Acute animal toxicity and genotoxicity of obliquumol, a potential new framework antifungal compound isolated from *Ptaeroxylon obliquum* (Rutaceae) leaf extracts.

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Abstract

Obliquumol (12-*O*-acetylptaeroxylinol) isolated from *Ptaeroxylon obliquum* leaves has excellent antifungal activity and low cellular toxicity. As a next step in the potential development of a framework antifungal product, the present work investigated the acute animal toxicity of obliquumol according to OECD 423 guidelines. Furthermore, the genotoxicity of *P. obliquum* acetone leaf extracts, fractions (hexane, chloroform and 30% H_2O in MeOH) and isolated compounds (obliquumol and a mixture of lupeol and β-amyrin) was determined using Ames test. . A single dose of obliquumol was orally administered to mice at levels of 50, 300 and 2000 mg/kg and observed for 14 days. The three

Salmonella typhimurium tester strains TA 98, TA 100 and TA 102 were used without metabolic activation to determine the genotoxicity. Even at the highest dose of obliquumol, the mass, behaviour and food intake of the animals were not affected. Gross necropsy and histopathological analysis on organs indicated hardly any effects. No samples had genotoxic activity against the *S. typhimurium* strains tested. Obliquumol had an LD_{50} >2000 mg/kg since there were no mortalities after 14 days. This encourages the possible development of a new class of antifungal compounds from the obliquumol framework.

Keywords: *Ptaeroxylon obliquum*, obliquumol, acute toxicity, genotoxicity, Ames test

1. Introduction

Medicinal plants have been used for years to treat and prevent various ailments including bacterial, fungal, parasitic and inflammatory infections or related symptoms (Botha and Penrith, 2008). This, however, does not necessarily mean they are safe and free from side effects. Generally, medicinal plants are considered safe because of their long traditional use without documented scientific toxicity. Toxicity of herbal medicine in some cases may result from misidentification, inadvertent or deliberate substitution of herbal material, incorrect or different preparation or inappropriate administration and unregulated dosages (Stewart et al., 1998: Van Wyk et al., 2002). Due to their structural diversity, plants are a valuable source of thousands of phytochemicals and some of them are toxic (Kuete et al., 2013). Numerous compounds with promising biological activities have been investigated, but some are not suitable for therapeutic use due to toxicity, carcinogenic or

mutagenic activities. The structure of active molecules may be changed to improve pharmacological activity and/or reduce toxicity (Kostova, 2005).

In vitro toxicity assays can be used to some extent to predict animal toxicity in the general screening of extracts, fractions, or compounds. Nonetheless, it is dangerous to extrapolate from *in vitro* to *in vivo* activity (Scheers et al., 2001). Although obliquumol had high cellular safety and selectivity index against different fungi (Ramadwa et al., 2021), it is essential to determine the *in vivo* safety and activity in an animal model. Genotoxicity assays are used to identify substances that have the potential ability to interact with nucleic acids at low concentrations. When a toxic substance interacts with DNA, it may lead to genetic mutations, chromosomal aberrations, and rearrangement of the chromosomes through translocation, deletion, and inversion (Varanda et al., 2002; Słoczyńska et al., 2014).

When choosing plants for ethnopharmacological studies, researchers are encouraged to search for available data on toxicological profiles of the plant of interest in the literature and where toxicity effects are not known, parallel toxicity studies, or inclusion of a panel of unrelated organisms are useful in detecting potential toxicity when screening plant extracts, fractions, or isolated compounds for biological activities (Cos et al., 2006). In a targeted analysis of the antimicrobial activity of leaves of South African tree species to find out if there was a relationship between taxonomy and antimicrobial activity (Pauw and Eloff, 2014), we found that acetone leaf extracts of *Ptaeroxylon obliquum* (Thunb.) Radlk had excellent antifungal activity.

Different parts of *P. obliquum*, a South African medicinal plant, also known as sneezewood, has been used for the treatment of parasitic infections in animals,

tuberculosis (TB) and related symptoms, and other microbial infections (Van Wyk et al., 1997). Further studies on the acetone leaf extract of this plant led to isolation of obliquumol from the non-polar fractions (Ramadwa et al., 2019). Obliquumol had better antifungal activity against *C. albicans* ATCC 10231, *C. neoformans* and *A. fumigatus* than with MICs of 2-16 µg/mL than the gold standard positive control amphotericin B. Obliquumol was not toxic against Vero African green monkey kidney cells or human liver (C3A) cells at the highest concentration tested (200 µg/mL) and had good selectivity index values against *C. albicans* and *Cryptococcus neoformans* (Ramadwa et al., 2021).

