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Investigation into the Genotoxicity of Water Extracts from Hypoxis Species and a Commercially Available Hypoxis Preparation

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We performed an *in vitro* evaluation of the genotoxic potential of water extracts from four Hypoxis species (*Hypoxis hemerocallidea, H. colchicifolia, H. rigidula, H. acuminata*) and a commercial preparation thereof using the neutral red uptake (NRU) assay, the alkaline comet assay and the cytome assay in human hepatoma HepG2 cells. The relative cytotoxicity of these samples was established by determining their NI_{50} values (50% inhibition of NRU), and these results were used for dose-finding in genotoxicity tests. None of the tested extracts were identified as genotoxic in both the alkaline comet assay and cytome assay. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: Hypoxis sp.; neutral red uptake test; comet assay; cytome test; toxicity; genotoxicity.

INTRODUCTION

According to estimations, about 80% of the South African population (27 million people) use traditional medicinal plant preparations for their health care (Mander, 1998). Some 3000 plants are apparently used on a regular basis (Van Wyk and Gericke, 2000). Because these plants are natural and have been used for ages, they are most often considered adequate and safe. Yet, many of them were never tested for their potential adverse health effects in the way our modern pharmaceutical preparations are. Acute toxicity as a result of usage of such medicinal plant extracts is more common than often assumed, especially among children (Stewart *et al*., 1999). It is estimated that between 8000 and 20000 annual deaths are recorded in South Africa due to incorrect use of medicinal plants (Thomson, 2000). Also long-term effects in terms of, for example, genotoxicity and/or carcinogenicity need to be carefully investigated. In this paper, we report on the toxicity and genotoxicity of water extracts from 4 different Hypoxis species (*H. hemerocallidea, H. colchicifolia, H. rigidula, H. acuminata*) and a commercial herbal mixture (*African potato extract*™). We used the neutral red uptake (NRU) test (as a toxicity and dose-finding test) and the alkaline comet assay and cytome test for the evaluation of genotoxicity. The comet assay is a rapid genotoxicity test which detects DNA single- and double strand breaks and alkali labile sites (Tice *et al*., 2000). The cytome assay (Fenech, 2002) detects different genotoxic events including chromosome breakage (clastogenicity), numeric chromosome aberrations (aneuploidy) and gene amplification amongst others.

Hypoxis species are used against different ailments (Hutchings *et al*., 1996; Van Wyk *et al*., 2009). Corm infusions of *H. hemerocallidea* are for example administered as emetics for dizziness and mental disorders, whereas hot aqueous extracts have been used to treat symptoms of benign prostate hypertrophy. Ground corm decoctions are administered orally or as enemas for patients who cannot speak as a result of shock and plant decoctions as tonics. Juices from the corms are applied to burns.

Major constituents of the plant are hypoxoside, phytosterol glycosides and cytokinins (zeatin, zeatin riboside, zeatin glucoside). Efficiency against prostate hypertrophy is ascribed to phytosterol glycosides, mainly β-sitosterol. The major constituents of the plant are also known to be cytotoxic to cancer cells and to have antimutagenic properties (Hutchings *et al*., 1996; Van Wyk *et al*., 2009; Aremu *et al*., 2010). Anti-cancer, anti-HIV and anti-inflammatory activity is ascribed to hypoxoside which, once in the human gut, readily converts to rooperol. This is a biologically active compound that balances the immune system. Besides anti-inflammatory and anti-cancer activities, antioxidant, antidiabetic and anticonvulsant (Drewes *et al*., 2008), as well as antimicrobial (Aremu *et al*., 2010) activities were also described. Furthermore other effects, e.g. increase of the bioavailability of an antiviral drug when taken concomitantly (Brown *et al*., 2008), uterolytic action (Nyinawumuntu *et al*., 2008), antidiarrhoeal activity (Ojewole *et al*., 2009) and cardiovascular effects (Ojewole *et al*., 2006) were described.

