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In vitro modulation of the innate immune response and phagocytosis by three *Hypoxis* spp. and their phytosterols

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ABSTRACT

Hypoxis forms an important part of South African traditional medicine. Crude *Hypoxis* extracts and hypoxoside have extensively been investigated for its biological activities. This study investigated the *in vitro* biological activities of *Hypoxis* extracts and its phytosterols on the innate immune system. Reactive oxygen species (ROS) and nitric oxide (NO) production in undifferentiated and/or differentiated human promonocytic U937 leukemia cells were investigated using the fluorescent dyes, 2,7-dichlorofluorescein diacetate and 5,6-diaminofluorescein diacetate, respectively. The phagocytic ability of U937 cells was investigated using pHrodo™ *E. coli* Bioparticle® Conjugate. Superoxide dismutase activation in Chang liver cells were measured following (phyto)sterol treatment. *Hypoxis stellipilis* extract has shown the highest ROS and NO production. *Hypoxis* extracts and β-sitosterol significantly increased phagocytic activity of pretreated differentiated U937 cells. For superoxide dismutase activation, only the (phyto)sterols had an effect. Phytosterols, in combination with other compounds found in *Hypoxis* extracts, might be responsible for increased ROS and NO production and phagocytosis seen in U937 cells, ultimately influencing the innate immune system response.

1. Introduction

Hypoxis (syn. African corms), belonging to the family *Hypoxidaceae* (Singh, 2007), is one of the most commonly used medicinal plants in South Africa (Drewes and Khan, 2004). In traditional medicine, it is used for the treatment of cancer, human immunodeficiency virus, diabetes mellitus, inflammation, urinary tract infection, prostatitis and benign prostate hyperthrophy (Drewes et al., 2008; Ncube et al., 2013). In addition to its medicinal properties, *Hypoxis* spp. are used as food in times of famine by the indigenous people of South Africa (Singh, 1999). Several glycosides have been identified and isolated from *Hypoxis* species (Nicoletti et al., 1992). The first glycoside to be isolated was hypoxoside [(E)-1,5-bis(4'-β-D-glucopyranosyloxy-3'-hydroxyphenyl) pent-4-en-1-yne] and its aglycone, rooperol (Drewes et al., 1984, 1989; Marini-Bettolo et al., 1982). Biologically inactive hypoxoside is catalyzed by β-glucosidase to form active rooperol (Drewes et al., 1984; Theron et al., 1994). Several studies have been conducted on the biological activity of rooperol (Albrecht et al., 1995; Boukes and van de Venter, 2012; Boukes et al., 2010; Guzdek et al., 1996; Laporta et al., 2007a, 2007b; Nair et al., 2007; van der Merwe et al., 1993).

Hypoxis spp. are rich in phytosterols, especially β-sitosterol and its sterolin, β-sitosterol glucoside (Boukes et al., 2008). Several commercial products, including Harzol®, Moducare®, Hypo-Plus and Prostone, contain sterols and sterolins first isolated or derived from *Hypoxis* spp. (Abegaz et al., 1999; Drewes and Khan, 2004). β-Sitosterol has cholesterol-lowering (Richelle et al., 2004; Vorster et al., 2003), anticancer (Awad and Fink, 2000), immune modulating (Bouic and Lamprecht, 1999; Breytenbach et al., 2001), anti-inflammatory, antioxidant and antidiabetic (Lagarda et al., 2006; Pegel, 1997) properties. Phytosterols are incorporated into functional foods to reduce the risk of cardiovascular diseases (Vorster et al., 2003). The main phytosterols found in plants include sitosterol, campesterol and stigmasterol (Moghadasian, 2000).

The human immune system is divided into innate (natural) and adaptive (acquired) immunity (Dale et al., 2008). The innate immune system detects pathogens and apoptotic cells via specific receptors and responds by activating immune competent (phagocytic) cells, including monocytes, macrophages, polymorphonuclear, natural killer and mast cells (Nair et al., 2004). The innate immune response is the first line of antimicrobial host defense and impacts the adaptive immune response. Phagocytes undergo increased metabolic activities (respiratory burst) during phagocytosis, which is associated with the generation of a superoxide radical (O_2^-), when oxygen is reduced by NADPH oxidase. O_2^- undergoes several reactions to form reactive oxygen intermediates, including hydrogen peroxide (H_2O_2), hydroxyl

Abbreviations: AA, arachidonic acid; CD, cyclodextrin; COX, cyclooxygenase; cPLA A_2, cytosolic phospholipase A_2; DAF-2 DA, 5,6-diaminofluorescein diacetate; DCFH-DA, 2,7-dichlorofluorescein diacetate; 1,25$(OH)_2D_3$, 1α,25-dihydroxycholecalciferol; MFI, mean fluorescence intensity; NO, nitric oxide; PMA, phorbol myristate acetate; ROS, reactive oxygen species; SOD, superoxide dismutase.

