Journal of Ethnopharmacology 145 (2013) 200–204

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

The cytotoxic effects of *Scilla nervosa* (Burch.) Jessop (*Hyacinthaceae*) aqueous extract on cultured HepG2 cells

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ARTICLE INFO

Article history:
Received 22 May 2012
Received in revised form
25 October 2012
Accepted 27 October 2012
Available online 2 November 2012

Keywords:
Scilla nervosa (*Hyacinthaceae*)
HepG2 liver cells
Caspase
Apoptosis
Genotoxicity
CYP3A4

ABSTRACT

Ethnopharmacological relevance: Bulbs of *Scilla nervosa*, a medicinal plant indigenous to Southern Africa, are traditionally used in aqueous decoctions to treat a diverse range of illnesses. The bulbs contain homoisoflavanones and stilbenoids. Little information is known about the plant's toxicity on the liver, a major detoxifying organ. This study investigated the effects of an aqueous extract of the bulbs in cultured HepG2 liver cells, a model system for investigating the toxicity of xenobiotics.

Materials and methods: The concentration that reduced cell viability to 50% (IC_{50}) after 24 h treatment was derived. Potential mechanisms of toxicity using the IC_{50} were investigated as changes in metabolic activity, apoptosis, oxidative damage and DNA fragmentation. In addition, cytochrome P450 3A4 (CYP3A4) activity, which is implicated in drug metabolism and interactions, was also assayed.

Results: Cell viability decreased in a concentration-dependent manner and the IC_{50} was determined as 0.03 mg/mL. Treating the cells at the IC_{50} for 24 h resulted in increased intracellular ATP levels, no significant change in phosphatidylserine externalisation, increased caspase-8 activity, decreased caspase-9 activity, no significant change in mitochondrial membrane potential, increased lipid peroxidation, evidence for genotoxicity as demonstrated by DNA fragmentation, and slightly induced CYP3A4 activity.

Conclusion: Results suggest that liver cells are sensitive to an aqueous extract of the bulbs and there is an increased potential to induce apoptosis, oxidative stress and genotoxicity in vitro.

1. Introduction

Scilla nervosa (Burch.) Jessop (*Hyacinthaceae*) [=*Schizocarphus nervosus* (Burch.) Van der Merwe] is a monocotyledonous perennial plant originally endemic to Botswana and has naturalised in the grasslands of the eastern parts of Southern Africa (Hutchings et al., 1996). The bulbs of the plant are considered to be a valuable medicinal species and have been used by traditional healers of different cultures to treat infertility in women, constipation, dysentery, nervous conditions, rheumatic fever and pain (Watt and Breyer-Brandwijk, 1962). In livestock it is used to treat gall sickness (Watt and Breyer-Brandwijk, 1962). It has been recently demonstrated that extracts prepared from the bulbs possess potent anti-inflammatory properties and these findings may therefore rationalise the traditional use of the plant as an analgesic for rheumatic fever (Du Toit et al., 2011). Previous studies have revealed that the bulbs contain homoisoflavanones and stilbenoids (Bangani et al., 1999; Silayo et al., 1999). The chemical structures, plant origins, ethnobotany and biological activities of homoisoflavanones have been reviewed by Du Toit et al. (2010). Studies by Bangani et al. (1999) demonstrated 5 homoisoflavanones and 2 stilbenoids, while Silayo et al. (1999) demonstrated 13 homoisoflavanones and 3 stilbenoids. These could individually or in combination be responsible for the observed therapeutic effects. However, little information is known about the plant's toxicity. Only one previous report suggested that 0.5–1 kg of the fresh plant in the flowering stages was toxic to sheep (Van der Walt and Steyn, 1946). Therefore, a current investigation of the potential toxicity of the bulbs on the liver, a major detoxifying organ, is required. The aim of this study was to investigate the toxic effects of an aqueous extract prepared from the bulbs in HepG2 liver cells, a model system to investigate the toxicity of xenobiotics in vitro. Specifically, the viability of HepG2 cells in the presence of varying concentrations of the extract for 24 h was investigated to determine the IC_{50}-value.

Abbreviations: $\Delta\Psi m$, mitochondrial membrane potential; CCM, complete culture medium; CYP3A4, cytochrome P450 3A4; IC_{50}, concentration of extract that reduced cell viability to 50%; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine; MDA, malondialdehyde; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide; PBS, phosphate buffered saline; RLU, relative light units

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http://dx.doi.org/10.1016/j.jep.2012.10.053

The IC_{50} was used as a reference concentration to investigate the potential mechanisms of toxicity by means of a series of tests. Changes in metabolic activity (intracellular ATP levels); apoptosis (phosphatidylserine externalisation; caspase-8 and -9 activity; and mitochondrial membrane depolarisation); oxidative stress through free radical-mediated lipid peroxidation; and DNA integrity were assessed. Cytochrome isozymes (CYP) are often implicated in drug–liver–drug interactions, impacting on the metabolism and plasma concentration of coexisting drugs in the body. CYP3A4 activity was therefore investigated to determine induction or inhibition.

2. Materials and methods

2.1. Chemicals

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Calbiochem (Johannesburg, South Africa (SA)) and emetine dihydrochloride from Sigma (St Louis, MO, USA). All tissue culture reagents were obtained from Whitehead Scientific (Johannesburg, SA). All other reagents were obtained from Merck (Johannesburg, SA).