Corms from *H. colchicifolia* are used for impotency and barrenness. Hot corm infusions are used as emetics against bad dreams caused by a bad hart. Infusions are taken as love charm emetics and are administered for hysterical fits. Rhizomes are used as diuretics and in the

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treatment of psychiatric disturbance. Powders from ground corms are mixed with food to kill vermin. Pentenyne-*bis*-glucosidophenol has been extracted from the corm and has some antitumor activity (Hutchings *et al*., 1996). Ethanolic corm extract of *Hypoxis colchicifolia* were also found active against epilepsy and convulsions (Risa *et al*., 2004).

H. rigidula and *H. acuminata* also contain hypoxoside (Boukes, 2010) and may therefore also balance the immune system. Pounded corm decoctions of another *Hypoxis* species, *H. gerrardii*, are used by the Zulu for stomach ailments and dysentery (Hutchings *et al*., 1996).

Investigating potential adverse health effects of *Hypoxis* spp. is of particular interest since preparations from them were highly promoted in the popular press in South Africa as agents that can also boost immunity in HIV/AIDS patients. Multiple websites, popular magazines, and even the South African Ministry of Health have supported this assertion (Mills *et al*., 2005a), but there are unconfirmed reports of toxic properties and hence warnings against the use of *Hypoxis* and their products (Nicus, 2006; Mills *et al*., 2005a,2005b). It is therefore of utmost importance to verify these warnings and to see whether these products can be safely used by the population at large. Investigating potential genotoxic properties contribute to this.

MATERIALS AND METHODS

Investigated plant extracts and commercial preparation. Corms of *Hypoxis* species, namely *Hypoxis acuminata* Baker, *Hypoxis colchicifolia* Baker (Syn = *H. distachya* Nel, *H. gilgiana* Nel, *H. latifolia* Hook., *H. oligotricha* Baker), *Hypoxis hemerocallidea* Fisch.Mey. & Ave-Lall. (Syn = *H. rooperii* T.Moore) and *Hypoxis rigidula* Baker (Syn: *H. cordata* Nel, *H. elliptica* Nel) were collected from the University of KwaZulu-Natal Botanical Garden in KwaZulu-Natal Province, South Africa. Plant voucher specimens were prepared and are kept at the University of KwaZulu-Natal Herbarium. Above mentioned plant materials were chosen based on their importance and alleged favourable and/or unfavourable bio-effects. A commercial preparation (*African potato extract*™*) was* bought at a traditional herbal (*muthi*) shop in Pietermaritzburg, KwaZulu-Natal (South Africa).

Extraction of plants. Crude extracts were made at the University of Kwazulu-Natal. Plants were dried at 50 °C for 3 days, and then the dried plant material was ground to powders. The ground plant materials were extracted with 20 mL/g water using a sonication bath for 1 h, the temperature being kept constant at 15 °C by adding ice to the water bath. The crude extracts were then filtered through Whatman No. 1 filter paper. The extracts were freeze dried and kept in airtight containers. The dried material was weighted and packed for transportation to the Toxicology Laboratory of the Scientific Institute of Public Health (Brussels, Belgium) for further analyses. Extracts were resuspended in 10% dimethyl sulfoxide (DMSO) to give an initial concentration of 5.0 mg/mL, and dilution series were prepared for testing from this stock.

Neutral red uptake test. The NRU test (Borenfreund and Puerner, 1985) is based on the ability of living cells to take up and bind NR. NR is a dye which easily penetrates cell membranes via non ionic diffusion. It accumulates in the lysosomes. Xenobiotics acting on lysosomal membranes are responsible for a decreasing NR uptake. Living cells can therefore be distinguished from dead or dying cells based on their different NR uptake. We performed the NRU test according to well-known standard methods as described by Repetto *et al*. (2008). Cell suspensions of human hepatocellular liver carcinoma cell line 2 (HepG2) in Dulbecco's modified Eagle's culture medium supplemented with 10% foetal calf serum were seeded into each well of a 96-well microtiter plate such that the cell density was forty thousand cells/well. Plates were incubated overnight at 37 °C and 5% CO_2. Humidity was maintained using a water bath containing milli-q water inside the incubator. After 24 h incubation, the cells were treated with dilutions of the extracts. Following another 24 h incubation time, cells were washed in PBS (to remove excess of extract) after which 200 μL of a 0.625 μg/mL NR-solution were added. Cells were washed 3 h later to remove excess of the dye. Then, 200 μL of a 50:1 ethanol-acetic acid solution was added to extract the dye from the cells. This was done in a microtiter plate shaker for approximately 1.5 h (until appearance of a homogenous purple color).

