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Toxicity Testing of Two Medicinal Plants, *Bridelia micrantha* and *Antidesma venosum*

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Abstract: *A. venosum* and *B. micrantha* are widely used ethnomedically and *B. micrantha* has furthermore indicated the potential to be developed into a drug due to the various biological activities previously reported. However, the safety of a plant must be determined before drug development. Cytotoxicity was determined using human adenocarcinoma cells of the cervix (HeLa), human breast cells (MCF-12A), lymphocytes (both resting and stimulated) as well as primary porcine hepatocytes. Acute systemic toxicity was determined using the luminescent bacteria, *Vibrio fischerii* and the vertebrate, *Poecilia reticulata* (guppy). Toxicity was found to be concentration dependent when HeLa and MCF-12A cells were exposed to the plant extracts. The IC_{50} was not reached at the concentrations tested (0.1 µg/ml – 1 mg/ml) for the hepatocytes as well as the resting and stimulated lymphocytes, indicative that both plant extracts showed little or no direct cytotoxicity against primary cultures. Both extracts resulted in 100% mortality of the guppies. This study illustrated that extracts of both *B. micrantha* and *A. venosum* are cytotoxic and possess acute systemic toxicity.

Key Words: Cytotoxicity, guppy, herbal remedy, *Poecilia reticulata,* systemic toxicity.

INTRODUCTION

Bridelia micrantha (Hochst.) Baill. (coastal goldenleaf) and *Antidesma venosum* E. Mey. ex Tul. (tassel-berry) belong to the Euphorbiaceae. Both *B. micrantha* and *A. venosum* are used by the Vhavenda for the treatment of gynaecological complaints [1]. Bark infusions of *B. micrantha* are taken by the Zulu as emetics [2], the East Africans to treat stomach ache, tapeworm infestations, diarrhoea and as tonics for children [3] and in Zimbabwe to treat infants' coughs [4]. *A. venosum* root-bark is taken for dysentery [5], and washed in to ease body pain [6]. In East Africa the roots of the latter are chewed after a snakebite, and root decoctions are used to treat abdominal pain [3], whereas in Tanzania they are used in the treatment of abdominal pain, dysmenorrhoea, malaria, schistosomiasis and to facilitate conception [7].

Previous studies have demonstrated antibacterial [8, 9], antimalarial [10] and antidiarrhoeic activity [11] for *B. micrantha.* Additionally, *B. micrantha* has been reported to inhibit β-lactamase activity [12], RNA-dependent-DNA polymerase and ribonuclease H activities of human immunodeficiency type 1 reverse transcriptase (HIV-1 RT) [13-15].

Compounds isolated from *B. micrantha* include: friedelin, taraxerone, epifriedelinol, taraxerol, gallic acid, ellagic acid [16, 17] and β-sitosterol [15]. Various alkaloids and specifically the glycine-derived acetogenic quinoline alkaloid, antidesmone, have been detected in *A. venosum* [18, 19].

B. micrantha and *A. venosum* are widely used as traditional remedies, however, due to uncertainty regarding their toxicity, confirmation is required in order to make decisions regarding whether they are safe to be used or not. In this study, we determined the cytotoxicity of the plant extracts using primary and transformed cell lines as well as acute systemic toxicity using guppies.

MATERIALS AND METHODS

Plant Specimens and Preparation

A. venosum root was collected from the Gardens at the Research Centre for Plant Growth and Development, University of KwaZulu-Natal and a voucher specimen (Fawole FAW 7) is deposited at this facility. A voucher specimen of the bark of *B. micrantha* (N.H. 1913), collected by Dr N Hahn, is accessioned to the Soutpansbergensis herbarium, Louis Trichardt.

The plant material was dried, ground and a decoction made by boiling 1g of plant material in 10 ml distilled water for 15 min. The supernatants were then first passed through 0.45 µm filters, followed by 0.22 µm filters (Millipore, Bedford, MA). Yields (w/v) for the extraction procedure as determined gravimetrically were 11.45 mg/ml for *B. micrantha* and 8.95 mg/ml for *A. venosum*.

Cytotoxicity

The MCF-12A (ATCC CRL-10782) cell line was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), insulin (10 µg/ml), hydrocortisone (500 ng/ml) and FCS (10%). HeLa (ATCC CCL-2) cells were cultured in Earl's minimum essential Eagle's medium (EMEM) supplemented with 1% penicillin/streptomycin solution and 5% heat inactivated FCS.

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Human lymphocytes were isolated from peripheral blood as described by Anderson *et al.* [20]. Primary hepatocytes were isolated from porcine liver following the method described by Frühauf *et al.* [21].

