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## Abstract

**Background:** *Arctotis arctotoides*, *Gasteria bicolor* and *Pittosporum viridiflorum* are commonly used in the Eastern Cape, South Africa by traditional healers for the treatment of opportunistic fungal infections in HIV/AIDS patients. Unfortunately, there is a dearth of published data regarding the toxicity of the selected plants, despite the fact that experimental screening of toxicity is crucial to guarantee the safety of the users.

**Materials and Methods:** Therefore, it was decided to evaluate the cytotoxicity of the hexane and acetone extracts of the medicinal plants against the Chang Liver cell line using the *in vitro* MTT assay. Different concentrations of the extracts were added into 24-hour cultured cells and incubated for 72 hours under specific condition (37 °C, 5% CO<sub>2</sub>). Cell survival was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

**Results:** Depending on the dosage and duration of treatment, the cytotoxic effects of *Gasteria bicolor* and *Pittosporum viridiflorum* were considered relatively weak (but not entirely absent) and less of a toxicity risk. *Arctotis arctotoides* extracts were the most toxic both in terms of IC<sub>50</sub> values as well as the steeper slope of the dose response curve. The IC<sub>50</sub> values for the acetone and hexane extracts of this plant were 17.4 and 12.4 µg/ml respectively.

**Conclusion:** These relatively low values raise concern for potential hepatotoxic effects and deserve further investigation or at least a warning to potential users.

**Keywords:** Cytotoxicity; medicinal plants; opportunistic fungal infections; Chang liver cell line

**List of non-standard abbreviations:** ATCC: American type culture collection; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.; OFI: Opportunistic fungal infections; PCR: Polymerase chain reaction

## Introduction

Plants commonly used in traditional medicine are assumed to be safe due to their long usage in the treatment of diseases according to knowledge accumulated. However, recent scientific findings have shown that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic (Ajaiyeoba et al., 2006). Toxicity is an expression of being poisonous, indicating the state of adverse effects caused by the interaction between toxicants and cells (Syahmi et al., 2010). Despite the advances in the understanding of the medicinal properties of many herbs and the increased acceptance and utilization of medicinal plants worldwide, the consumer today is confronted with the lack of information or with misinformation concerning the safety of these herbs that rivals the heyday of the patent medicine era (Teshome et al., 2010). Hence, many medicinal plants are used indiscriminately without recourse to any safety test. Thus, the need for toxicity tests to determine the safe dose for oral consumption (Teshome et al., 2010).

### *In vitro* cytotoxicity assays

Cell-based *in vitro* cytotoxicity assays are generally designed for studying individual compounds of known structure and concentration. It is important to keep in mind that cell-based cytotoxicity analysis of botanical extracts are difficult to interpret in terms of predicting toxicity *in vivo*, because these extracts contain mixtures of compounds and their concentrations will differ depending on which compounds reach the liver or other organs after oral/dermal administration.

### Medicinal plants used in this study

In South African traditional medicine, the use of plants in the form of infusions or decoctions is widespread among people of rural communities. South Africa has a large floral biodiversity and is home to over 30,000 species of higher plants with 3,000 of these used in traditional medicine across the country (Van Wyk et al., 1997). The following medicinal plants were used in this study: *Arctotis arctotoides* (L.f.) O. Hoffm. (Asteraceae), *Gasteria bicolor* Haw. (Asphodelaceae) and *Pittosporum viridiflorum* Sims. (Pittosporaceae). *A. arctotoides* is a decumbent herb commonly found as a roadside weed in most coastal districts of South Africa (Afolayan et al., 2007). The plant forms carpets of light grey-green foliage and butter-yellow daisy flowers almost all year round. The Xhosa-speaking people in the Eastern Cape Province apply the juice from the leaf as a topical paste to treat wounds (Afolayan, 2003). The succulent genus, *Gasteria*, which comprises 16 species, is endemic to South Africa and has its main centre of distribution in the Savannah Region in the Eastern Cape (Dagne et al., 1996). Previously, this genus was classified in the large heterogeneous Liliaceae family but is now classified under the family Asphodelaceae (Sultana and Afolayan, 2003). *P. viridiflorum* is a small tree, 3-6m tall, with glabrous branches and leathery leaves, 6-15 cm long. It is native to South Africa and is widely distributed in the Eastern half of the country (Matshinyalo and Reynolds, 2006). The stem and bark have been used medicinally in various regions of the world.

