistry, 28*, 153–156.

Erlejman, A. G., Verstraeten, S. V., Fraga, C. G., & Oteiza, P. I. (2004). The interaction of flavonoids with membranes: potential determinant of flavonoid antioxidant effects. *Free Radical Research, 38*, 1311–1320.

Fagelman, E., & Lowe, F. C. (2002). Herbal medications in the treatment of benign prostatic hyperplasia (BPH). *Urologic Clinics of North America, 29*, 23–29, vii.

Geerinck, D. (1968). Considérations taxonomiques au sujet des Haemodoraceae et des Hypoxidaceae (Monocotyledones). *Bulletin de la Société Royale de Botanique de Belgique, 101*, 265–278.

Gomez-Fernandez, J. C., Villalain, J., Aranda, F. J., Ortiz, A., Micol, V., Coutinho, A., et al. (1989). Localization of alpha-tocopherol in membranes. *Annals of the New York Academy of Sciences, 570*, 109–120.

Gordon, M. H., Paiva-Martins, F., & Almeida, M. (2001). Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. *Journal of Agricultural and Food Chemistry, 49*, 2480–2485.

Gottlieb, H. E., Kotlyar, V., & Nudelman, A. (1997). NMR chemical shifts of common laboratory solvents as trace impurities. *Journal of Organic Chemistry, 62*, 7512–7515.

Guzdek, A., Nizankowska, E., Allison, A. C., Kruger, P. B., & Koj, A. (1996). Cytokine production in human and rat macrophages and dicatechol rooperol and esters. *Biochemical Pharmacology, 52*, 991–998.

Jager, A. K., Hutchings, A., & van Staden, J. (1996). Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. *Journal of Ethnopharmacology, 52*, 95–100.

Kruger, P. B., Albrecht, C. F., Liebenberg, R. W., & van Jaarsveld, P. P. (1994). Studies on hypoxoside and rooperol analogues from *Hypoxis rooperi* and *Hypoxis latifolia* and their biotransformation in man by using high-performance liquid chromatography with in-line sorption enrichment and diode-array detection. *Journal of Chromatography B-Biomedical Applications, 662*, 71–78.

Liebenberg, R. W. (1969). Pharmaceutical corm extract of *Hypoxis*, antiinflamatory and for the treatment of prostate gland hypertrophy. In: German Offenlegungsschrift Patent 2015877.

Mahomed, I. M., & Ojewole, J. A. (2003). Hypoglycemic effect of *Hypoxis hemerocallidea* corm (African potato) aqueous extract in rats. *Methods and Findings in Experimental and Clinical Pharmacology, 25*, 617–623.

Marini-Bettolo, G. B., Patamia, M., & Nicoletti, M. (1982). Hypoxoside, a new glycoside of uncommon structure from *Hypoxis obtusa* Busch. *Tetrahedron, 38*, 1683–1687.

Marsh, D. (1990). *Handbook of lipid bilayers*. Boca Raton, USA: CRC Press.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science (London), 84*, 407–412.

Miller, N. J., & Ruiz-Larrea, M. B. (2002). Flavonoids and other plant phenols in the diet: their significance as antioxidants. *J. Nutr. Environ. Med., 12*, 39–51.

Mills, E., Cooper, C., Seely, D., & Kanfer, I. (2005). African herbal medicines in the treatment of HIV: hypoxis and sutherlandia. An overview of evidence and pharmacology. *Nutrition Journal, 4*, 19.

Mills, E., Foster, B. C., van Heeswijk, R., Phillips, E., Wilson, K., Leonard, B., et al. (2005). Impact of African herbal medicines on antiretroviral metabolism. *Aids, 19*, 95–97.

Nicoletti, M., Galeffi, C., Messana, I., & Marini-Bettolo, G. B. (1992). Hypoxidaceae. Medicinal uses and the norlignan constituents. *Journal of Ethnopharmacology, 36*, 95–101.

Ojewole, J. A. (2002). Antiinflammatory properties of *Hypoxis hemerocallidea* corm (African potato) extracts in rats. *Methods and Findings in Experimental and Clinical Pharmacology, 24*, 685–687.

Ojewole, J. A. (2005). Antinociceptive, anti-inflammatory and antidiabetic properties of *Hypoxis hemerocallidea* Fisch. & C.A. Mey. (Hypoxidaceae) corm ['African Potato'] aqueous extract in mice and rats. *Journal of Ethnopharmacology*.

Ou, B., Hampsch-Woodill, M., Flanagan, J., Deemer, E. K., Prior, R. L., & Huang, D. (2002). Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *Journal of Agricultural and Food Chemistry, 50*, 2772–2777.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*, 4619–4626.

Paiva-Martins, F., Gordon, M. H., & Gameiro, P. (2003). Activity and location of olive oil phenolic antioxidants in liposomes. *Chemistry and Physics of Lipids, 124*, 23–36.

Parker, C. A. (1968). *Photoluminiscence of solutions*. Amsterdam: Elsevier.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*, 933–956.

Saija, A., Scalese, M., Lanza, M., Marzullo, D., Bonina, F., & Castelli, F. (1995). Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radical Biology and Medicine, 19*, 481–486.

Saija, A., Trombetta, D., Tomaino, A., Lo Cascio, R., Princi, P., Uccella, N., et al. (1998). In vitro evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. *International Journal of Pharmaceutics, 166*, 123–133.

Smit, B. J., Albrecht, C. F., Liebenberg, R. W., Kruger, P. B., Freestone, M., Gouws, L., et al. (1995). A phase I trial of hypoxoside as an oral prodrug for cancer therapy – absence of toxicity. *South African Medical Journal, 85*, 865–870.

Steenkamp, V., Gouws, M. C., Gulumian, M., Elgorashi, E. E., & van Staden, J. (2005). Studies on antibacterial, anti-inflammatory and antioxidant activity of herbal remedies used in the treatment of benign prostatic hyperplasia and prostatitis. *Journal of Ethnopharmacology*.

Van den Berg, R., Haenen, G., Van den Berg, H., & Bast, A. (1999). Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chemistry, 66*, 511–517.

Van der Merwe, M. J., Jenkins, K., Theron, E., & van der Walt, B. J. (1993). Interaction of the di-catechols rooperol and nordihydroguaiaretic acid with oxidative systems in the human blood. A structure–activity relationship. *Biochemical Pharmacology, 45*, 303–311.

Van Wyk, B. E., Van Oudshoorn, B., & Gericke, N. (1997). *Medicinal plants of South Africa*. Pretoria: Briza Publications.

Wilt, T. J., Ishani, A., Rutks, I., & MacDonald, R. (2000). Phytotherapy for benign prostatic hyperplasia. *Public Health Nutrition, 3*, 459–472.