Then, absorbance against a blank reference was measured at 540 nm using a micro plate spectrophotometer. For all wells, optical density (OD) values were calculated as the measured value minus the control value (Vc). Results were expressed as percentage of the OD determined from the average of the blank control culture read at 540 nm and set at 100%. The NI_{50} (50% inhibition of NRU) was determined from the dose response curve of the mean OD values of the different concentrations. For the positive control, a separate plate was used where cells were treated with different concentrations of sodium dodecyl sulphate (SDS; 0–0.42 mM), and the NI_{50} was determined as for the herbal extracts described above. The NI_{50} should be within limits that were determined from ten independent experiments from which average NI_{50} values, and standard deviations were calculated (unpublished data). The calculated NI_{50} for the positive control in an experiment should be within ±2.5 SD of these historical data for SDS. If this is not the case, the results cannot be accepted, and the test should be repeated. The reported results were all in accordance with the requirements.

The alkaline comet assay. The test was performed according to standard methods (Olive and Banáth, 2006). In short, cells were grown in 24-well plates (1 mL/400,000 cells). After a 24 h growth period, plant extracts were added in different concentrations. Concentrations were chosen based on the results of the NRU test. Cells were trypsinized after another 24 h, brought to PBS and kept on ice to prevent further DNA damage. A 10 μL cell suspension + 300 μL 0.8% LMP agarose was brought on pre-coated slides (1% NMP agarose). Slides were kept on ice for 5 min and then brought in lysis buffer (2.5 M NaCl; 100 mM EDTA; 10 mM TRIS; 1 v% Triton X-100 and 10 v%DMSO). The pH was adjusted to pH = 10 with NaOH pellets. The slides remained overnight into the lysing solution. The next day, slides were brought into denaturation buffer (0.3 M NaOH, 1 mM EDTA in water, t = 17 °C, pH = 13)

in which electrophoresis (20 min, 1 V/m, 300 mA) occurred. After lysis, histones and nucleosomes were removed leaving supercoiled DNA behind. DNA damage results in broken DNA fragments and loops that will unwind and migrate in the agarose gel. A 'comet-like' figure is formed that can be visualized after staining with a fluorescent dye. Slides were therefore dried, renaturated in 200 μL H_2O (10 min) and stained for another 10 min with 100 μL gelred (1:3300 stock solution). Afterwards, slides were analyzed with an Axio Imager.Z2 (Zeiss) fluorescence microscope with Metacyte and Metafer 4 (version 3.8.5) software from Metasystems (Altlussheim, Germany). The percentage DNA in the comet tail was used as the measure of DNA damage. Ethyl methane sulfonate (0.75 mM) was included as a positive control. Two slides were prepared per exposure, and a total of 100 cells (DNA comets) were measured (50 per slide).

The cytome assay. The 'cytochalasin B micronucleus assay' is one of the most used cytogenetic tests in genotoxicology studies. In this test, dividing cells are blocked by cytochalasin B in their telophase stage of cell division (to be sure that cell division occurred) and then screened for the presence of micronuclei. These are small nuclei containing chromosome fragments or whole chromosomes that are not incorporated in the main nuclei and that are therefore indicative of respectively chromosome breakage or chromosome loss (clastogenicity or aneuploidy). A couple of years ago, an extension of the 'classical' micronucleus test was presented, which is now known as the *cytome assay* (Fenech, 2002, 2006, 2007, 2009). In this assay, not only the frequency of cells with micronuclei are scored but also other morphological features that allow a better insight into (genetic) effects of pollutants and their mode of action. Amongst them, nucleoplasmic bridges (NPBs) and nuclear buds (NBs), trinucleated cells, apoptosis and necrosis can be scored.

