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Does the African potato (Hypoxis hemerocallidea) activate the aryl hydrocarbon receptor in H4IIE-luc cells?

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Introduction

Plants produce several biologically active phytochemicals that provide protection against disease, damage and predation; contribute to flavour, colour and aroma; and provide benefits for human and animal health (Saxena et al., 2013). Due to their antibacterial, antifungal and antiviral properties, as well as protection against inflammation, phytochemicals are used to treat a variety of ailments (Aboul-Soud et al., 2020).

Hypoxis hemerocallidea is a yellow star-shaped tuberous perennial of the Hypoxidaceae family, native to South Africa and other parts of sub-Saharan Africa (Mwinga et al., 2019, Van Wyk & Wink, 2018). This plant is used by traditional healers to treat conditions such

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as haemorrhoids, headaches, dizziness, bladder disorders, allergies, ulcers, heart weakness, and infertility (De Wet & Ngubane, 2014, Matyanga et al., 2020). Traditional medicine users often assume incorrectly that chemicals derived from medicinal plants are safe for regular or extended use (Wachtel-Galor & Benzie, 2011). South Africa has no guidelines for traditional medicine uses, nor are there existing quality control measures to protect consumers (Ngcobo et al., 2012).

Hypoxis hemerocallidea contains flavonoids with antioxidant properties that are beneficial to human and animal health (Ncube et al., 2011). Flavonoids are also known as ligands of the aryl hydrocarbon receptor (AhR) (Zhang et al., 2003). The AhR is a ligand-activated transcription factor, which plays a role in multiple vertebrate signalling pathways which can be beneficial or harmful: development of autoimmune diseases and hypersensitivity; shielding gut immune cell survival; suppression of B cell differentiation; increased occurrence of hepato-biliary cancer due to long-term exposure to the potent AhR agonist, 2,3,7,8- tetrachlorodibenzo- *para*-dioxin (2,3,7,8-TCDD); and interferences with nervous system function are largely mediated by AhR (Tian et al., 2015). AhR is the main pathway through which the body attempts to degrade dioxin-like pollutants through conjugation and excretion (Larsson et al., 2014). The AhR pathway is a potential target for chemical treatment of nervous, liver, and autoimmune diseases (Tian et al., 2015). In this study aqueous extracts of *H. hemerocallidea* leaves and cormwere investigated for the presence of AhR ligands by usingthe H4IIE-*luc* rat hepatoma cell line (Garrison et al., 1996).

Materials and methods

2.1 Sampling and decoction preparation

Hypoxis hemerocallidea corm and leaves were collected during February (Austral summer) from the North-West University's (NWU) botanical garden in Potchefstroom, South Africa. The plant's identity was confirmed by Mr. Chris van Niekerk (curator), and a specimen (PUC0015089) was deposited to the AP Goosens Herbarium at the NWU. Decoctions were prepared by boiling 1 g of air-dried plant material for 45 minutes in 200 mL deionised water (18.2 MΩ.cm). Water was used as the extraction solvent, because it is similar to methods used by traditional healers.

2.2 Maintenance of cells

The H4IIE-*luc*cell line, provided by the University of Saskatchewan (Canada) is a recombinant cell line that exhibits AhR-mediated expression of luciferase. It is used to measure

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the potency of extracts to activate the AhR signal transduction pathway, which is correlated with induction of CYP1A enzyme activity and can be quantified as 2,3,7,8-TCDD-quivalents (Day et al., 2006). Luciferase activity is quantified by measuring light emission when the luciferin substrate is presented. Cells were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) (D2902-1L, Sigma-Aldrich) supplemented with 0.04 M sodium bicarbonate (NaHCO3) (Sigma- Aldrich, S5761-1KG) and 10% foetal bovine serum (FBS) (S181G-500, Biowest). Cells were maintained at 37ºC in humidified air supplemented with 5% CO2 (Whyte et al., 2004).

2.3 H4IIE-*luc* reporter gene assay

The reporter gene assay was performed using DMEM supplemented with hormone-free FBS, to prevent false-positive results. Hormones were removed by treating the FBS with dextran-coated charcoal (C6241-20G; Sigma-Aldrich). Cells were seeded at 80 000 cells/mL into 96-well white-walled microplates with transparent bottoms (Greiner Bio-one; 655098) in 250 µL aliquots per well containing 1% antibiotic-antimycotic (Biowest, L0010-100). To create a constant micro-environment across all cell-containing wells, cells were seeded into the inner 60 wells while the outer wells were filled with Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich, D5652-10L). Plates were incubated for 24 h, allowing cell attachment (Larsson et al., 2014). On the day of exposure, the nutrient medium was replaced with medium containing plant extract at various concentrations: 0.001, 0.0038, 0.023, 0.139, 0.833, and 5 mg/mL. Each concentration was tested in triplicate. The media containing the decoctions were sterilised by filtration (Pall Corporation, Acrodisc®, PN4187, 0.22 µm syringe filter). Positive control cells were exposed to 0.006, 0.037, 0.22, 1.33, 8, and 48 ng/mL 2,3,7,8-TCDD. Each plate had three wells with growth medium only (blank control) and three wells were dosed with hexane (solvent control, SC). Cells were incubated with exposure medium for 72 h according to Whyte et al., 2004.