In our efforts to synthesize obliquumol and its derivatives, it became clear that the correct structure of obliquumol is not the angular compound (eranthin acetate) as previously reported (Ramadwa et al., 2019), but the linear isomer ptaeroxylinol acetate (Malefo et al., 2020). Because obliquumol represents a potential novel framework molecule for the development of new bioactive compounds the biological activity was patented in Europe and the USA (Van Wyk et al., 2017). Since the patenting we discovered additional biological activities (Khunoana et al., 2022; Ramadwa et al., 2022; Ramadwa et al., 2023: Ramadwa et al., 2024). The high biological activities and low cytotoxicity motivates the investigating of the toxicity of obliquumol compound in animal studies as a required step before efficacy studies in animal models and potential development of new medicinal products.

In the search for new potential drugs, before *in vivo* studies on the efficacy of the compounds or extracts, it is important to determine the acute toxicity in an animal model before ethical approval can be obtained for efficacy studies in animals. The use of animals in toxicity testing is regulated in the protocols of the Organization for Economic

Cooperation and Development (OECD) which is enforced by South African law. The present study aimed to determine the *in vivo* safety of obliquumol isolated from *P. obliquum* acetone leaf extract by measuring the acute toxicity in mice and to determine the potential genotoxicity.

2. Materials and Methods

2.1. Plant collection, identification, and storage

The leaves of *P. obliquum* used in the study were collected during the summer of 2019 from a tree growing in the South African National Biodiversity Institute (SANBI), Pretoria (25° 44.0548' S, 28° 16.815' E). Plant material was collected in open woven orange bags, dried at room temperature in the shade and powdered using a mill. The powders were then stored in closed containers in the dark until needed. Voucher specimens were prepared and kept at the HGWJ Schweickerdt Herbarium of the University of Pretoria (PRU130510).

2.2. Isolation of large quantities of obliquumol from *P. obliquum* leaves

Acetone leaf extracts were partitioned by liquid-liquid fractionation into water, butanol, 30% water in methanol (30% H_2O in MeOH), chloroform and hexane fractions, and pure compounds (obliquumol and a mixture of lupeol and β-amyrin) were isolated using open column silica gel chromatography and hexane-ethyl acetate gradient as previously described (Ramadwa et al., 2019). Obliquumol was dissolved in analytical grade propylene glycol (Sigma-Aldrich) and prepared in a range of doses (50, 300 and 2000 mg/kg) to determine the acute toxicity at these concentrations.

2.3. Acute toxicity

The animal experimental protocol was submitted and approved by the Animal Ethics Committee (AEC) of the University of Pretoria (Approval Number V113-16). The acute oral toxicity test of obliquumol isolated from *P. obliquum* extracts was evaluated in mice according to the procedures outlined by the OECD guidelines No.423 (OECD, 2001). The experiment was conducted at the OVARU, at the Faculty of Veterinary Science in a conventional experimental animal room, according to the OECD 423 guidelines with slight modifications. The experiment was performed twice.

The female Swiss albino mice/CDI used for the study were marked for proper individual identification and kept in the cages for at least 7 days prior to dosing to allow for proper acclimatization to the laboratory conditions. A total of 18 mice were used for each group and 6 mice were randomly allocated to each step per group. All animals were approximately 8 weeks old at the start of the study. Mice were housed in groups of 3 per standard Type II rodent cage and were provided with sawdust bedding and standard enrichment items (autoclaved toilet rolls, tissues, wooden sticks, and egg containers). All the mice were fed EPOL rodent pellets *ad libitum* and reverse osmosis water was always freely available. Animals were under the constant care of a registered para-veterinarian and under the supervision of a veterinarian. The compound was administered in a single dose by gavage using an intubation cannula/dosing needle. The initial dose tested was 50 mg/kg and, upon no signs of toxicity to the mice, the dose was increased to 300 mg/kg for another group of mice, and then to 2000 mg/kg for the third group of mice, which is the highest dose recommended by the OECD. The mice were fasted for 3 h by withholding food prior to gavage, but water was supplied with no restriction. After fasting, the animals

were weighed and obliquumol was administered by oral gavage. Food was further withheld for 3 h after administration. The mice were observed individually at least once during the first 30 minutes after dosing followed by periodic inspection during the first 24 h and daily thereafter for a total of 14 days per dosing group. Animals were monitored for 14 days and were euthanized at the end of the study by means of isoflurane overdose and subjected to full necropsy and histopathology.