The test was conducted according to Fenech (2007). Cells were seeded in 24-well plates (500,000 cells/well) and incubated for 24 h (as before). Then, test sample was added for another 24 h followed by addition of cytochalasin B (4.5 μg/mL). After a further 24 h incubation (total of 72 h), cells were fixed three times with ice cold 3:1 methanol-acetic acid. Formaldehyde (37%) was added at the first fixation only. Cells were kept at −20 °C prior to slide preparation. Slides were stained in a filtered 50% May-Grunwald solution for 2 min, followed by a second staining step (6 min in 10% Giemsa). Slides were visually inspected with a Zeiss light microscope. We investigated 2000 randomly selected cells per concentration. Concentrations were based on the results of the NRU and alkaline comet tests. Determinations of the 'cytochalasin B proliferation index' (CBPI) were also performed (in 500 cells per culture) as an additional cytotoxicity parameter (Kirsch-Volders *et al.*, 2003). It is given by the following formula:

$$\mathrm{CBPI} = \frac{1\text{x mononuclear cells} + 2\text{x binucleated cells} + 3\text{x polynucleated cells}}{\text{total number of cells}}$$

This allows identification of a cytotoxicity index that is given as:

$$\mathrm{CI} = 100 - 100[(CBPI_T - 1)/(CBPI_C - 1)]$$

where T = test compound and C = control.

We here report only the results on the frequencies of cells with micronuclei, NPBs and NBs as these were the most important in this study. We used 4-nitroquinoline-oxide (0.53 mM) as a positive control.

Statistical analysis. No statistics is needed for the NRU test. Results of the comet assay were analyzed with the Mann–Whitney U-test whereas Kastenbaum & Bowman tables (Kastenbaum and Bowman, 1970) were used for determining statistically significant deviations from (unexposed) control frequencies in the cytome test. This binomial test was found adequate for the purpose of this investigation.

RESULTS

NRU Test

Following a preliminary range-finding experiment, we tested six concentrations of the Hypoxis extracts which allowed the determination of the NI_{50} values (Table 1). SDS NI_{50} falls within the required limits (as for example seen in Table 1), which means that the tests were acceptable. The NI_{50} of the 4 Hypoxis extracts are an order of magnitude higher than that of our positive control. For the African potato extract™, no NI_{50} could be determined due to solubility problems. The NRU cytotoxicity test thus showed that *H. colchicifolia* and *H. rigidula* were the least toxic (NR_{50} = 3.3 and 3.4 mg/mL), whereas *H. hemerocallidea* was the most toxic (NR_{50} = 1.95 mg/mL). For our genotoxicity tests, we used concentrations showing at least 70% viability. This means that the concentrations used may be well below the NI_{50}. Figure 1 shows for example the viability found for a number of tested concentrations of Hypoxis sp. and expressed as percentage from the unexposed controls. The concentrations used for our genotoxicity evaluation was determined according to such values.

Table 1. NI_{50} values (50% viability) of *Hypoxis* extracts and SDS (positive control) in HepG2 cells showing the relative *in vitro* toxicity of four hypoxis species. The value found for the positive control is within the limits determined from ten independent experiments

Extract	NI_{50} (mg/mL)	positive control	NI_{50} (mM)
H. hemerocallidea	1.95	SDS	0.267
H. colchicifolia	3.4	0.2426 ≤ 0.267 ≤ 0.3314	= OK
H. rigidula	3.3		
H. acuminata	2.23		

