Anthelmintic and cytotoxic activities of extracts of *Markhamia obtusifolia* Sprague (Bignoniaceae)

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Abstract

The anthelmintic activity of *Markhamia obtusifolia* Sprague (Bignoniaceae) leaf extracts was evaluated against the ruminant gastrointestinal nematode parasite *Trichostrongylus colubriformis* (Nematoda: Strongylida) using the *in vitro* egg hatch test. Also, the cytotoxic activity of aqueous extracts of *M. obtusifolia* was evaluated in cell line cytotoxicity assays. The results indicated that the effective concentration (EC_{50}) for the water extract of *M. obtusifolia* leaves (0.46 mg/mL; Confidence Interval [CI] 0.3-0.5 mg/mL) was significantly lower than the EC_{50} for the acetone extract of *M. obtusifolia* (0.8 mg/mL; CI 0.7-1 mg/mL). Aqueous extracts were twice as potent as the acetone extracts. The ED_{90} (0.2 mg/mL; CI 0.1-0.02) for thiabendazole (positive control) was significantly lower than the EC_{90} for the water extract of *M. obtusifolia*

(10.7 mg/mL; CI 8.3-13.7 mg/mL). In the cytotoxicity bioassay, the lethal concentration (LC_{50}) for the aqueous extract of *M. obtusifolia* was 0.476 mg/mL, which was relatively high (low toxicity) in comparison to the highly toxic berberine (LC_{50} = 9.80 µg/mL). The current study showed that *M. obtusifolia* plant extracts possess anthelmintic activity and are relatively non-cytotoxic, thus providing support for their use in traditional veterinary practices.

Keywords: Anthelmintic; *Markhamia obtusifolia*; *Trichostrongylus colubriformis*; Cytotoxicity; Livestock health

1. Introduction

In most developing countries, gastrointestinal nematodes impact negatively on livestock production (Satrija et al., 2001). Gastrointestinal parasites cause clinical and sub-clinical infections that reduce animal survival and depress growth rates, wool and milk production, and reproductive performance (Alawa et al., 2010). Parasitism has been ranked high among factors that threaten livestock production worldwide (Perry et al., 2001).

Moreover drugs needed for the treatment of endoparasites of humans or livestock are usually expensive and often out of reach for the populations of developing countries (Amin et al., 2009). Furthermore, there is rapid development of anthelmintic resistance (Melo et al., 2003). According to Alawa et al. (2003), declining funding and rising costs of veterinary services cause difficulties in access to these services by resource-poor communities. Alternative methods of control are thus required that are both practical and economical for farm production systems. Anthelmintics derived from plant parts that are used traditionally for treatment of parasitic infections in human and animals

may offer an alternative to minimize some of these problems (Akhtar et al., 2000). In addition, the growth of organic livestock farming globally, which is claimed to be less toxic to man and the environment might favor the continuous use of traditional medicinal plants in Africa for treatment of endoparasitic infections caused by intestinal worms.

Suitable anthelmintic bioassays are being used to evaluate extracts from different plant species in order to validate some of the traditional claims about their efficacy. For example, the species *Vernonia amygdalina* Delile (Asteraceae) and *Annona senegalensis* Pers (Anonnaceae) are used by local livestock farmers in Nigeria as anthelmintics and their aqueous extracts showed *in vitro* anthelmintic activities (Alawa et al., 2003). *Spilgelia anthelmia* L. (Loganiaceae), which is used for the treatment of gastrointestinal helminthiasis in folk medicine of Brazil, Panama and Costa Rica also showed *in vitro* ovicidal and larvicidal activity against *Haemonchus contortus* Cobb (Nematoda: Strongylida) (Assis et al., 2003).

Even though the use of herbal remedies to treat animal diseases is widespread, there is a possible threat of herbal preparations being toxic (van der Merwe, 2001 and Masika et al., 2000). Plant extracts may simultaneously have high anthelmintic activities and high non-selective cytotoxic activities. Results from numerous studies suggest that tannin components in plant extracts have anthelmintic and cytotoxic activities (Takechi et al., 1985; Bizimenyera et al., 2006; Jiang et al. 2008; Ramalingam et al., 2010). Tannins have been linked to a case of poisoning in avian species (Kinde, 1988). Methods such as cell line cytotoxicity assays are widely used for determining toxic effects of plant extract *in vitro* (McGaw et al., 2007).

Markhamia obtusifolia is a perennial shrub belonging to the family Bignoniaceae, and grows naturally in southern and eastern Africa (Germishuizen and Meyer, 2003). Ethnomedically, roots of *M. obtusifolia* are used for the treatment of hookworm in parts of Tanzania (Chhabra and Mahunnah, 1994). However, no experimental evidence of the anthelmintic activity of the species has been found in the available literature. This study was carried out to validate the anthelmintic activity of *Markhamia obtusifolia* on *Trichostrongylus colubriformis* (Nematoda: Strongylida) by evaluating efficacy of extracts of the plant to inhibit egg hatching. The *in vitro* cytotoxic effects of extracts were also determined to investigate the possible relationship between cytotoxicity and anthelmintic efficacy.