MCF-12A (4 x 10^4 cells/ml), HeLa (5 x 10^4 cells/ml), stimulated and resting lymphocytes (2 x 10^6 cells/ml) and primary hepatocytes (2 x 10^5 cells/ml) were seeded into 96-well plates and incubated for 24h, after which cells were treated with various concentrations of plant extract (0.1 µg/ml – 1 mg/ml). MCF-12A, HeLa cells and hepatocytes were incubated for 7 days and the lymphocytes for 3 days at 37°C in a 5% CO_2 incubator. Lymphocytes were tested in the resting state or stimulated by the addition of phytohemagglutinine (PHA). Cisplatin, a known anti-tumor agent, was used as positive control. The MTT assay was performed to determine cell death for each treatment as previously described by Mossmann [22]. GraphPad was used to draw the figures and for determination of the 50% inhibition of proliferation (IC_{50}) value. The tests were carried out in triplicate.

Acute Systemic Toxicity

i) BioTox™ Test

Freeze dried *Vibrio fisheri* reagent (NRRL B-11177; Bio-Orbit, Oy, Finland) was reconstituted with 2% NaCl (pH 7.0), and equilibrated for 1h. The luminescent bacteria (0.5 ml) were placed into cuvettes and the light intensity measured using a temperature-controlled luminometer (Bio-Orbit Sirius, 1257). Various dilutions (0.015, 0.0625, 0.125, 0,25, 0.5, 1.0 mg/ml) of plant specimen (0.5 ml) were combined with *V. fischeri*. The decrease of light intensity was measured after 30 min. The inhibitory effect of the samples were compared to a toxin free control and thus gave the percentage inhibition (BioTox™ Software version 1.1). Experiments were carried out in triplicate.

ii) Vertebrate Test

Ethical clearance was obtained from the Animal Use and Care Committee of the University of Pretoria. *Poecilia reticulata* were cultured in the laboratory under optimal conditions. Five fish, 8-15 days in age, were transferred into glass beakers containing 200 ml of various concentrations of the plant extracts (0.015, 0.0625, 0.125, 0, 25, 0.5, 1.0 mg/ml) or controls. Samples and controls were incubated at 21°C $\pm$ 2°C. Potassium dichromate (250 mg/ml) and EPA water was included as positive (reference toxicant) and negative controls, respectively. Mortalities were recorded after an exposure period of 96 h and expressed as percent mortality i.e. the percentage of test organisms that died per sample concentration. Tests were performed in triplicate.

RESULTS AND DISCUSSION

A. venosum and *B. micrantha* are widely used ethnomedically and *B. micrantha* has furthermore indicated the potential to be developed into a drug due to the various biological activities previously reported. However, the safety of a plant must be determined before drug development. We determined the cytotoxicity of these plants against the HeLa cancer cell line, since these cells are widely used for the evaluation of drugs [23], MCF-12A cells which are transformed cells and are sensitive to drugs, and both porcine hepatocytes and lymphocytes as these represent normal, primary cell lines and are used as control for cancer cell lines.

The effects of *A. venosum* and *B. micrantha* on HeLa, MCF-12A and both resting and PHA-stimulated lymphocytes are presented in Figs. (**1**, **2**, **3A** and **3B**), respectively. The 50% inhibition of proliferation (IC_{50}) of *A. venosum* and *B. micrantha* in HeLa cells was 25.4 µg/ml and 8.9 µg/ml, respectively (Fig. **1**). The IC_{50} was 44.0 µg/ml and 24.2 µg/ml for *A. venosum* and *B. micrantha* in MCF-12A cells (Fig. **2**). The positive control, cisplatin, had IC_{50} values of 0.14 µg/ml and 0.21 µg/ml for the HeLa and MCF-12A cells, respectively. Toxicity was found to be concentration dependent when HeLa and MCF-12A cells were exposed to the plant extracts. For the hepatocytes (results not shown) as well as the resting and stimulated lymphocytes (Fig. **3**) the IC_{50} value was not reached at the concentrations tested. The latter is indicative that both plant extracts showed little or no direct cytotoxicity against primary cultures. At low concentrations, the extracts seem to have a hormetic effect, as there was an increase in the proliferation above 100% for all cell lines.

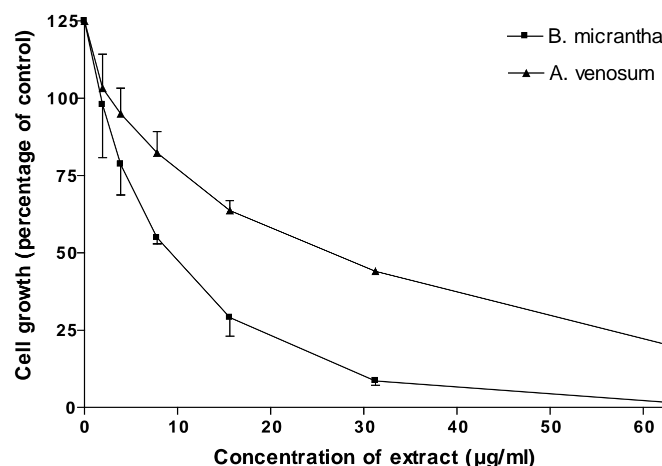

Fig. (1). Effects of *B. micrantha* and *A. venosum* on the growth of HeLa cells.

