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

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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*, *O. pretoriensis*, *O. pulchra*, *O. gamostigmata*, and *O. serrulata* (Ochnaceae) were determined in monkey kidney (Vero) cells, human hepatocellular carcinoma cells (C3A) and bovine demis 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. The LC_{50} values of the extracts on the various cell lines ranged from 26 to 99 µg/ml. None of the plants 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 which we calculated the selectivity index values respectively. The plant extracts had low selectivity index (SI) values ≤ 1.307. This is a clear indication of non-selective toxicity, i.e. extracts are 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 removal of toxic compounds and concentration of active compounds. The extracts may then be useful for development into treatments of topical bacterial infections.

Keywords: *Ochna*; Cytotoxicity; Ames test; Safety of medicinal plants, Antibacterial activity

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 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

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

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 minutes at 360 X g (Hettich Centrifuge, Rotofix 32A, Labotec Johannesburg, South Africa). The extracts were decanted into preweighed glass vials through Whatman No. 1 filter papers 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 5°C in tightly stoppered glass tubes.

2.2. Cytotoxicity assay

African green monkey kidney cells (Vero cells), human hepatocellular carcinoma cells (C3A) 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 x g for 5 minutes and resuspended in growth medium to 5 x 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 minimize 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. 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) were included. After incubation, 30 µl of 5 mg/ml MTT (Sigma) in phosphate buffered saline PBS was added to each well and the plates were incubated for a further 4 hours at 37°C. 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 column 1 and 12, containing medium and MTT but no cells was 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 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 is directly proportional to the number of live cells present (Mosmann, 1983).

From the published 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

$$\mathbf{SI = LC_{50} / MIC}$$

The selectivity index values indicate the plant extract's relative safety. The index value is a measure of the extract's beneficial affects at a low dose versus its harmful effects a high dose. A high selectivity index is an indication of a large safety margin between beneficial and toxic dose.

2.3. Genotoxicity testing (Ames test)

The potential genotoxic effects of five *Ochna* species were investigated using the *Salmonella 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 16h at 37°C on a rotative shaker. Of this overnight culture, 0.1 ml were 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).

3. Results and Discussion

Most reports of toxic effects due to the use of herbal medicines and dietary supplements are associated with hepatotoxicity, although reports of other toxic effects

including kidney, nervous system, blood, cardiovascular and dermatologic effects, mutagenicity and carcinogenicity have also been published (Temple and Himmel, 2002). It was for these reasons that we investigated the cytotoxic effects of extracts of five *Ochna* species. Table 2 shows the antibacterial activity and cytotoxic effects of

Table 2. Minimal inhibitory concentrations (MIC) of leaf acetone extracts of five *Ochna* species against *E. coli, P. aeruginosa, S. aureus and E. faecalis*, and cytotoxic effects against Vero, dermal and hepatic cells.

	Antibacterial activity MIC values (mg/ml) (Makhafola and Eloff, 2012)					
Organisms	*O. pretoriensis*	*O. pulchra*	*O. serrulata*	*O. gamostigmata*	*O .natalitia*	Gentamicin
E. coli	0.065 ±0.02	0.312 ±0.00	0.312 ±0.00	0.312 ±0.00	0.520 ±0.18	0.0039
P. aeruginosa	0.104 ±0.04	0.065 ±0.02	0.156 ±0.00	0.104 ±0.04	0.833 ±0.36	0.00019
S. aureus	0.104 ±0.04	0.078 ±0.00	1.250 ±0.00	0.416 ±0.18	0.416 ±0.18	0.00048
E. faecalis	0.039 ±0.00	0.078 ±0.02	0.520 ±0.18	0.312 ±0.00	0.625 ±0.00	0.0039
	Cytotoxic effects LC_{50} (mg/ml)					
Cell line	*O .pretoriensis*	*O. pulchra*	*O. serrulata*	*O. gamostigmata*	*O. natalitia*	Doxorubicin
Vero	0.040 ±0.003	0.040 ±0.001	0.026 ±0.002	0.059 ±0.003	0.046 ±0.003	0.0170
bovine dermis	0.051 ±0.001	0.037 ±0.001	0.044 ±0.014	0.066 ±0.004	0.070 ±0.004	0.0042
C3A	0.051 ±0.001	0.055 ±0.006	0.036 ±0.003	0.029 ±0.003	0.099 ±0.013	0.0006

the five *Ochna* species. Furthermore, selectivity index values demonstrating selective activity of the extracts were calculated (Table 3). The extracts were almost equally toxic to all three cell lines with LC_{50} values ranging from 0.026-0.099 mg/ml. *O. natalitia* was the least toxic against all the cell lines, followed by *O. gamostigmata*, *O. pretoriensis*, *O. pulchra* and lastly *O. serrulata*. Each of the extracts had similar cytotoxic effects on all the cell lines irrespective of the differences in cell properties and physiological origins.

The extracts were almost equally toxic to the three cell lines and the pathogens used in the previous study with selectivity index values ranging from 0.074 to 1.307. With selectivity index values of less than 10 against all the bacteria, it appears that the observed antibacterial activity may be a result of general metabolic toxicity. Moreover, the almost equal LC_{50} values may be due to a similarity in the chemical composition of active constituents in the crude extracts prepared from species of the

Table 3. Selectivity index values of acetone leaf extracts of five *Ochna* species against Vero, dermal and hepatic cells.