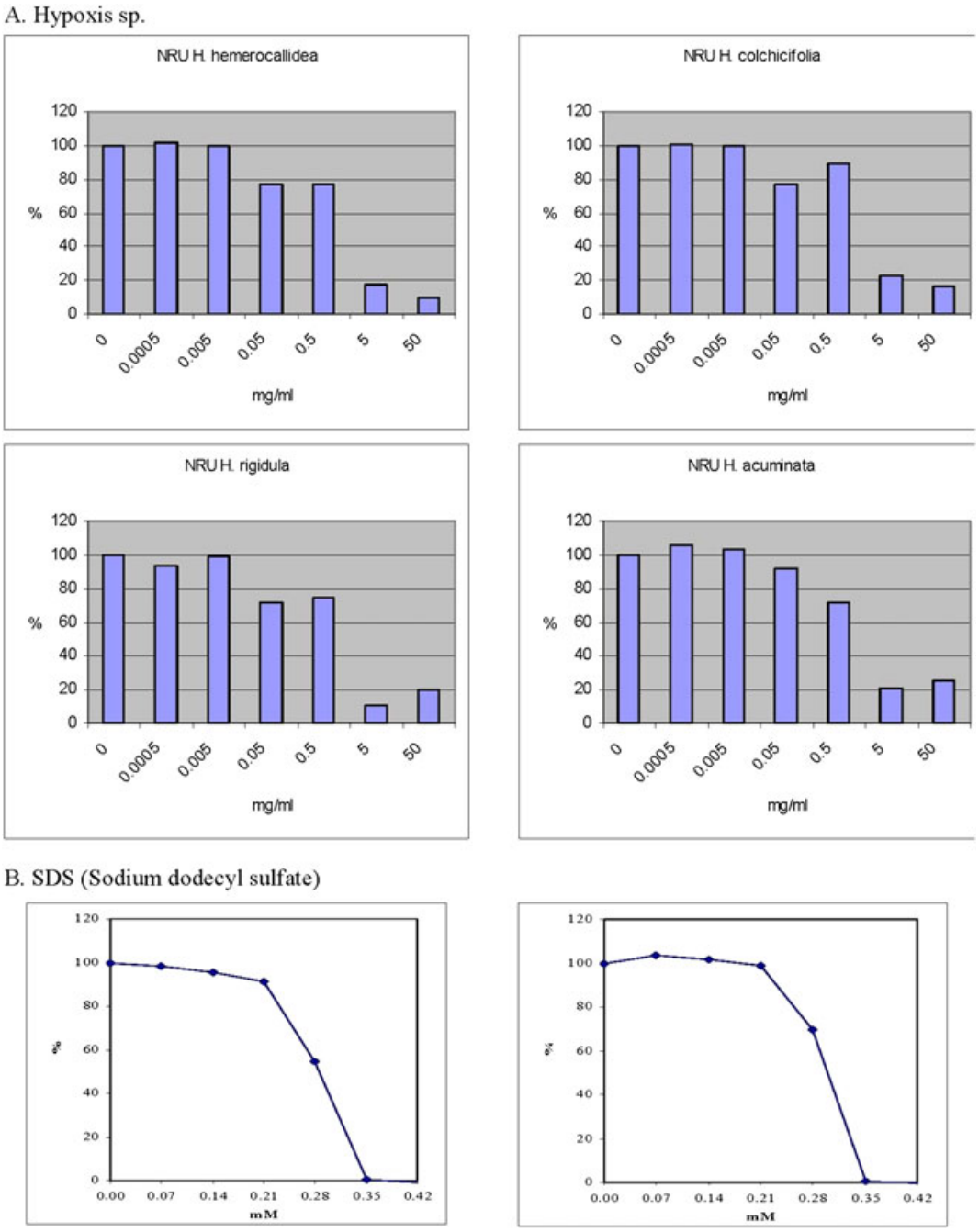

Figure 1. Viability of *Hypoxis* sp. expressed as percentage of the unexposed controls for a number of tested concentrations (A) and two replicates of NI_{50} determination curves for the positive control SDS (B). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Alkaline comet assay