2. Materials and Methods

2.1. Plant Material

Fresh leaves of *M. obtusifolia* were harvested from the Lowveld National Botanical Gardens (Nelspruit), South Africa in April 2005. The origin of the tree is recorded in the database of the garden's herbarium with number 15/94. Leaves were dried under shade at room temperature (21-27°C) for a period of one month. The dried leaves were ground using a Jankel and Kunkel Model A 10 mill into fine powder.

2.2. Plant Extraction

2.2.1. Preparation of Plant Material and Extraction

The powdered leaf material (2 g) was separately extracted with acetone and water (20 mL) in a centrifuge tube by shaking for 15 minutes and centrifuging at 300x g for 5 minutes. The insoluble materials were separated from the supernatant by filtering

through Whatman No. 1 filter paper. Extraction was repeated two times per extractant by replacing solvent after each extraction. The combined filtrate obtained following acetone extraction was transferred into a pre-weighed glass beaker and the solvent evaporated by a stream of air at room temperature. Dry aqueous extract was obtained by subjecting the combined aqueous suspension to freeze-drying.

2.3. Egg Preparation

Egg recovery and preparation was based on the procedure described by Bizimenyera et al. (2006). Faeces were collected from lambs with monospecific infections of *T. colubriformis*. The *T. colubriformis* strain was highly susceptible to anthelmintic agents and was provided by Dr Jan van Wyk from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. The lambs were housed on a concrete floor indoors, fed hay and commercial concentrate pellets and had free access to water. This study was approved by the Animal Ethics Committee, University of Pretoria.

2.4. Egg hatch assay

The *in vitro* egg hatch assay was based on the method described by Coles et al. (1992) with slight modifications. It is used to test ovicidal effects of plant extracts (Ademola *et al*., 2004; Alawa *et al*., 2003; Assis *et al*., 2003; Molan *et al*., 2003). Briefly, approximately 100 eggs in 200 μL of egg suspension were pipetted into each well of a 48-well microtitre plate. In one set of test wells, 200 μL of the acetone plant extract in a mixture of acetone and water (1:3) in concentrations of 50, 10, 2, 0.5, 0.4, 0.08 mg/mL were added. One hundred percent (100%) acetone was found to be toxic to *T. colubriformis*. A similar volume (200 μL) of aqueous plant extract in distilled water at

concentrations of 100, 10, 1, 0.2, 0.04 and 0.008 mg/mL was added to a second set of wells. There were three replicates per concentration. Thiabendazole (Sigma®) in sterile distilled water solution with 25% acetone at concentrations of 25.8, 5.16, 1.032, 0.2064, 0.04128 and 0.008256 µg/mL in triplicate was used as a positive control while the diluents distilled water or the acetone-water mixture (1:3) were negative controls. The plates were incubated under humidified conditions at ambient temperature (23°C) for 48h. A drop of Lugol's iodine solution was added to each well to stop further hatching, and all the unhatched eggs and L_1 larvae in each well were counted. Percent inhibition of egg hatching was calculated using the following equation (Rabel et al., 1994):
% inhibition = ([number of eggs exposed – the number of eggs hatched] / number of eggs exposed) X 100

2.5. Cytotoxic assay

The plant extracts were tested for cytotoxicity against the Vero monkey kidney cell line (obtained from the Department of Veterinary Tropical Diseases, University of Pretoria) as described by McGaw *et al*. (2007). The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). To prepare the cells for the assay, cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. After overnight incubation at 37°C in a 5% CO_2 incubator, the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. A stock solution of the aqueous plant extract was prepared by reconstitution to a concentration of 100 mg/mL and appropriate dilutions of the extract were prepared in growth medium and applied to the cells. The viable cell growth after 120 h incubation with plant extract was determined

using the tetrazolium-based colorimetric assay (MTT assay) (Mosmann, 1983). The absorbance was measured on a Versamax microplate reader at 570 nm. Berberine chloride (Sigma) was used as a positive control.

2.6. Statistical Analysis

The effective concentration of acetone and aqueous *M. obtusifolia* extracts and thiabendazole required to inhibit 50 % (EC_{50}) and 90% (EC_{90}) egg hatchability was determined using probit analysis at 95% confidence level. The statistical analysis was performed using Minitab 15 Statistical Software (2007). Regression analysis was used to determine the lethal concentrations of aqueous *M. obtusifolia* extract and berberine chloride that killed 50% of Vero monkey kidney cells (LC_{50}). The selectivity index for aqueous extracts of *M. obutsifolia* was calculated as follows: Selectivity index = LC_{50}/EC_{50}. The selectivity index formula is a modification of that reported by Shai et al. (2008).