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The choice of these plants was based on the following criteria: firstly, these plants have ethnopharmacological data indicating their traditional utilisation in the treatment of opportunistic fungal infections in HIV/AIDS patients (Otang et al., 2012a) Secondly, in the domain of HIV/AIDS management, there is no study in the Eastern Cape of South Africa that deals with these plants. The third and fourth criteria were based on bioactivity and availability of the plant materials. From a pool of 33 plant species (26 families and 32 genera) cited as being used to treat one or more opportunistic fungal infections (OFIs) in the study area, 10 plants were subjected to a broad antifungal screening initiative amidst a panel of 10 opportunistic fungi comprising of yeasts and moulds (including dermatophytes) (Otang et al., 2012b). Based on their relatively higher antifungal activity and availability of the plant materials, *A. arctotooides*, *G. bicolor* and *P. viridiflorum* were selected for toxicological analysis. To be medically useful as antifungal agents, the selected medicinal plants should be non-toxic or of low toxicity to human cells. Unfortunately, there is a dearth of published data regarding the toxicity of the selected plants, despite the fact that experimental screening of toxicity is crucial to guarantee the safety of the users and could lead to innovative strategies in the management of OFIs in HIV/AIDS patients. Therefore, following our ethnobotanical and antifungal studies, it was decided to evaluate the cytotoxicity of the hexane and acetone extracts of *A. arctotooides*, *G. bicolor* and *P. viridiflorum* against the Chang Liver cell line using the *in vitro* MTT assay.

### The Chang Liver cell line

The Chang liver cell line originated from a normal liver obtained from a Chinese boy (Chang, 1954) and was later transformed *in vitro*. There exists a controversy in the literature regarding the current authenticity of this cell line as it is believed to have been contaminated by HeLa cells prior to its deposition in cell banks such as ATCC and possibly others. The presence of HeLa markers (DNA sequences) and the absence of PCR amplifiable Y chromosome DNA sequences are considered as evidence that these cells now represent a derivative of HeLa. Despite these findings, there exist a number of publications which suggest that these cells possess characteristics associated with hepatocyte function (Luduena et al., 1997; Matsuguchi et al., 1990). Therefore the use of this cell line as a model for normal human liver should be interpreted with caution as there is no definitive proof as to the authenticity of our present Chang liver cell line. On the other hand, even if they do not represent hepatocytes, Chang liver cells provide an acceptable model to explore general cellular cytotoxicity, as many of the known drug-induced cytotoxic mechanisms, including inhibition of mitochondrial function, disruption of intracellular calcium homeostasis, activation of apoptosis, oxidative stress, inhibition of specific enzymes and transporters and formation of reactive metabolites that cause direct toxicity, are common to most cells.

### MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

A modification of the original MTT assay (Mossmann, 1983) was used to evaluate the cytotoxicity of the extracts. MTT, a yellow tetrazolium salt, is converted by dehydrogenase enzymes in viable cells to an insoluble, purple formazan. The amount of purple formazan that is extracted from the cells is proportional to the number of viable cells. Since the assay quantifies the number of viable cells at the end of the treatment period, it is not possible to distinguish between treatments that have a cytotoxic (cell killing) or cytostatic (inhibition of cell growth) effects. It has been shown that plant extracts with strong antioxidant activity can also convert MTT to the purple formazan (Muraina et al., 2009). This may mask cytotoxic effects and therefore the method was performed in such a way that masking effects would be excluded. The cells are incubated with the plant extract for 48 hours and the extract is then removed from the cells before MTT is added.

## Materials and methods

### Plant material

*A. arctotooides* was collected at the Agricultural Research Farm of the University of Fort Hare, South Africa, while *G. bicolor* and *P. viridiflorum* were supplied by an herbalist. The plants were authenticated at the Griffin Herbarium of the Department of Botany, University of Fort Hare, where voucher specimens were deposited (*A. Arctotooides*: W28, *G. bicolor*, W31, *P. viridiflorum*: W9).

### Extraction procedure

Leaf samples of the three plants were chopped dried in an oven at 40 °C and ground into fine powder. The ground samples were put into separate conical flasks containing acetone or hexane and shaken for 24 h on an orbital shaker. After filtering with a Buchner funnel and Whatman No. 1 filter paper, the hexane and acetone filtrates were concentrated to dryness under reduced pressure at a maximum of 40 °C using a rotavapor (Van Wyk et al., 1997). Aqueous filtrates were freeze-dried. Each extract was resuspended in the respective solvent of extraction to yield a 20 mg/ml stock solution (Afolayan et al., 2007).