The tested concentrations (>70% viability) were based on the results of the NRU test. Two replicate studies were performed, essentially giving the same results. We here will only present the results of one representative experiment (Table 2). *H. hemerocallidea* was tested in the concentrations 0.125 – 1 mg/mL (>70% viability). Other extracts could only be tested at concentrations ≤0.5 mg/mL as higher concentrations had lower than 70% viability. Although 1 mg/mL of the *H. hemerocallidea* extracts showed somewhat increased DNA damage compared to the unexposed controls, the difference was statistically not significant. Also, no statistically significant deviations from the controls were found for *H. colchicifolia*, *H. rigidula* and *H. acuminata*. In all cases, except one, repeat experiments gave the same overall results and conclusions. *H. colchicifolia* appeared to damage DNA at 0.25 and 0.5 mg/mL ($p < 0.05$) in one experiment (not shown), but this was not confirmed in two subsequent repeats. We therfore conclude that none of the extracts damaged DNA according to the alkaline comet assay at non-toxic exposure levels.

Results for the African potato extract are given in Fig. 2. The figure gives results from two independent experiments. Both experiments gave very similar results. As before, subtoxic concentrations were tested (>70% viability). There were no significantly different DNA damage levels compared to the unexposed controls in none of the samples and concentrations tested. The positive control (0.75 mM ethyl methane sulfonate) did always produce substantial DNA damage as expected (not shown) indicating that the tests were correctly conducted.

Table 2. DNA damage expressed as % DNA in DNA comets from HepG2 cells that were exposed to extracts from four *Hypoxis* sp

	% DNA in comet tail (median)	% DNA in comet tail (average)	Standard deviation
H. hemerocallidea			
Negative control	2.04	4.54	6.79
0.125 mg/mL	1.03	6.51	9.9
0.25 mg/mL	1.73	4.54	8.75
0.5 mg/mL	1.18	2.82	4.14
1.0 mg/mL	3.62	6.84	8.79
H. colchicifolia			
Negative control	1.69	3.96	5.59
0.25 mg/mL	1.83	6.8	10.94
0.5 mg/mL	1.65	4.09	5.32
H. rigidula			
Negative control	2.31	8.23	13.97
0.25 mg/mL	2	8.03	12.21
0.5 mg/mL	2.17	9.89	13.33
H. acuminata			
Negative control	2.31	8.23	13.97
0.125 mg/mL	3.44	9.99	15.54
0.25 mg/mL	1.12	6.52	11.46
0.5 mg/mL	2.6	6.96	10.93

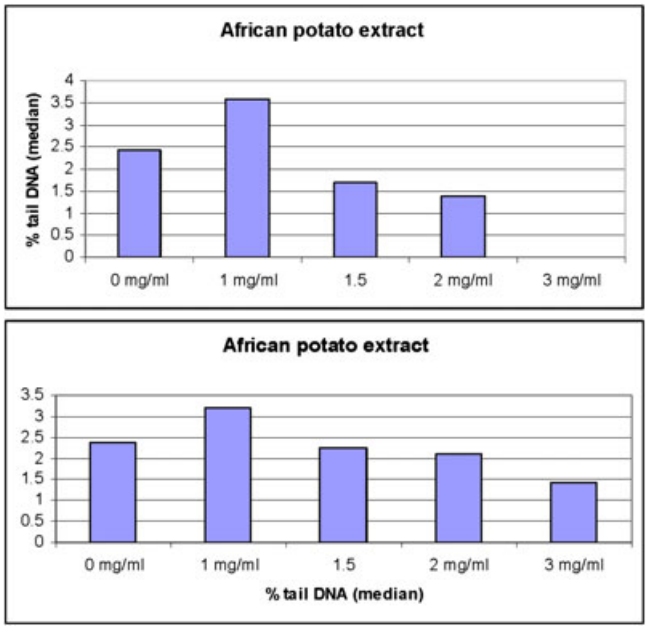

Figure 2. Results from the alkaline comet assay in two independent experiments on the commercial African potato extracts (mixture of *H. hemerocallidea* and *H. colchicifolia*). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Cytome assay