2.4. Histopathology

A necropsy was carried out on the day the mice were euthanized. The animals were identified with a unique reference number allocated to each individual mouse. During necropsy morphological findings were recorded, and specimens were collected from each animal and fixed in 10% buffered formalin. The following histological organ specimens were collected: adrenal gland, brain, heart, intestines (large and small), kidney, liver, lung, lymph node, oesophagus, pancreas, spleen, stomach, testes, ovary/thymus, and urinary bladder. Sections were cut and processed according to routine histological tissue processing in an automated tissue processor following standard operating procedures. Following tissue processing, sections were cut of 5-6 µm and the slides produced were stained in an automated haematoxylin and eosin tissue stainer before histological evaluation.

2.5. Ames Test

The *Salmonella* microsome assay with slight modifications was used to determine the genotoxicity of the acetone leaf extracts, fractions and isolated compounds from *P. obliquum* leaves (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). The crude

extract and fractions were dissolved in 10% dimethyl sulphoxide (DMSO) to give three concentrations of 5000, 500 and 50 µg/mL. The isolated compounds were dissolved to concentrations of 2000, 200 and 20 µg/mL. The Ames test was performed with three *Salmonella typhimurium* tester strains, TA 98, TA 100, and TA 102, without metabolic activation. Bacterial stocks (100 µL) were incubated in 10 ml of Oxoid nutrient broth No.2 at 37°C on a rotary shaker for 16 h. The cultured bacteria (100 µL) were added to 100 µL of the test sample with 500 µL of phosphate buffer and 2 ml of top agar containing biotin–histidine (0.5 mM). The top agar mixture was then poured over the surface of a minimal agar plate and incubated at 37°C for 48 h. The positive control used was 4-nitroquinoline1-oxide (4-NQO) at a concentration of 2 µg/mL. Sterile distilled water and 10% DMSO were used as negative controls. All the samples were tested in triplicate and the experiment was repeated twice. The results were expressed as the mean number of revertant colonies per plate.

3. Results

3.1. Acute toxicity

3.1.1. Influence of obliquumol on mass of mice.

There was no weight loss in any of the treatments of the mice with the different concentrations of obliquumol. In some cases, there was a slight gain in mass.

3.1.2. General post-mortem observations

The following general macroscopic observations were made by a qualified pathologist as part of the service contract with University of Pretoria Onderstepoort Veterinary Animal Research Unit (OVARU). The mice were all intact, adult females in good body condition,

the stomachs were empty, and a moderate quantity of granular material was noted in segments of the small intestine. Well formed, dark green, granular faecal material was noted in the large intestines. The livers were dark red and normal in size, shape, and consistency. The lungs were pink and well aerated. The hearts were within normal limits.

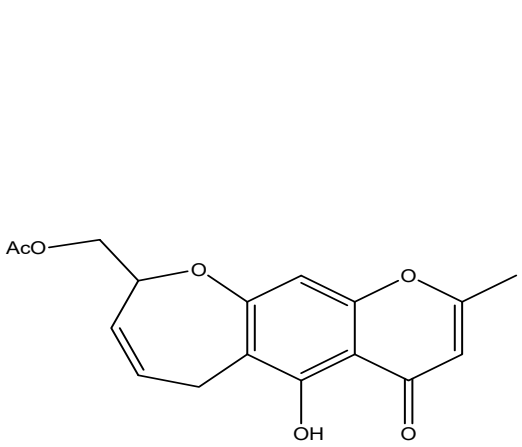
3.2. Histopathology

There was no microscopic pathology detected on the adrenal gland, cerebellum, cerebrum, heart, large intestines, lungs, lymph node, oesophagus, ovary, pancreas, small intestines, spleen, stomach, thymus and uterus. The livers of all the tested mice had some evidence of mild, sublethal, non-specific hepatocellular injury. However, there was no sign of necrosis or inflammation. The cause could not be established as there were no specific or pathognomic lesions and it was not dose associated. A few mice did show background lesions such as interstitial nephritis, but these were all mild and were expected to have had little to no clinical effect on the mice.

