South African Journal of Botany 91 (2014) 9–13

Contents lists available at ScienceDirect

South African Journal of Botany

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

In vitro cytotoxicity and genotoxicity of five *Ochna* species (Ochnaceae) with excellent antibacterial activity

Tshepiso J. Makhafola, Lyndy J. McGaw *, Jacobus N. Eloff

Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa

ARTICLE INFO

Article history:
Received 15 April 2013
Received in revised form 13 November 2013
Accepted 14 November 2013
Available online 14 December 2013

Edited by L Verschaeve

Keywords:
Ames test
Antibacterial activity
Cytotoxicity
Mutagenicity
Ochna
Safety of medicinal plants

ABSTRACT

Extracts and fractions of some *Ochna* species had excellent antibacterial activity. Before considering the potential therapeutic use of these extracts it is important to determine the safety of extracts. The cytotoxicity of *Ochna natalitia*, *Ochna pretoriensis*, *Ochna pulchra*, *Ochna gamostigmata,* and *Ochna serrulata* (Ochnaceae) was determined in monkey kidney (Vero) cells, human hepatocellular carcinoma (C3A) cells and bovine dermis cells using the mitochondrial viability MTT assay. Their potential mutagenic effects were also determined using the Ames test with strains *Salmonella typhimurium* TA98 and TA100 with and without metabolic activation. The LC_{50} values (the lethal concentration at which 50% of the cells are killed) of the extracts on the various cell lines ranged from 26 to 99 μg/ml. None of the plant species was mutagenic (mutagenic index values ≤ 1.59 for TA98 and ≤0.92 for TA100). In a previous study, we determined the antibacterial activity of the five extracts against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. From this we calculated the selectivity index (SI) values by dividing the LC_{50} value by the minimum inhibitory concentration (MIC) to obtain the ratio of toxicity to bioactivity of each extract. The plant extracts had low SI values ≤ 1.307. This is a clear indication of non-selective toxicity, i.e. extracts are almost equally toxic to the bacteria and mammalian cell lines used in the assays. As a result, the extracts may have limited application as ingestible or intravenous therapeutic agents based on the in vitro findings. However, it may be necessary to also evaluate in vivo toxicity of the extracts in animal models as in vitro toxicity does not always equate to in vivo toxicity because of the difference in physiological microenvironment in live animals and tissue culture. Additionally, if it is the case that the toxic compounds are not the same as the active compounds, it may be possible to potentiate the extracts by the removal of toxic compounds and concentration of active compounds. The extracts may then be useful for development into treatments for topical bacterial infections.

1. Introduction

The reliance on medicinal plants as an alternative form of health care warrants scientific validation of their safety. Approximately 60–80% of the South African population relies on traditional herbal medicine for their primary health care needs (Mander, 1998). Despite the known pharmacological/therapeutic effects of traditional medicinal plants, it is crucial to validate their safety as well as their efficacy when studying their traditional uses. Medicinal plants and pharmaceutical drugs may be therapeutic at one dose and toxic at another (McGaw et al., 2007). Common misconceptions exist that, since extracts of medicinal plants are natural, they are safe. Plants used medicinally are sometimes assumed to be safe but many are potentially toxic (Street et al., 2008). Secondary metabolites, which are the basis of biological activity of medicinal plants, are not benign molecules. Plants have evolved many chemical defences to deter, stun, poison or kill threatening species (Gurib-Fakim, 2006).

In this study, we investigate the potential cytotoxic and genotoxic effects of five *Ochna* species previously reported to have good antibacterial activity against four nosocomial pathogenic bacteria, namely *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Makhafola and Eloff, 2012). We used the MTT (methyltetrazolium) cytotoxicity assay (Mosmann, 1983) which assesses the cells' mitochondrial competence. The MTT assay is widely used to assess the viability and the metabolic state of the cells (Freshney, 2000). Genotoxicity was tested using the *Salmonella* microsome mutagenicity assay which is commonly used to detect substances or chemicals that can produce genetic damage that leads to gene mutations. It has a high predictive value for in vivo carcinogenicity (Mortelmans and Zeiger, 2000; Morandim-Giannetti et al., 2011).