### Preparation of extracts

Aliquots of the plant extracts were transferred to pre-weighed Eppendorf tubes and the samples dried by speed vac (Savant instruments INC, USA). Dried extracts were reconstituted in DMSO to a concentration of 100µg/µl and diluted with complete medium to the concentrations indicated.

### Assay method

Cells were seeded into 96-well culture plates (Nunc) at 10,000 cells/well in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and left for 24 hours. Plant extracts were added and the cells incubated for a further 48 hrs after which the medium was replaced with 200 µl MTT (Sigma) (0.5 mg/ml in EMEM). After a further 4 hr incubation at 37°C, the MTT was removed and the purple formazan product dissolved in 200µl DMSO and absorbance measured at 560nm using a multiwell scanning spectrophotometer (Multiscan MS, Labsystems). All incubation steps were carried out in a 37°C humidified incubator with 5% CO<sub>2</sub>. IC<sub>50</sub> values were calculated from 5-point dose-response curves using Graph Pad Prism 4 software package (Graph Pad software, San Diego California, USA) (Afolayan, 2003).

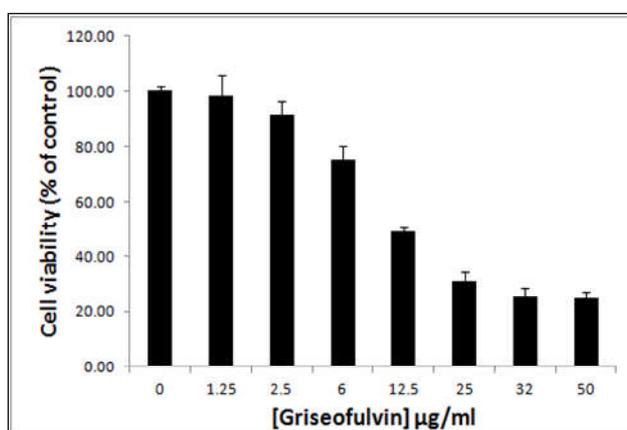
**Results**

IC<sub>50</sub> values obtained on the Chang liver cell line for each extract and the control drug are summarised in Table 1. The extracts and the control drug induced cell cytotoxicity in a concentration-dependent manner, as illustrated in Figures 1-6. Results are the mean of 3 determinations (mean±SD) and are expressed as % cell survival.

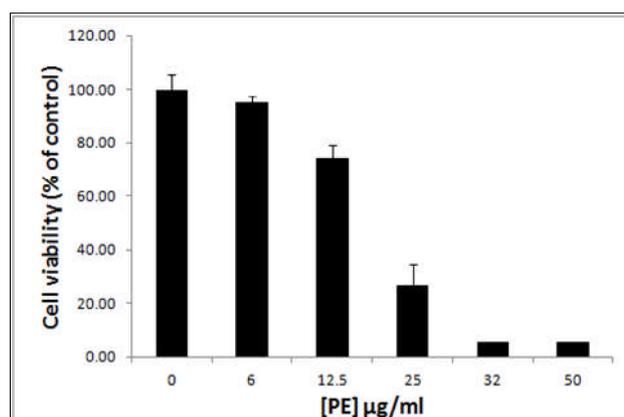
**Table 1:** IC<sub>50</sub> values (mean ± SD) for extracts and reference compound against Chang liver cells obtained using the MTT assay for cytotoxicity.

Plant extract/compound	IC <sub>50</sub> (µg/ml)	Hill slope	r <sup>2</sup>
Griseofulvin	9.02±2.4	-1.78	0.98
<i>A. artotoides</i> (acetone)	17.46±1.5	-3.37	0.98
<i>A. artotoides</i> (hexane)	12.48±0.7	-2.78	0.98
<i>P. viridiflorum</i> (acetone)	246.95±25.2	-1.92	0.97
<i>P. viridiflorum</i> (hexane)	225.50±18.4	-1.71	0.99
<i>G. bicolor</i> (acetone)	156.00±12.6	-0.89	0.98
<i>G. bicolor</i> (hexane)	278.80±19.1	-2.16	0.98

With regards to the plant extracts screened, *A. actotoides* extracts were the most toxic both in terms of IC<sub>50</sub> values as well as the steeper slope of the dose-response curve (Figures 2 and 3). The IC<sub>50</sub> values for the acetone and hexane extracts were 17.4 and 12.4 µg/ml respectively (Table 1).



**Figure 1:** Dose dependent inhibition of Chang liver cell viability by Griseofulvin (0-50µg/ml).



**Figure 2:** Dose dependent inhibition of Chang liver cell viability by the acetone extract of *A. arctotoides* (PE = plant extract).