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radicals and hypoclorous acid, which are microbicidal agents. Respiratory burst can be induced by intact bacteria, opsonized particles, endotoxins, cytokines, N-formylated chemoattractant peptides and protein kinase C activators (Guzdek et al., 1997). Macrophages activate nitric oxide synthase, leading to nitric oxide (NO) production. NO has cytostatic or cytotoxic activity against viruses, bacteria, fungi, helminthes, protozoa and tumor cells (Dale et al., 2008; Jiménez et al., 1999) and is involved in the production of free radicals (Jiménez et al., 1999). Interaction between reactive oxygen and nitrogen intermediates forms peroxynitrite, which increases cytotoxicity and inflammation (Guzdek et al., 1997).

In this study, novel findings are reported on the effect of three *Hypoxis* spp. extracts on reactive oxygen species (ROS), NO production, and phagocytosis. Furthermore, the role of phytosterols as purified compounds in the *Hypoxis* extracts was investigated in the above-mentioned biological activities as well as in superoxide dismutase (SOD) activation. Together, these biological activities can explain the effect of *Hypoxis* extracts and its phytosterols on the innate immune system.

2. Materials and methods

2.1. Materials/reagents/chemicals

Human promonocytic U937 leukemia cells and Chang liver cells were purchased from Highveld Biological, South Africa. Phorbol myristate acetate (PMA), 2,7-dichlorofluorescein diacetate (DCFH-DA), β-sitosterol, campesterol, cholesterol and stigmasterol were purchased from Sigma (St. Louis, MO, USA). 5,6-Diaminofluorescein diacetate (DAF-2 DA) was purchased from Fluka (St Louis, MO, USA). $1,25(OH)_2D_3$ (syn. calcitriol, 1α,25-dihydroxycholecalciferol) was purchased from Alexis Biochemicals (Lausen, Switzerland). Phagocytosis kit, pHrodo™ *E. coli* BioParticles® Conjugate, was purchased from Invitrogen (San Diego, CA, USA).

2.2. Plant material and preparation of extracts

Corms of *Hypoxis hemerocallidea* (voucher number: PEU 14798) and *Hypoxis stellipilis* (PEU14841) were purchased in Port St John's and Port Elizabeth (Xhosa traditional medicine shop), respectively, in the Eastern Cape, South Africa. Corms of *Hypoxis sobolifera* var *sobolifera* (PEU 14840) were collected near Plettenberg Bay in the Southern Cape, South Africa. Corms of the three *Hypoxis* spp. were planted in the same soil type and exposed to the same environmental conditions for at least 6 months before they were harvested and used fresh. *Hypoxis* spp. were identified by Dr. Y. Singh from the South African National Biodiversity Institute, and voucher specimens were deposited in the Nelson Mandela Metropolitan University herbarium. Chloroform extracts of *H. hemerocallidea*, *H. stellipilis* and *H. sobolifera* corms were prepared as described by Boukes et al., 2008. Extracts were dried *in vacuo* to remove all traces of chloroform before addition to cells. Chloroform was used as an organic solvent to ensure extraction of non-polar compounds, especially phytosterols and sterolins. The amounts of β-sitosterol quantified in *H. hemerocallidea*, *H. stellipilis* and *H. sobolifera* chloroform extracts, using gas chromatography, were 29.38, 10.05 and 74.69 μg per 5 mg of extract, respectively (Boukes et al., 2008). This equates to 1.77, 0.61 and 4.50 μM of β-sitosterol per 125 μg/mL of *H. hemerocallidea*, *H. stellipilis* and *H. sobolifera* treatment, respectively. Trace amounts (<2 μg/mL) of campesterol were identified in *H. hemerocallidea*, *H. stellipilis* and *H. sobolifera*, while trace amounts of stigmasterol were identified in *H. hemerocallidea* and *H. sobolifera* (Boukes et al., 2008). Chloroform extracts of *Hypoxis* were tested for endotoxins using the Limulus Amebocyte Lysate Endochrome™ kit. Endotoxin levels in the extracts were less than the maximum level (<1 ng/mL of LPS) allowed in samples for immunological experiments (data not shown).