2. Materials and methods

2.1. Plant collection and extraction

Leaves of the five plant species were collected in summer at the National Botanical Gardens in Pretoria, the Lowveld National Botanical

* Corresponding author. Tel.: +27 12 529 8351; fax: +27 12 529 8304.
E-mail address: lyndy.mcgaw@up.ac.za (L.J. McGaw).

0254-6299/$ – see front matter
http://dx.doi.org/10.1016/j.sajb.2013.11.010

Gardens in Nelspruit and the University of Pretoria Botanical Gardens. The origin of each tree is documented in the database of the botanical garden and voucher specimens were deposited in different herbaria (Table 1). Leaves were dried in the dark at room temperature and pulverised into fine powder and stored in closed glass bottles in the dark until used. The dry leaf powder (2 g) was mixed with 20 ml acetone (technical quality; Merck Pharmaceuticals, Pretoria, South Africa). The resulting suspension was shaken vigorously in 50 ml polyester centrifuge tubes and centrifuged for 15 min at 360 ×*g* (Hettich Centrifuge, Rotofix 32A, Labotec, Johannesburg, South Africa). The extracts were decanted into preweighed glass vials through Whatman No. 1 filter paper and concentrated to dryness under a stream of air. The dried extracts were made up to a concentration of 100 mg/ml in acetone to be used in subsequent assays and stored at 4 °C in tightly stoppered glass tubes.

2.2. Cytotoxicity assay

African green monkey kidney (Vero) cells, human hepatocellular carcinoma (C3A) cells and bovine dermis cells were used in this experiment. The C3A cells were obtained from the American Type Culture Collection (ATCC CRL-10741), and the other two cell lines were obtained from the culture collection of the Department of Veterinary Tropical Diseases (University of Pretoria). The cells were maintained in Minimal Essential Medium (MEM, Highveld Biological) supplemented with 0.1% gentamicin and 5% foetal bovine serum (FBS, Adcock-Ingram) for the Vero and bovine dermis cells, and 10% FBS for the C3A cells.

The cells of a subconfluent culture were harvested using trypsin-EDTA (Sigma) and centrifuged at 200 ×*g* for 5 min and resuspended in growth medium to 5×10^4 cells/ml. A total of 200 μl of the cell suspension was pipetted into each well of columns 2 to 11 of a 96 well culture plate. The same amount of the growth medium was added to wells of columns 1 and 12 to maintain humidity and minimise the edge effect. The plates were incubated at 37 °C in a 5% CO_2 incubator overnight until the cells were in the exponential phase of growth. After incubation, the MEM was aspirated from the cells and replaced with 200 μl of different concentrations of the test samples prepared in MEM. Each dilution of the test sample was tested in quadruplicate. The plates were again incubated for 2 days at 37 °C in a 5% incubator. A negative control (untreated cells) and positive control (cells treated with different concentrations of doxorubicin chloride, Sigma) as well as an acetone solvent control, were included. After incubation, the MEM containing test substances was removed from the cells, the cells were washed with phosphate buffered saline (PBS, Sigma) and then fresh MEM (200 μl) was added to each well to avoid interaction of the plant extract with MTT. Following this washing step, 30 μl of 5 mg/ml MTT (Sigma) in PBS was added to each well and the plates were incubated for a further 4 h at 37 °C (Mosmann, 1983). After incubation with MTT, the medium in each well was removed and the formazan crystals formed were dissolved by adding 50 μl of DMSO to each well of the plates. The plates were gently shaken until the crystals were dissolved. The amount of MTT reduction was measured immediately by detecting the absorbance using a microplate reader at a wavelength of 570 nm (VersaMax, Molecular Devices). The wells in columns 1 and 12, containing medium and MTT but no cells were used to blank the microplate reader. The percentage of cell viability was calculated using the formula below:

$$\%\text{cell viability} = \frac{\text{Mean Absorbance of sample}}{\text{Mean Absorbance of control}} \times 100.$$

The LC_{50} values (the lethal concentration at which 50% of the cells are killed) were calculated as the concentration of the test sample that resulted in 50% reduction of absorbance compared to untreated cells. The intensity of the MTT formazan produced by living metabolically active cells, as determined by the correlating absorbance reading, is directly proportional to the number of live cells present (Mosmann, 1983).