2.2. Plant material and extract preparation

Scilla nervosa was purchased from the Fig Tree Indigenous Nursery (Westville, Durban, SA). Plants were identified and a voucher (Du Toit2, NU) was lodged at the University of KwaZulu-Natal Herbarium (Pietermaritzburg, SA) (Du Toit et al., 2011). To minimise seasonal variation and changes in metabolic content, all the plants were of the same developmental stage and were harvested at the same time (June 2008). The fresh bulbs (1.5 kg) were chopped into small pieces and dried overnight. Pieces were soaked in methanol (3 days) to release the polar and non-polar components of the material. The extract (42.3 g) was filtered and dried (65 °C) in a rotary evaporator (Heidolph, Darmstadt, Germany). Small amounts (1 g) of the dry material were dissolved in double distilled water through continuous agitation (10–15 min), filtered and dried. The dry material was reconstituted in double distilled water at a stock concentration of 10 mg/mL and stored at 4 °C until further use.

2.3. Cell culture

The human HepG2 hepatocellular carcinoma cell line was obtained from American Type Culture Collection (ATCC number HB-8065). Cells were cultured in a humidified environment (37 °C; 95% O_2, 5% CO_2) to confluency in complete culture medium (CCM) consisting of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) L-glutamine and 1% (v/v) Penstrep-Fungizone® solution. Cells were trypsinised and seeded according to the type of assay.

2.4. Cell viability assay

Cell viability was measured using the methylthiazol tetrazolium assay. Cells were seeded into a 96-well microtitre plate (15,000 cells/well) and treated in triplicate (three wells per condition) with varying concentrations of the extract in CCM for 24 h. The known cytotoxic drug emetine was used as a positive control. Emetine is an alkaloid anti-amoebic drug which has been reported to enhance cytotoxicity (Lee and Wurster, 1995). Thereafter, cells were incubated with a MTT salt solution (5 mg/mL in phosphate buffered saline (PBS)) and incubated (4 h; 37 °C). Culture fluid was then aspirated and dimethyl sulphoxide (DMSO) (100 μL/well) was added to solubilise the formazan crystals and the plate was incubated (1 h; 37 °C). Optical density was measured (570 nm; reference 690 nm) with an ELISA plate Reader (Bio-Tek μQuant). The concentration of the extract that reduced cell viability to 50% (IC_{50}) was determined and used as a reference concentration to investigate potential mechanisms of cytotoxicity. Cells were treated with the extract (0.03 mg/mL; 24 h; 37 °C) in CCM and seeded according to the type of subsequent assay. The negative control was untreated cells that received CCM only.

2.5. Metabolic activity assay

Changes in energy exchange required to drive cellular processes were investigated by determining the intracellular levels of ATP with a CellTiter-Glo® kit (Promega, Madison, WI, USA). Treated cells were seeded into a white luminometer plate (20,000 cells/well) in duplicate followed by the addition of 10 μL/well of the supplied reagent. The plate was agitated and incubated in the dark (10 min; room temperature) to lyse the cells. Thereafter, the luminescent signal was measured on a microplate luminometer (Turner Biosystems, USA). The level of ATP, in which luminescence was quantified based on an enzymatic luciferase-based reaction, was expressed as relative light units (RLU).

2.6. Phosphatidylserine externalisation

The loss of cell membrane asymmetry and subsequent phosphatidylserine externalisation during apoptosis was determined by measuring the amount of annexin-V bound to phosphatidylserine. An Annexin-V-FITC kit (Roche Diagnostics, Penzberg, Germany) was used according to the manufacturer's instructions. Briefly, 500,000 cells per condition in duplicate were transferred into polystyrene cytometry tubes. Cells were stained with 100 μL of the Annexin-V-FLUOS labelling solution and incubated in the dark (15 min; room temperature). Flow cytometry analysis was conducted using a FACS Calibur flow cytometer (BD Biosciences, Johannesburg, SA) set at an excitation wavelength of 488 nm. Data was collected from 2500 events per sample and cells were gated to exclude necrotic cells.

2.7. Caspase-8 and -9 activity assay

The activities of the initiator caspases of apoptosis, caspase-8 and -9, were investigated using the Caspase-Glo® 8 and Caspase-Glo® 9 assay kits respectively (Promega). Reagents were prepared according to the manufacturer's instructions. Briefly, cells were seeded into a white luminometer plate (20,000 cells/well) in duplicate followed by the addition of 10 μL/well of either the Caspase-Glo® 8 or Caspase-Glo® 9 reagent. The plate was agitated (300–500 rpm; 30 s) and incubated in the dark (30 min; room temperature). Caspase activity (expressed as RLU) was measured by the level of luminescence signal emitted following a luciferase based reaction, measured on a microplate luminometer (Turner Biosystems, USA).

2.8. Mitochondrial membrane depolarisation assay

An increase in mitochondrial membrane depolarisation and subsequent decrease in mitochondrial membrane potential, which contribute to mitochondrial dysfunction in apoptosis was investigated by measuring fluorescence of the cationic lipophilic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1) as part of the JC-1 Mitoscreen assay (BD Biosciences). Briefly, 500,000 cells per condition in duplicate were transferred into polystyrene cytometry tubes. Cells were