After exposure, plates were visually inspected and rinsed with DPBS supplemented with 0.25 g/L Mg2+ (Sigma-Aldrich, 23,039-1) and 0.11 g/L Ca2+ (Sigma-Aldrich, C-2661). Lysis buffer (Promega, ADE3971) was added and the plates were stored at -80ºC for 20 minutes to lyse the cells. Plates were thawed and luminescence was measured with a Berthold multimode microplate reader (model Tristar LB941). A volume of 100 µL Luciferase Assay Reagent (LAR) was added to each well followed by quantification of luminescence (Villeneuve et al., 1999). The luciferin in the LAR cleaves the luciferase produced in the cells in response to ligand-binding of the AhR and subsequent transcription of the gene, producing light. The light produced in each well is measured and is expressed as relative light units (RLU).

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2.4 MTT viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay was done concurrently with the reporter gene assay with the same controls and exposure periods. The MTT (0.5 mg/mL) was prepared in DMEM (Mossman, 1983). Precautions against light exposure were taken because MTT is light sensitive.

The media were removed and the plates were washed in triplicate with DPBS before adding 100 µl MTT solution to each well (Mossman, 1983). Metabolically active cells reduce the yellow MTT solution through mitochondrial dehydrogenases producing water-insoluble violet formazan precipitate. The plates were incubated for 30 minutes and the formazan crystals were dissolved with dimethyl sulfoxide (200 µl per well). After 30 minutes, the optical density (OD) was measured at 560 nm. The OD of the solubilised formazan per well is directly proportional to the number of living cells of that well.

2.5 Data processing and statistical analysis

2.5.1 Reporter-gene assay

Dose-response curves were constructed for the sample and 2,3,7,8-TCDD after expressing the RLU response of the samples in terms of the maximum light elicited by the reference compound (%2,3,7,8-TCDD -max) (y-axis) (Villeneuve et al., 1999) and the logarithm of the 2,3,7,8-TCDD concentration for the reference curve and the logarithm of the plant concentration (sample curves) on the x-axis. A bio-assay equivalent was calculated by dividing the effects con- centration at 20, 50 and 80% (EC 20–80) of the 2,3,7,8-TCDD by that of the decoctions using linear sections of the dose-response curves.

2.5.2 MTT viability assay

Viability was expressed as a percentage of the OD of the exposed cells to that of the blank control cells. A percentage lower than 70% was subjected to non-parametric tests (Mann-Whitney U) to determine statistical significance ($p<0.05$).

Results

None of the decoctions exhibited measurable cytotoxicity (Figure 1). The two greatest *H. hemerocallidea* leaf extract concentrations increased viability significantly (Mann-Whitney U; $p < 0.05$). Cells that received corm decoctions showed greater viability compared to the SC, ($p > 0.05$). The lowest concentration of *H. hemerocallidea* corm extract was slightly

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cytotoxic, although not statistically significant compared to the SC. A reference dose-response curve was obtained for 2,3,7,8-TCDD. Neither leaf nor corm decoctions activated the AhR at the concentrations investigated (Figure 2).