2.3. Monocyte to monocyte-macrophage differentiation

Human promonocytic U937 leukemia cells were differentiated to monocyte-macrophages using 100 nM $1,25(OH)_2D_3$ for 24 h as described by Boukes and van de Venter (2012).

2.4. Increasing solubility of phytosterols using cyclodextrin

2-Hydroxypropyl-β-cyclodextrin [CD; Shimoda Biotech (PTY) Ltd.] was dissolved in saline (0.9% NaCl, 50 mM) and filter sterilized. Prior to treatment, β-sitosterol, campesterol, cholesterol and stigmasterol were dissolved in absolute EtOH (5 mg/mL), sonicated for 15 min and diluted in CD, absolute EtOH and complete medium. Dilutions were made to reach concentrations of 30 μM of the respective (phyto)sterol, 4 mM CD and 5% EtOH. These concentrations have not shown any cytotoxicity (data not shown).

2.5. Reactive oxygen species (ROS) production

Undifferentiated and differentiated U937 cells were counted and resuspended in complete medium at cell densities of 1×10^6 cells/mL. U937 cells were uniformly stained by adding 1 mM DCFH-DA stock solution to reach a final concentration of 1 μM. Cells were mixed and incubated in the dark for 30 min at 37 °C. Excess DCFH-DA was removed and cells washed twice with complete medium (i.e., RPMI supplemented with 10% fetal bovine serum) by centrifuging at 250×*g* for 5 min at room temperature. Fifty thousand cells were added to polypropylene tubes and treated with DMSO (2.5%, v/v), *H. hemerocallidea* (125 μg/mL), *H. stellipilis* (125 μg/mL), *H. sobolifera* (125 μg/mL), CD/EtOH (4 mM/5%, v/v), β-sitosterol (30 μM), campesterol (30 μM), cholesterol (30 μM), stigmasterol (30 μM) or PMA (10 nM) for 1 h at 37 °C. DMSO and CD/EtOH were vehicle controls. After treatment, cells were pelleted and washed by centrifugation with PBS (1 mL). Cells were resuspended in PBS (500 μL) and samples read on a Beckman Coulter Cytomics FC500 flow cytometer. Mean fluorescence intensity (MFI) of treated cells was expressed as a percentage of the MFI of control cells.

2.6. Superoxide dismutase (SOD) activity

U937 cells have low levels of endogenous MnSOD mRNA and protein levels (Suresh et al., 2003), and CuZn-SOD mRNA levels are decreased during differentiation (Saito et al., 1989), hence the use of Chang liver cells. Chang liver cells were seeded at densities of 40,000 cells/mL in 24-well plates and left to attach overnight at 37 °C in a humidified incubator and 5% CO_2. Cells were treated with DMSO (0.25%, v/v), *H. hemerocallidea* (125 μg/mL), *H. stellipilis* (125 μg/mL), *H. sobolifera* (125 μg/mL), CD/EtOH (4 mM/5%, v/v), β-sitosterol (30 μM), campesterol (30 μM), cholesterol (30 μM), stigmasterol (30 μM), ciprofibrate (PPARα agonist; 1 mM) or rosiglitazone (PPARγ agonist; 1 mM) for 24 h. DMSO and CD/EtOH were vehicle controls. Supernatants were transferred to 1.5 mL Eppendorf tubes, cells pelleted by centrifuging at 2500×*g* for 5 min at room temperature and supernatant was discarded. Adherent cells were washed with PBS, and CytoBuster™ protein extraction reagent (100 μL; Novagen) was added and allowed to extract for 5 min at room temperature. Cells were scraped with a cell scraper and combined with supernatants in the appropriate Eppendorf tubes. Extracts were centrifuged at 15,000×*g* for 5 min at 4 °C and the supernatants transferred to new Eppendorf tubes and kept on ice for analysis or frozen at −20 °C. SOD activity was determined using the SOD determination kit (Fluka/Sigma). In brief, samples and blanks were prepared as described in the protocol, incubated at 37 °C for 20 min and absorbance read at 450 nm using a BioTek® PowerWave XS spectrophotometer.