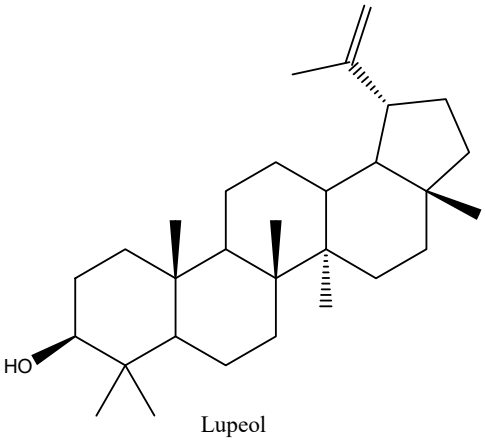
3.3. Genotoxicity

The Ames test was performed with *S. typhimurium* strain TA 98 which detects frame-shift mutations, TA 100 which detects base-pair substitutions and TA 102 which detects oxidative and DNA cross-linking damage. In the Ames test, for the acetone leaf extracts, fractions and isolated compounds to be considered mutagenic, the samples must exhibit a dose-dependent increase in the mean number of revertant colonies. Additionally, the number of revertant colonies produced in each plate of the extracts, fractions and isolated compounds must be at least two times higher than those of the negative control (Maron and Ames, 1983; Madikizela et al., 2014). The acetone leaf extract, hexane, chloroform

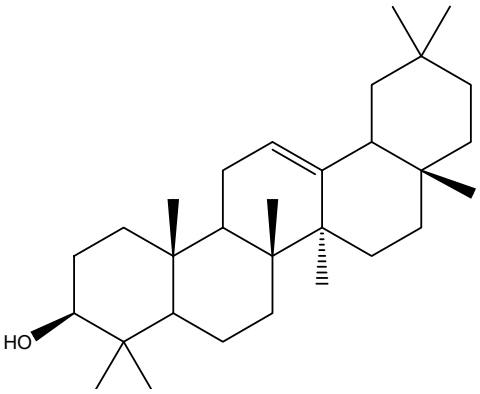
and 30% H_2O in MeOH fractions, obliquumol and a mixture of lupeol and β-amyrin as presented in **Figure 1** were all not genotoxic against any of the tested *S. typhimurium* TA 98, TA 100, and TA 102 strains in the absence of metabolic activation as presented in **Table 1**.



Obliquumol (12-*O*-acetylptaeroxylinol)



Lupeol



β-amyrin

Figure 1: Structures of obliquumol and, lupeol and β-amyrin mixture isolated from *P. obliquum* leaves (Ramadwa et al., 2019).

Table 1. Mean number of revertant colonies per plate (± SD) of *S. typhimurium* strains TA 98, TA 100, and TA 102 exposed to different concentrations (µg/ml) of the acetone leaf extracts, fractions, and isolated compounds from *P. obliquum.*

Samples	**Bacterial strains**			
	Conc (µg/ml)	**TA 98**	**TA 100**	**TA 102**
Acetone leaf extracts	5000	13.00 ± 4.36	154.66 ± 9.29	226.50 ± 7.78
	500	12.66 ± 4.04	143.00 ± 21.92	232.33 ± 16.26
	50	17.66 ± 3.51	118.66 ± 4.16	209.66 ± 23.54
Hexane fraction	5000	13.77 ± 2.64	122.33 ± 19.79	214.50 ± 10.61
	500	15.55 ± 2.08	139 ± 7.21	219.00 ± 24.25
	50	14.55 ± 4.16	144.66 ± 14.74	240.33 ± 17.79
Chloroform fraction	5000	17.33 ± 2.88	137.66 ± 14.85	226.50 ± 13.43
	500	9.66 ± 0.57	117.33 ± 1.41	220.50 ± 10.61
	50	11.00 ± 20	116.66 ± 0.71	196.33 ± 9.86
30% H_2O in MeOH fraction	5000	11.66 ± 5.50	111.00 ± 8.48	245.50 ± 6.36
	500	14.33 ± 6.11	125 ± 13.23	220.66 ± 10.69
	50	13.33 ± 5.51	115.00 ± 12.73	207.00 ± 35.76
Obliquumol	2000	11.66 ± 1.53	133.66 ± 9.81	243.00 ± 14.57
	200	8.33 ± 2.52	129.33 ± 5.65	216.00 ± 4.24
	20	7.33 ± 1.53	116.33 ± 17.15	212.00 ± 10
Lupeol & β-amyrin mixture	2000	7.00 ± 1.65	122.33 ± 22.14	224.00 ± 20.66
	200	5.33 ± 1.15	166.00 ± 12.02	208.00 ±3 9.60
	20	7.33 ± 0.58	126.00 ± 9.85	221.66 ± 17.47
10% DMSO		7.00 ± 1.73	121.00 ± 19.16	209.00 ± 11.31
H_2O		11.33 ± 2.31	125.66 ± 5.65	236.50 ± 14.85
4-nitroquinoline1-oxide		44.66 ± 11.72	535.00 ± 54.29	751.33 ± 103.35