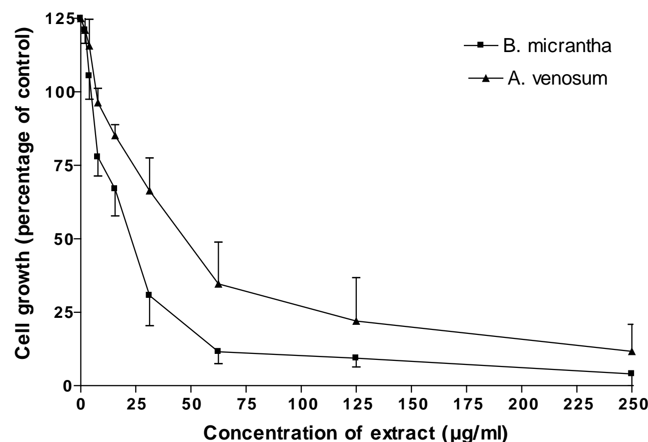

Fig. (2). Effects of *B. micrantha* and *A. venosum* on the growth of MCF-12A cells.

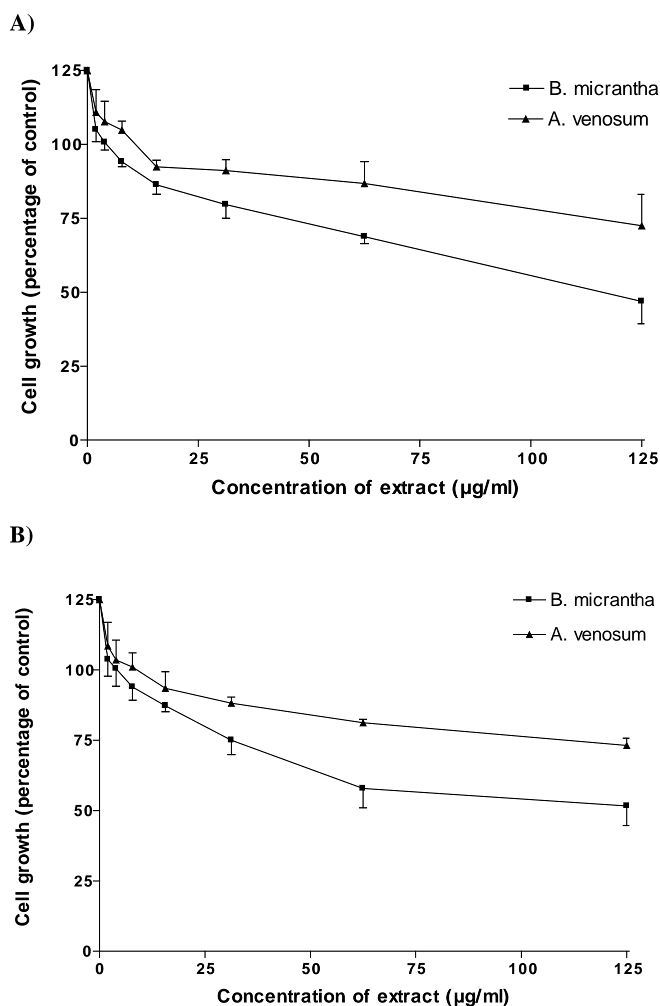

Fig. (3). Effects of *B. micrantha* and *A. venosum* on the growth of (**A**) resting and (**B**) PHA-stimulated lymphocytes.

Bacteria are amongst the most sensitive organisms in aquatic ecosystems and *Vibrio fischeri* is highly sensitive to environmental toxins. Luminescence analysis requires small specimen volumes, allows for rapid measurement, and measures analytes even in turbid solutions. However, this test is extremely sensitive to the pH of the liquid medium and when the pH was below 6 or above 8 there was an increased rate of light decay, which created inconsistency of results and therefore these results were not accepted as an indication of toxicity.

Both extracts were found to be highly toxic resulting in 100% mortality of the guppies at the concentrations tested, with the exception of the 15µg/ml extract of *A. venosum* which caused 80% mortality. Potassium dichromate (positive control) also caused 100% mortality, whereas the negative control (EPA water) maintained 100% survival. The vertebrate toxicity test thus confirmed the toxicity evident in the HeLa and MCF-12A cells.

The death of a patient has been reported, four hours after the ingestion of a cough mixture made from *B. micrantha* [24] while *A. venosum* has been reported as genotoxic [25, 26]. However, Ajaiyeoba *et al.* [27] reported that *B. micrantha* was the least toxic of 20 plants tested when using the brine shrimp lethality assay, with the 50% lethal concentration being greater than 90 mg/ml. This is contrary to the findings of the present study and at concentrations of orders of a magnitude difference when taking the vertebrate and cytotoxicity (HeLa and MCF-12A) results into account.

This study illustrated that extracts of both *B. micrantha* and *A. venosum* are cytotoxic and possess acute systemic toxicity. Using *Poecilia reticulata* allows for the biotransformation of phytochemical compounds, the latter that can have pronounced effects on toxicity, due to the formation of more or less toxic metabolites.

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