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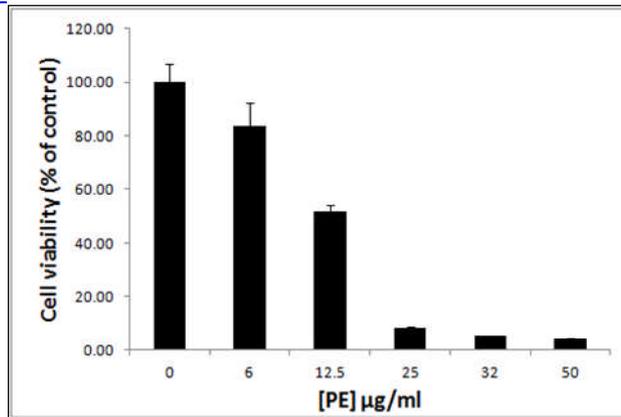


Figure 3: Dose dependent inhibition of Chang liver cell viability by the hexane extract of *A. arctoides* (PE = plant extract).

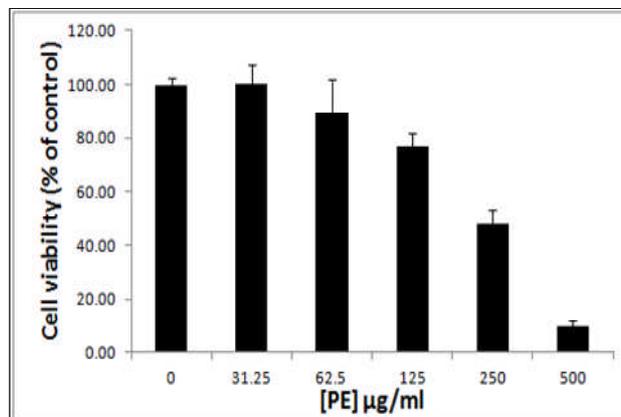


Figure 4: Dose dependent inhibition of Chang liver cell viability by the acetone extract of *P. viridiflorum* (PE = plant extract).

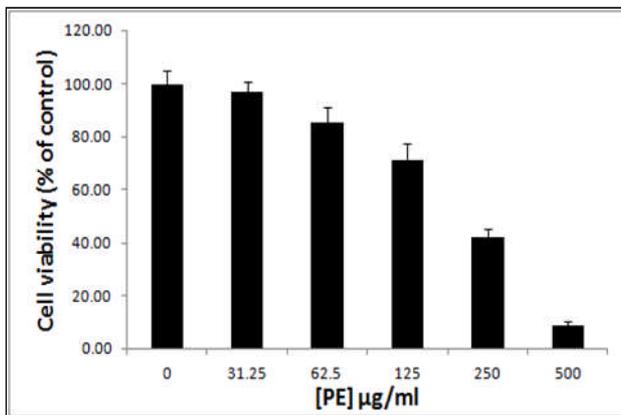
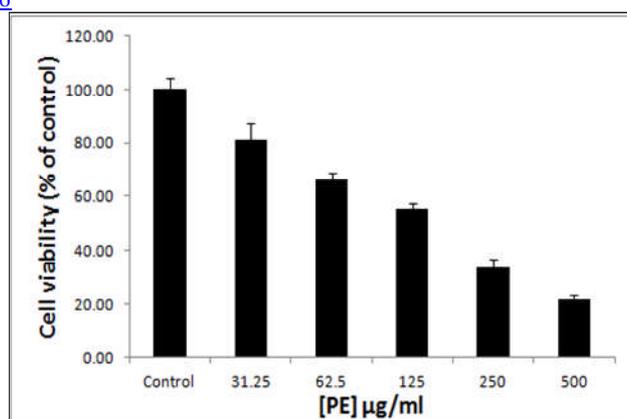
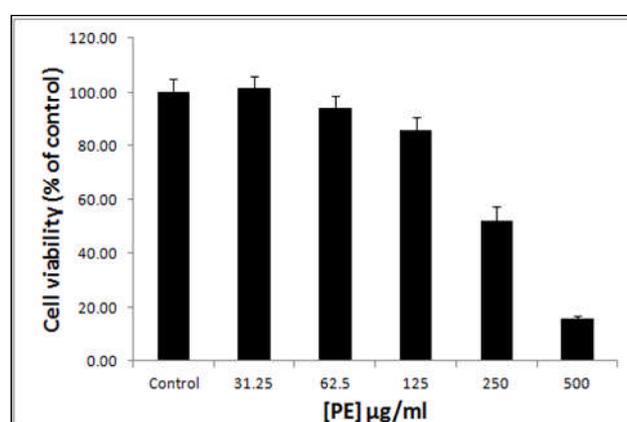


Figure 5: Dose dependent inhibition of Chang liver cell viability by the hexane extract of *P. viridiflorum* (PE = plant extract).