From the published minimum inhibitory concentration (MIC) values (Makhafola and Eloff, 2012) and LC_{50} values calculated in this study, the selectivity index values against the three cell lines were calculated using the formula below

$$\text{SI} = \text{LC}_{50/}\text{MIC}.$$

The selectivity index values indicate the plant extract's relative safety, reflecting the ratio between toxicity and activity. A high selectivity index is an indication of a large safety margin between the concentration of the extract that is able to kill the bacteria and the concentration that is toxic to mammalian cells in this case.

2.3. Antibacterial activity testing against Salmonella typhimurium

In an initial step to assess the potential antibacterial activity of the extracts on the Ames tester strains, a serial dilution assay (Eloff, 1998) was conducted to determine the MIC values of the crude extracts against *S. typhimurium* test strains TA98 and TA100. Each extract was assayed at an initial concentration of 5 mg/ml and serially diluted two-fold in 96-well microtitre plates with equal volumes of water. One hundred microlitres of each bacterial culture was added to each well. A negative control and gentamicin as the positive control were included. The plates were incubated overnight at 37 °C. To indicate bacterial growth, 40 μl of 0.2 mg/ml INT was added to each well after incubation and the plates incubated further at 37 °C for 30 min. The MIC was recorded as the lowest concentration of the compounds at which the bacterial growth was inhibited. The experiment was performed in triplicate and repeated twice for verification.

2.4. Genotoxicity testing (Ames test)

The potential genotoxic effects of five *Ochna* species were investigated using the *S. typhimurium* test strains TA98 and TA100 (Maron and Ames, 1983). Briefly, 0.1 ml of bacterial stock was incubated in 20 ml of Oxoid Nutrient broth (Fluka) for 16 h at 37 °C on a rotative shaker. Of this overnight culture, 0.1 ml was added to 2.0 ml of top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (test sample, solvent control or positive control) and 0.5 ml phosphate buffer. The top agar mixture was poured over the surface of a minimal agar plate and incubated for 48 h at 37 °C. After incubation the number of revertant colonies (mutants) was counted. The positive control used in this study was 4-nitroquinoline 1-oxide (4-NQO) (Sigma) at concentrations of 2 and 1 μg/ml for *S. typhimurium* TA98 and TA100 respectively. All cultures were made in triplicate (except the solvent control where five replicates were made).

The above assay was repeated with the inclusion of freshly prepared S9 mixture (Sigma-Aldrich) in the place of phosphate buffer to assess the effect of metabolic activation of the plant extracts on the *S. typhimurium* test strains. The S9 mixture contained 4% (v/v) S9 fraction pooled from Sprague–Dawley male rats. Aflatoxin B_1 (1 μg/ml, Romer Labs Diagnostic GmbH, Austria) was used as a positive control

Table 1
Names of plants, herbarium and voucher specimen numbers of the five species of *Ochna* (Ochnaceae) investigated.

Plant species	Herbarium and voucher number
Ochna natalitia (Meisn.) Walp	Lowveld NBG Herbarium 30/1969
Ochna pretoriensis E.Phillips	HGWJ Schweickerdt Herbarium 114801
Ochna pulchra Hook.f	HGWJ Schweickerdt Herbarium 148021
Ochna serrulata (Hochst) Walp	HGWJ Schweickerdt Herbarium 114820
Ochna gamostigmata Du Toit	HGWJ Schweickerdt Herbarium 114796