Table 3 gives the CBPI and % toxicity for the highest tested concentrations. In all cases, no cytotoxicity (CI <60%) was found. All tests were therefore performed in cells that were exposed to non-toxic concentrations of the extracts. Figure 3 summarizes the results of the cytome test for the different *Hypoxis sp*. The frequencies of micronucleated cells and of cells with NPB and NB were not found statistically significant in any of the treatments (except the positive control, not shown).

Table 3. CBPI values and corresponding cytotoxicity (%) of *Hypoxis* sp. and a commercial preparation at the highest tested concentrations (mg/mL)

Sample	Concentration (mg/mL)	CBPI	Cytotoxicity (%)
Negative control	0	1.59	-
H. hemerocallidea	1	1.39	33.9
H. colchicifolia	0.5	1.46	22.03
H. rigidula	0.5	1.57	3.39
H. acuminata	0.5	1.41	30.51
African potato extract™	3	1.43	27.12

Figure 4 gives the results for the commercial preparation. Although somewhat increased frequencies of anomalies were found, differences with the unexposed controls were never statistically significant.

DISCUSSION

There are up to about 40 *Hypoxis* species in southern Africa, but of these, *H. hemerocallidea* and *H. colchicifolia* are those mostly used and to some extent also *H. rigidula* and *H. acuminata*. A problem is that all the *Hypoxis* species are referred to and traded as 'African potato', thus they are always cross traded because of the similarities in the corms. *H. rigidula* looks for example similar to *H. colchicifolia* except for very few detailed leaf morphological differences for which the gatherers do not check. *H. hemerocallidea* and *H. colchicifolia* have relatively bigger corms compared to the others, making them more likely to be used in traditional medicine than the others. The commercial African potato herbal mixture that we used is made from a mixture of *H. hemerocallidea* and *H. colchicifolia*.

Hypoxoside is thought to be the major, non-toxic compound in extracts of the African potato (*Hypoxis* species), which has led to the claimed anticarcinogenic properties of this plant. Hypoxoside itself has no anti-cancer activity but it is, upon hydrolysis, converted to rooperol, which is cytotoxic (hence the anti-cancer claim) and does possess potent antioxidant properties. Although *Hypoxis* sp. are considered of uttermost importance in the treatment of cancer and other life-threatening diseases, there are so far no robust scientific data that support this claim (Nicus, 2006; Mills *et al*., 2005a). Nevertheless, they are widely used, and the safety of the plant extract has been questioned and remains a cause of serious concern. Especially, its use against aids is so far questionable as it even may put patients at risk of treatment failure, viral resistance or drug toxicity (Mills *et al*., 2005b; Nicus, 2006). Therefore, HIV/AIDS patients should better avoid any such supplements, until their safety and efficacy have been fully documented.

The aim of this paper was to contribute to the safety evaluation of *Hypoxis* spp. NR_{50} values allow comparisons of *in vitro toxicity* and were used for *in vitro*

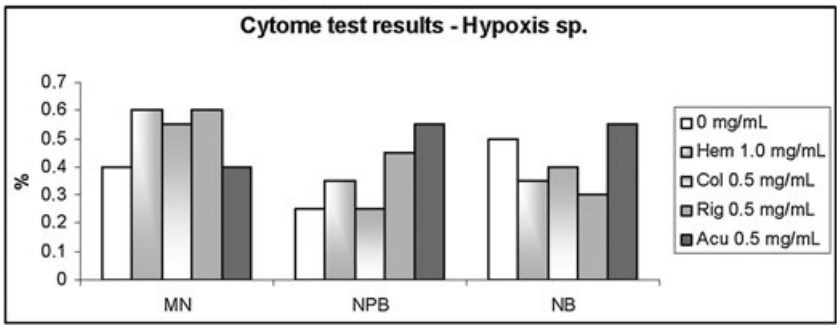

Figure 3. Frequencies of micronucleated cells (MN), nucleoplasmic bridges (NPB) and nuclear buds (NB) in resp. negative control cultures (0 mg/mL) and top concentrations of extracts from *Hypoxis hemerocallidea* (Hem), *Hypoxis colchicifolia* (Col), *Hypoxis rigidula* (Rig) and *Hypoxis acuminata* (Acu).