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**Figure 6:** Dose dependent inhibition of Chang liver cell viability by the acetone extract of *G. bicolor* (0-50µg/ml).



**Figure 7:** Dose dependent inhibition of Chang liver cell viability by the hexane extract of *G. bicolor* (PE = plant extract).

## Discussion

Griseofulvin is an orally active nontoxic antifungal drug that has been used for many years for the treatment of skin infections in both humans and animals. Its mechanism of action is believed to occur through the “selective” inhibition of fungal cell mitosis concomitant to its accumulation in the keratin layers of the epidermis. At first glance the relatively strong cytotoxic effect observed in the Chang liver cells (Fig.1) is somewhat unexpected considering its accepted non-toxic clinical use. This apparent discrepancy may to some extent be due to the limitations of *in vitro* cytotoxicity screening and the difference between therapeutic dosage and that used to determine the IC<sub>50</sub> value. While there exists a number of reports on the antiproliferatory activity of Griseofulvin against various cancer cell lines, these antiproliferatory effects occur at concentrations well above that required to inhibit fungal mitosis. One of the reasons for the relative safety of Griseofulvin at clinically useful doses may be the fact that it accumulates to high levels in the keratin layers of the skin, where it acts on the growth of dermatophytes. Such an effect is completely lost in the *in vitro* Chang liver model. The IC<sub>50</sub> value of 9.07µg/ml (approximately 25µM) obtained for the Chang liver cells is similar to that reported for HeLa (20µM) (Panda et al., 2005) and K562 cells (44µM) (Zhong et al., 2010). In contrast, the inhibitory concentrations of Griseofulvin against various dermatophytes *in vitro* range from 0.4 to 1.7µM, concentrations which are well below the range required to attenuate cell viability in Chang liver cells. Therefore the apparent cytotoxic effects displayed by Griseofulvin are unlikely to manifest in the clinical context as the dosage will be below that required to produce a toxic effect.

It is also important to point out that the MTT assay does not distinguish between cytotoxic and cytostatic effects and that Griseofulvin has been shown to induce cell cycle arrest in various cell lines (Teshome et al., 2010). Therefore, the decrease in cell viability of Griseofulvin-treated Chang liver cells is most likely due to the cytostatic effects of the drug and to a lesser extent to direct toxicity. This is supported by the Griseofulvin dose-response curve which does not extend to zero viability at the higher drug concentrations; instead a plateau is reached in the region of 30% viability which probably represents the initial cell numbers at time zero.

The IC<sub>50</sub> values for the acetone and hexane extracts were 17.4 and 12.4 µg/ml respectively (Table 1). In our experience these relatively low values raise concern for potential hepatotoxic effects and deserve further investigation or at least a warning to potential users. Similar to the principles described above for griseofulvin, it is the dosage at which these plants are used as ethnopharmacological agents and the pharmacokinetics of the toxic compounds which will ultimately determine the clinical relevance of these toxic effects.

According to Gertsch (2005) *in vitro* bioassays of biological extracts that possess IC<sub>50</sub> values greater than 200µg/ml are not considered meaningful as it is unlikely that such concentrations will be reached *in vivo* and due to the probability that high concentrations of plant extracts will significantly change the physicochemical environment in terms of ionic strength, pH and osmolarity which will impact on the cell viability independent of the extract itself leading to artificial toxicity. Depending on the dosage and duration of treatment, the cytotoxic effects of *Gasteria bicolor* and *Pittosporum viridiflorum* may be considered relatively weak (but not entirely absent) and less of a toxicity risk.

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## Conclusion

Depending on the dosage and duration of treatment the cytotoxic effects of *G. bicolor* and *P. viridiflorum* may be considered relatively weak (but not entirely absent) and less of a toxicity risk. *A. arctotoides* extracts, however, were the most toxic both in terms of IC<sub>50</sub> values as well as the steeper slope of the dose-response curve. This raises concern for potential hepatotoxic effects and deserves further investigation or at least a warning to potential users. Screening of the extracts against other cell lines such as HepG2 and Vero cells as well as the used of multiple end point assays targeted toward specific cytotoxic mechanisms may be used to improve the predictability for potential toxic effects.

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