Selectivity Index values					
Vero monkey kidney cells					
Organisms	*O. pretoriensis*	*O. pulchra*	*O. serrulata*	*O. gamostigmata*	*O .natalitia*
E. coli	0.615	0.128	0.083	0.189	0.088
P. aeruginosa	0.385	0.615	0.166	0.567	0.055
S. aureus	0.385	0.513	0.021	0.142	0.111
E. faecalis	0.975	0.513	0.050	0.189	0.074
Bovine dermis					
Organisms	*O. pretoriensis*	*O. pulchra*	*O. serrulata*	*O. gamostigmata*	*O .natalitia*
E. coli	0.785	0.119	0.141	0.212	0.135
P. aeruginosa	0.490	0.569	0.282	0.635	0.084
S. aureus	0.490	0.474	0.035	0.159	0.168
E. faecalis	1.307	0.474	0.085	0.212	0.112
Human hepatocytes (C3A)					
Organisms	*O. pretoriensis*	*O. pulchra*	*O. serrulata*	*O. gamostigmata*	*O .natalitia*
E. coli	0.785	0.176	0.115	0.093	0.190
P. aeruginosa	0.490	0.846	0.231	0.279	0.119
S. aureus	0.490	0.705	0.029	0.069	0.238
E. faecalis	1.307	0.705	0.069	0.093	0.158

same genus. We did however previously report that there are major differences in the phytochemical constituents of these species (Makhafola and Eloff, 2012).

Although the extracts had low selectivity index values (indicating similar cytotoxicity and antibacterial activity) it may be worthwhile to evaluate the *in vivo* toxicity of the extracts in animal models where the activity is high enough to warrant it. The *in vitro* toxicity does not always equate to *in vivo* toxicity because of the difference in physiological microenvironment in live animals and tissue culture. Other factors related to chemical kinetics such as rate of absorption, biotransformation, distribution and excretion, which influence the exposure at the level of target cells *in vivo*, cannot at present be adequately simulated *in vitro* (Freshney, 2000).

Table 4 shows the results obtained from the mutagenicity testing of the five *Ochna* species represented as number of revertants per plate and mutagenic index value. The bacterial-based Ames test is primarily used to confirm gene safety of test substances. There is a direct relationship between mutagenesis and carcinogenesis. Because of this relationship, evaluation of the mutagenic effects of medicinal plants is highly

Table 4. Mutagenic effects of acetone leaf extracts of five *Ochna* species in the *Salmonella*/microsome assay using *S. typhimurium* TA98 and TA100 tester strains without metabolic activation. The results are expressed as mean number of revertants, standard deviation and mutagenic index values (in parentheses).

***Salmonella typhimurium* TA98**					
Conc. (mg/ml)	*O. pretoriensis*	*O. pulchra*	*O. serrulata*	*O. gamostigmata*	*O .natalitia*
5	24.66 ±2.08 **(1.011)**	34.33 ±2.52 **(1.407)**	24.33 ±1.55 **(0.997)**	35.00 ±3.00 **(1.434)**	25.33 ±2.52 **(1.038)**
0.5	36.00 ±4.36 **(1.475)**	36.67 ±1.15 **(1.503)**	27.00 ±1.73 **(1.107)**	27.00 ±4.00 **(1.107)**	29.33 ±0.58 **(1.202)**
0.05	39.00 ±3.30 **(1.598)**	33.00 ±1.00 **(1.352)**	33.67 ±0.57 **(1.379)**	31.00 ±1.73 **(1.270)**	26.33 ±3.00 **(1.079)**
0	24.4 ±3.58 **(1)**	**4-NQO (2 µg/ml)**	301.33 ±3.21 **(12.35)**		
***Salmonella typhimurium* TA100**					
Conc. (mg/ml)	*O. pretoriensis*	*O. pulchra*	*O. serrulata*	*O. gamostigmata*	*O .natalitia*
5	121.33 ±2.52 **(0.792)**	104.33 ±4.04 **(0.681)**	94.67 ±1.53 **(0.618)**	101.00 ±1.00 **(0.659)**	127.33 ±1.53 **(0.831)**
0.5	140.00 ±4.04 **(0.914)**	110.33 ±1.53 **(0.720)**	118.00 ±1.00 **(0.770)**	110.67 ±2.08 **(0.722)**	123.67 ±1.53 **(0.807)**
0.05	141.67 ±3.06 **(0.925)**	124.00 ±4.36 **(0.809)**	120.67 ±2.89 **(0.788)**	128.67 ±6.66 **(0.839)**	136.33 ±2.082 **(0.889)**
0	153.20 ±2.86 **(1)**	**4-NQO (1 µg/ml)**	1154.67±5.77 **(7.54)**		

recommended for detecting potential genotoxicants (Morandim-Giannetti et al., 2011). From the results obtained in this study, no extract of the five *Ochna* species had any mutagenic effects on *Salmonella typhimurium* TA98 and TA100 tester strains. In all cases, the mutagenic index values (MI = # of revertants in test plate/# of revertants in negative control) were less than 1. A test sample is considered to be mutagenic when the numbers of revertants in the test plate are two times or more than the number of revertants in the negative control. Moreover, a clear dose-response should be evident for the various concentrations assayed (Varella et al., 2004, Verschaeve and Van Staden, 2008).

4. Conclusion

Based on the results obtained from this study, the previously reported high antibacterial activity of the five *Ochna* species may be a result of non-selective toxicity i.e. general toxicity. Because there were such good antimicrobial activities *in*

vitro it may be worthwhile to confirm the toxic effects *in vivo* if the extracts are to be used in a therapeutic context. If the extracts also have high *in vivo* toxicity, the extracts may still be useful in topical applications to treat infections and wounds. It is also possible that the safety/antimicrobial activity can be altered by solvent-solvent fractionation (Eloff et al., 2005, Masoko et al., 2010). The extracts were not mutagenic in the Ames test without metabolic activation. These results show the importance of determining the cytotoxicity and mutagenicity of medicinal plant extracts or phytocompounds before they are considered as remedies.

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