4. Discussion

Identifying and categorizing compounds or plant extracts according to their ability to cause acute harm to living organisms at high doses, particularly anatomical, pathological and lethal injuries, is done through the evaluation of acute toxicity. This methodology can also help establish toxicity parameters and provide other information on the toxicity of substances to human health (Valadares, 2006; Zatta et al., 2009). The initial step in the toxicological analysis of herbal medications is to investigate the acute toxicity (Déciga-Campos et al., 2007). Safety evaluation investigations provide significant details about the toxicity of herbal products before moving on to efficacy studies and clinical trials. Even though it has been demonstrated that herbal extracts or bioactive compounds offer a variety of biological activities and the potential for a wide range of uses, the possible adverse consequences of herbal extracts or bioactive compounds are at times underestimated. When obliquumol was administered in all the tested doses there was no mortality in all the treated animals. Moreover, no significant variations were recorded in the body weight of all the animals which indicated that the obliquumol had an LD_{50} above 2000 mg/kg. Histopathological analysis of the liver and renal tissues supported these findings. In animals, the liver is one organ involved in the biotransformation of hazardous chemicals. In addition, the kidneys are an organ connected to the toxicokinetic process of toxokinetic elimination. The renal tissues and liver of the treated mice were found to be normal and only had insignificant lesions expected to have had little to no clinical effect on the mice.

Since it has been discovered that nearly all chemicals that can cause bacterial mutations in animals are also carcinogenic to humans, mutagenic compounds, fractions, or plant

extracts pose potentially hazardous risks to humans and should be carefully investigated based on risk and benefit (Déciga-Campos et al., 2007). The standard plate incorporation test created by Ames et al. (1973) is a highly helpful approach to identify mutagenic chemical compounds. This test determines whether any samples cause the genetically modified DNA of *S. typhimurium* strains to mutate (Ames et al., 1973). The Ames test uses *S. typhimurium* strains that are auxotrophic for histidine to find specific types of gene alterations, such as base pair substitutions and frameshift mutations (Mortelmans, 2019). The acetone crude extracts, hexane, chloroform, and 30% H_2O in MeOH fractions, obliquumol, and a mixture of lupeol and β-amyrin, were not genotoxic to any of the tested *S. typhimurium* TA 98, TA 100, and TA 102 strains in the absence of metabolic activation. This demonstrated the potential of obliquumol as an antifungal framework molecule because the Ames test is a key tool for toxicological genetics and forms part of a battery of preclinical studies designed to determine the mutagenic potential of new pharmaceuticals and alternative therapies (Zwarg et al., 2010).

5. Conclusion

The isolated compounds, fractions, and *P. obliquum* acetone leaf extracts were tested for their potential genotoxicity and none of the samples were genotoxic. In the acute toxicity test, obliquumol had an LD_{50} above 2000 mg/kg. After establishing the *in vivo* efficacy of obliquumol, it may be interesting to determine chronic toxicity of obliquumol. It appears that it may be possible to develop a new class of antifungal or antiparasitic compounds based on the obliquumol framework (Ramadwa et al., 2021).

Declaration of Competing Interest

The authors declare that none of the work reported in this study could have been influenced by any known competing financial interests or personal relationships.

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CRediT authorship contribution statement

TER analyzed the data and wrote the first draft. BM assisted with Ames test assay. JNE LJM supervised the work. All authors read and approved the final manuscript.

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