3. Results

A negative concentration-response relationship was observed (y = -1.05x + 1.05). Egg hatchability in *T. colubriformis* reduced as concentration of acetone and aqueous extracts of *M. obtusifolia* increased (Table 1). The EC_{50} for the water extract of *M. obtusifolia* (0.46 mg/mL; confidence interval [CI] 0.3-0.5 mg/mL) was significantly lower than the EC_{50} for the acetone extract of *M. obtusifolia* (0.8 mg/mL; 0.7-1 mg/mL). The EC_{90} (0.2 mg/mL; CI 0.1-0.02) for thiabendazole was significantly lower than the EC_{90} for the water extract of *M. obtusifolia* (10.7 mg/mL; CI 8.3-13.7 mg/mL) (Figure 1). However, relative potency of the acetone extract was half as potent or 0.51 times as high as aqueous extracts (Table 2). In the cytotoxicity bioassay, the LC_{50} for

the aqueous extract of *M. obtusifolia* was 0.476 mg/mL, which was relatively high (low toxicity) in comparison to the highly toxic berberine (LC_{50} = 9.80 µg/mL). The selectivity index of water extracts of *M. obtusifolia* was 1.04.

Table 1: Percentage inhibition of egg hatching of *Trichostrongylus colubriformis* eggs

Markhamia extract	Concentration (mg/mL)	Mean % inhibition (Std)	Count
Acetone	0.0488	5.6(1.3)	3
	0.1953	37.1(3.6)	3
	0.7813	58.0(10.1)	3
	3.1250	74.2(2.5)	3
	12.500	81.1(4.8)	3
	50.000	87.3(3.4)	3
Aqueous	0.098	4.7(1.3)	3
	0.4	60.3(3.1)	3
	1.6	84.6(2.4)	3
	6.3	89.1(3.9)	3
	25.000	95.3(0.5)	3
	100.00	96.3(1.8)	3
Negative control	0	0.01(0.01)	6

Std denotes standard deviation. Count denotes number of replicates.

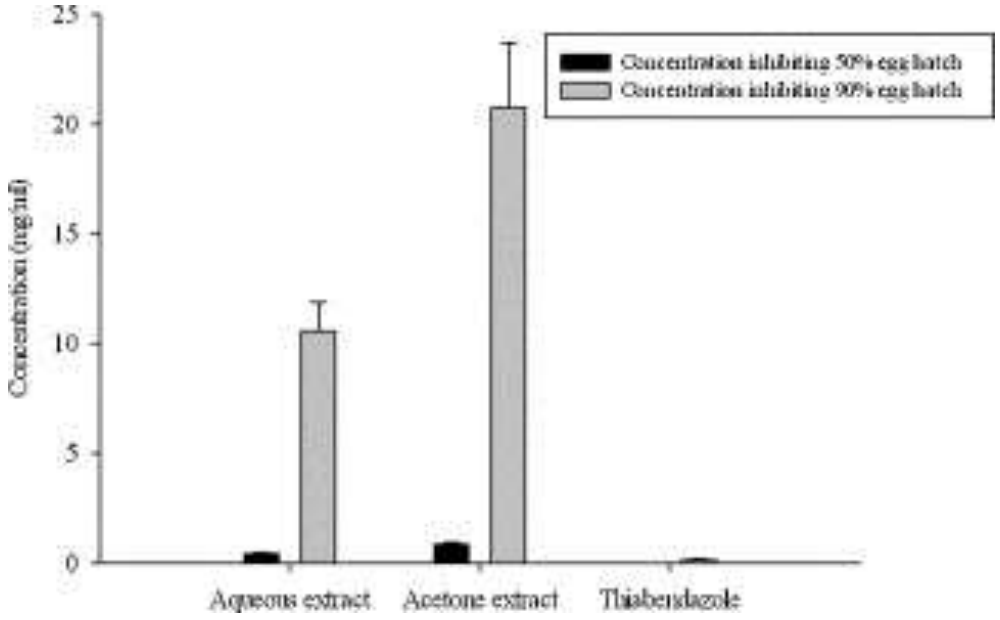

Fig. 1: Concentrations (mg/mL) of aqueous and acetone extracts of *Markhamia obtusifolia* required to inhibit 50 and 90% eggs of *T. colubriformis* from hatching and the standard error of this concentration

Table 2: Relative potency of egg hatch inhibition between thiabendazole and two *Markhamia obstusifolia* extracts (aqueous and acetone) as well as between the two plant extracts

Comparison	Relative potency	95% fiducial limits	
		Lower	Upper
TBZ vs MK ACE	136.5	91.1	202.9
TBZ vs MK AQ	69.6	45.7	104.5
MK ACE vs MK AQ	0.51	0.4	0.7

TBZ, Thiabendazole; MK, Markhamia; vs, versus; ACE, Acetone; AQ, Aqueous.