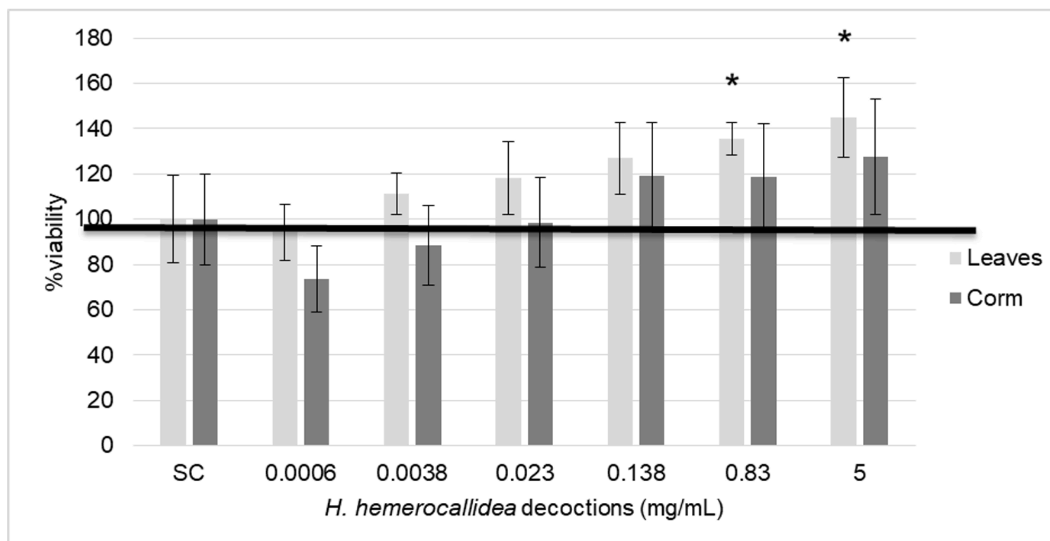

Figure 1: Cell viability after exposure to H. hemerocallidea leaves and corm aqueous decoctions, with error bars as standard deviation. Asterisks indicate the concentrations which were statistically significantly different from the solvent control (SC) ($p<0.05$).

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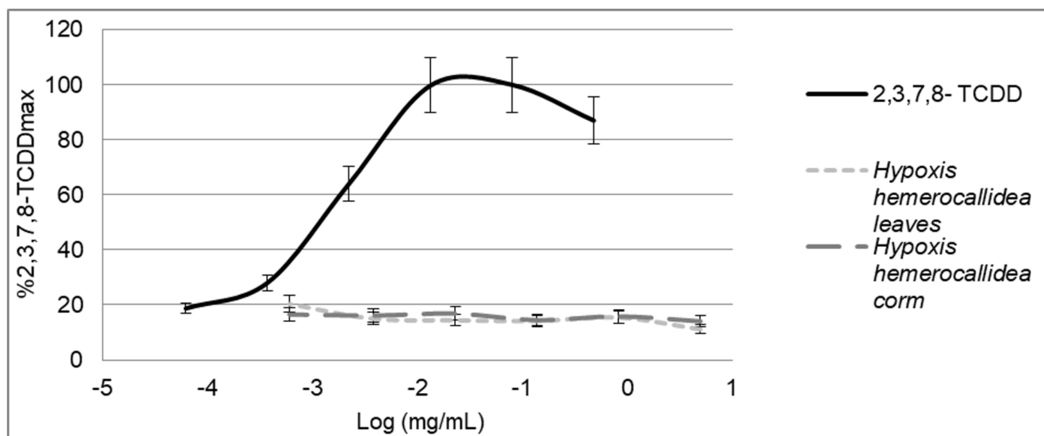

Figure 2: Dose-response curve of the AhR-antagonist (2,3,7,8-TCDD) and Hypoxis hemerocallidea leaves and corm exposures expressed in terms of 2,3,7,8-TCDD concentration that caused maximum response. Error bars indicate standard deviation.

Discussion

Although *H. hemerocallidea* contains flavonoids (Ncube et al., 2011), which are known AhR ligands, neither the aqueous decoctions of the corm nor leaves activated the signal transduction pathways under control of the AhR. The flavonoid concentrations could have been too low since plants were harvested during summer and flavonoid concentrations are generally greatest during autumn and winter (Ncube et al., 2011). The mass of dried plant material per volume tested in this study was comparable to the 1.6 mg/L in the Ncube et al (2011) study, as well as the nature of their solvent: aqueous methanol vs 100% water in this study. It is also possible that the decoctions contained flavonoids with a mixture of agonistic and antagonistic effects on the AhR (Zhang et al., 2003) and the 'no activation' result we report is due to the synergistic effect. Also, it has been shown that cell lines respond differently to various flavonoids despite them all containing the AhR: galangin, genistein, and diosmin were active in transiently transfected Hepa-1 mouse cells, and cantharidin elicited a response from stably transfected human MCF-human breast cells and HepG2 human liver cells (Zhang et al., 2003). Future research would involve harvesting plant material in winter/spring and screening the decoctions for flavonoids (Gul et al., 2017) before measuring AhR activation. It would be beneficial to screen the decoctions for other reporter gene activity such as androgen, oestrogen and thyroid activity since the plant is prescribed for hormone-related ailments such as diabetes and infertility (Bassey et al., 2014, De Wet & Ngubane, 2014).

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Conclusion

These preliminary results show that *H. hemerocallidea* preparations with similar concentrations to this study, would not contribute to adverse effects mediated through the AhR should a consumer already be exposed to dioxin-like pollutants. Simultaneously, it would also not contribute significantly to the health benefits that may be mediated by other, natural occurring AhR ligands although there was evidence of improved cell viability.

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