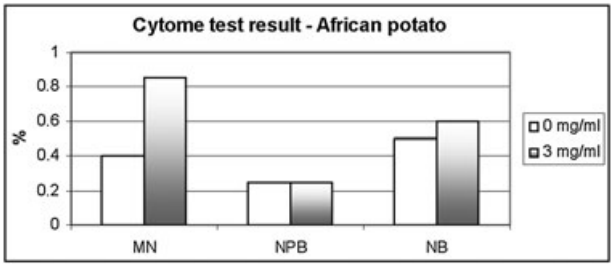

Figure 4. Frequencies of micronucleated cells (MN), nucleoplasmic bridges (NPB) and nuclear buds (NB) in resp. negative control cultures (0 mg/mL) and the top concentration (3 mg/ml) of the *Hypoxis* commercial product: African potato.

dose-finding purposes. Regarding the alkaline comet assay, it should be noted that we used the % DNA in the comet tail to estimate DNA damage levels. This means that we can theoretically expect levels from 0% up to 100% (apoptotic) with most cells having low % tail DNA in the control cultures and a shift to more cells with more substantial DNA damage for increasing concentrations of a genotoxic compound. Although most cells have more or less similar (and relatively low) DNA damage levels, some have excess damage. Inclusion of these (up to 70% damage) results in high standard deviations which are therefore not unusual and normal.

All four *Hypoxis* spp. were not genotoxic according to the comet and cytome tests. There are no previous reports on DNA damage as assessed in the comet assay from *Hypoxis* spp. in the literature but we already investigated dichloromethane and methanol extracts from *Hypoxis colchicifolia* and *Hypoxis hemerocallidea* with the classical micronucleus test in human peripheral blood lymphocytes (Taylor *et al.*, 2003). Our present negative results are in accordance with these earlier findings. Extracts from *H. colchicifolia* (corm) and *H. hemerocallidea* (twig) were also investigated with the bacterial Ames test (Elgorashi *et al.*, 2003). Both were also negative (non mutagenic) according to this well-known standard mutagenicity test. In another investigation on South African medicinal plants, *H. hemerocallidea* (corm and leaf) extracts did again show absence of mutagenicity (Reid *et al.*, 2006). Our negative findings are thus completely in accordance and complementary with previous literature data where other genetic endpoints were tested. There are no data available in the literature on the genotoxicity of *H. acuminata* and *H. rigidula*.

The commercial African potato extract was also not genotoxic according to the present investigation.

Our data are thus reassuring, but further research including *in vivo* studies are still necessary given the importance of *Hypoxis* spp. in South African society. Such studies should involve cooperative or synergistic effects with other agents, e.g. antiretroviral drugs. Indeed, many patients infected with the human immunodeficiency virus are taking traditional herbal medicines (a.o. *H. hemerocallidea*) in conjunction with their modern antiretroviral medication. Drug–herb interactions can occur in these cases. *H. hemerocallidea* increase the transport of the antiviral drug nevirapine across human intestinal epithelial cells when taken concomitantly (Brown *et al.*, 2008), but other combined effects are possible. The same holds true for other combinations of agents involving medicinal plant extracts. Antimutagenic, but also co-mutagenic effects involving many traditional medicinal plants were for example described (Verschaeve and van Staden, 2008).

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Conflict of Interest

There is no conflict of interest. The authors alone are responsible for the content and writing of the paper. All the authors have approved the final article.

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