4. Discussion

The results obtained in this study demonstrate that the aqueous extract of *M. obtusifolia* has potential in the management of helminth infections. The plant has an effect on the hatchability of nematode eggs. Further assays, e.g. larval development assay, larval mobility inhibition assay, will have to be done to better define its potential.

Although the efficacy of the aqueous extracts was significantly lower than that of the conventional anthelmintic thiabendazole, further optimization and characterization of extracts may enhance the activity of constituent substances (Tariq et al., 2008). In this study, the aqueous extracts were twice as potent as the acetone extracts. These results are in contrast to findings of other studies, which suggest water extracts of some plant species are not effective anthelmintics (Worku et al., 2009). However, Amin et al. (2009) reported high *in vitro* efficacy of water extracts of 20 plant species against adult intestinal nematodes of cattle. The acetone fraction of *M. obtusifolia* had the highest antifungal activity against *Candida albicans* and the three antifungal compounds isolated (ursolic acid, pomolic acid and 2-epi-tormentic acid) were from the intermediate polarity fraction (Nchu et al., 2010). This indicates that these antifungal compounds are not responsible for the anthelmintic activity observed in this study.

Anthelmintic activities in plant extracts have been attributed to high levels of tannins in some extracts (Al-Shaibani et al., 2009; Hoste et al., 2009). However, tannins often have high cytotoxic activities (Takechi et al., 1985; Jiang et al., 2008), which may be non-specific. Although generally speaking condensed tannins are less toxic than hydrolysable tannins, instances of toxicity to livestock due to condensed tannins have been reported (Singleton, 1981; Oelrichs et al., 1994). In the current study, water extracts were evaluated for cytotoxic activities in order to rule out cytotoxicity as the mechanism of action of *M. obtusifolia* extracts. Chemotherapeutic drugs against helminth infection act mainly through three different mechanisms, namely disruption of the neuromuscular physiology, blocking the energy metabolism, or disrupting the highly efficient reproductive system of the parasites (Geary et al., 1992). The results obtained in the current study indicate relatively lower levels of cytotoxicity compared to berberine. Berberine is an alkaloid with well-studied pharmacological and cytotoxic effects, derived from plants of the families Berberidaceae and Ranunculaceae (Bahar et al., 2011). The purified compound is inexpensive and readily available commercially, and published LC_{50} values for its activity against Vero cells are available (McGaw et al., 2007; Chin et al., 2010). The Vero cell line was selected for cytotoxicity evaluation as it is widely available, easy to culture and has been adopted by many laboratories as a model for screening plant extracts and other compounds for cytotoxicity (Mahapatra et al., 2007; McGaw et al., 2007; Liao et al., 2010; de Toledo et al., 2011). Using this cell line provided an indication of cellular toxicity of the *M. obtusifolia* plant extract which is easily comparable with published cytotoxicity data of other plant extracts.

The hexane and dichloromethane extracts of *Curtisia dentata* (Burm.f) were more toxic than the aqueous extracts of *M. obtusifolia* in this study (Shai et al. 2008). Other

evidence suggests that ethanol extracts are more cytotoxic than aqueous extracts of plants. Aqueous extracts of *Athrixia elata* and *A. phylicoides* prepared in the laboratory, and decoctions and infusions of the plant material prepared following the traditional approach are less toxic than ethanol extracts (McGaw et al. 2007). Although they reported lower cytotoxicity for aqueous extracts of *A. elata* and *A. phylicoides* (>1 mg/mL) compared to *M. obtusifolia* (0.476 mg/mL) obtained in this study, the calculated selective index for aqueous extracts of *M. obtusifolia* is close to 1. This suggests that cytotoxicity may not explain the efficacy of the extracts and it is likely other mechanisms of action by water extracts of *M. obtusifolia* in inhibiting egg hatchability in *Trichostrongylus* spp. are involved. In a previous study, extracts of *M. obtusifolia* were toxic (LC_{50} = 8.94 µg/mL) against brine shrimp larvae, another assay used for pharmacological and cytotoxicity determination (Moshi et al., 2004). No clear explanation could be provided for the discrepancies in cytotoxicity results obtained in the two studies, but toxicity to a crustacean on the one hand and mammalian cells on the other may be difficult to correlate. It is worth mentioning that some drugs can undergo extensive metabolism in mammals following oral administration and the end-products of metabolism may be less toxic to the environment (Lanusse et al., 2009; Monosson, 2010).

This *in vitro* study supports the ethnomedical use of water extracts of *M. obtusifolia* to control worms; but further research is necessary to confirm if this is also the case *in vivo*.

Conflict of interest statement

The authors declare that there were no competing interests and none of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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