Curtisia dentata (Cornaceae) leaf extracts and isolated compounds inhibit motility of parasitic and free-living nematodes

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ABSTRACT

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Haemonchus contortus and *Trichostrongylus colubriformis* are among the most important parasitic nematodes of small ruminants. *Caenorhabditis elegans*, a free-living nematode, is used as a model for evaluating anthelmintic activity of a variety of test substances. Extracts of several medicinal plants are useful *in vitro* and *in vivo* against nematode development. Extracts of *Curtisia dentata*, a South African medicinal plant, and compounds isolated from leaves of this plant were investigated for anthelmintic activity against *T. colubriformis, H. contortus* and *C. elegans*. The acetone and dichloromethane extracts were active against all nematodes at concentrations as low as 160 µg/mℓ. Betulinic acid and lupeol were active against the parasitic nematodes only at the high concentrations of 1 000 and 200 µg/mℓ, respectively. All compounds were effective against *C. elegans* with active concentrations as low as 8 µg/mℓ. Betulinic acid was less active than lupeol and ursolic acid against *C. elegans.* The acetone and dichloromethane extracts were also active against *C. elegans* with a concentration of 0.31 mg/mℓ resulting in almost 80 % inhibition of larval motility. The use of free-living nematodes may provide information on the activity of potential anthelmintics against parasitic nematodes. Extracts of various medicinal plant species may provide solutions to ill-health of small ruminants caused by parasitic nematodes in poor communities of southern Africa.

Keywords: Anthelmintic, betulinic acid, *Curtisia dentata*, lupeol, ursolic acid

INTRODUCTION

Helminthosis, caused by parasitic gastrointestinal nematodes, remains a major constraint to livestock production across the tropical and subtropical regions, with *Haemonchus contortus* and *Trichostrongylus colubriformis* listed among the top ten most important parasites of ruminants (Perry & Randolph 1999; Chiejina 2001; Hounzangbe-Adote, Paolini, Fouraste, Moutairou & Hoste 2005). *Trichostrongylus colubriformis* causes parasitic enteritis that predisposes ruminants to diarrhoea, weakness and death. This parasite is frequently found infecting cattle and sheep in South Africa, causing loss in production (Horak 2003; Horak, Evans & Purnell 2004). *Haemonchus contortus* causes anaemia, haemorrhagic gastroenteritis, hypoproteinaemia partly manifested as submandibular oedema or 'bottle jaw' and sudden death, or chronic emaciation

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in affected livestock (Urquhart, Armour, Duncan, Dunn & Jennings 1996). In southern Benin, the prevalence of *H. contortus* was estimated at 92.5 % in goats and sheep (Salifou 1996).

The usual mode of control of these parasitic diseases relies on the repeated and strategic use of proprietary anthelmintic drugs. However, these drugs are often so highly priced that they are not accessible to subsistence and small-scale livestock farmers in developing countries (Hounzangbe-Adote *et al.* 2005). Extensive use of drugs by commercial farmers has created anthelmintic resistance that has become cosmopolitan. The emergence of resistance of gastrointestinal nematodes to conventional anthelmintics and resulting economic and production losses emphasize the need to search for alternative methods of worm control (Prichard 1990; Wolstenholme, Fairweather, Prichard, Von Samson-Himmelstjerna & Sangster 2004; Jabbar, Iqbal, Kerboeuf, Muhammad, Khan & Afaq 2006).

One of the alternative methods of worm control is the use of medicinal plants. In many parts of the developing world, small-scale and subsistence farmers rely on traditional methods of deworming that include the administration of remedies derived from plants (Hammond, Fielding & Bishop 1997; Danø & Bøgh 1999). The scientific evidence of the efficacy and safety to support widespread use of plant-based remedies as anthelmintics is still scanty (Hammond *et al.* 1997). Extracts of several African plant species are active against parasitic and free-living nematodes (Hammond *et al.* 1997; Enwerem, Okogun, Wambebe, Okorie & Akah 2001; Bizimenyera, Githiori, Eloff & Swan 2006). Extracts of *Artemisia* sp. have shown activity against *Haemonchus* sp. (Idris, Adam & Tartour 1982; Iqbal, Lateef, Ashraf & Jabbar 2004) and *Trichostrongylus* species (Sharma 1993). Furthermore, Hördegen, Hertzberg, Heilmann, Langhans & Maurer (2003) demonstrated anthelmintic activity of ethanolic extracts of several plant species in an *in vivo* sheep model.

Some compounds and plant extracts that possess anthelmintic activity also have antifungal efficacy. In 1993, Padmaja, Thankamany & Hisham reported that extracts of the root bark of *Uvaria hookeri* and *Uvaria narum*, together with the isolated acetogenins, have antifungal, antibacterial and anthelmintic activities. Benzimidazole carbamates, a class of anthelmintics used in human and veterinary medicine have antifungal activity (Murray, Hudson & Yassa 1992; Katiyar, Gordon, McLaughlin & Edlind 1994). Polyphenols isolated from the leaves of *Piper betle* had both antifungal and anthelmintic activity (Evans, Bowers & Funk 1984). The extracts of *C. dentata* leaves showed antifungal activity against *Candida albicans* and several other fungal species (Shai 2007; Shai, McGaw, Masoko & Eloff 2008). We investigated anthelmintic activity in *C. dentata* extracts and compounds isolated from the leaves of the plant to discover potential correlations with known antifungal activity.

Curtisia dentata is traditionally used in humans for stomach ailments and diarrhoea, as a blood strengthener and an aphrodisiac (Hutchings, Scott, Lewis & Cunningham 1996; Pujol 2000). It is also used in the treatment of heartwater in cattle in the Eastern Cape Province (Dold & Cocks 2001) and as a pimple treatment (Grierson & Afolayan 1999). The anthelmintic activity of *C. dentata* extracts has not been reported in available literature. Betulinic acid is one of the compounds isolated from *C. dentata* leaves (Shai 2007). Betulinic acid is active against *Caenorhabditis elegans,* a free-living nematode, at a concentration of 500 µg/mℓ after 7 days of incubation (Enwerem *et al.* 2001). The dichloromethane and acetone extracts of *C. dentata* leaves, containing high concentrations of betulinic acid, were selected for investigation of their anthelmintic activity against the parasitic nematodes *T. colubriformis* and *H. contortus*, and the free-living *C. elegans*. Betulinic acid and two other compounds isolated from the leaves of *C. dentata*, ursolic acid and lupeol, were also tested for anthelmintic activity. This study was aimed at investigating whether the extracts of the leaves of *C. dentata* and compounds from it, which have shown antifungal activity (Shai 2007), also have anthelmintic activity.

MATERIALS AND METHODS

Plant extracts and compounds

Plant leaves were collected from the Lowveld National Botanical Garden in Nelspruit, South Africa in woven sacks and dried at room temperature prior to powdering in a laboratory mill. A herbarium specimen, Shai 002, was deposited in the medicinal plant herbarium at the University of Pretoria (Department of Paraclinical Sciences). Powdered leaves (837 g) of *C. dentata* were serially extracted with hexane, dichloromethane, acetone and methanol, and compounds were isolated from the dichloromethane extract as previously described (Shai 2007). Aliquots of the dichloromethane and acetone extracts were dissolved in DMSO to a concentration of 100 mg/mℓ. Stock solutions of 2 mg/mℓ of the isolated compounds betulinic acid, lupeol and ursolic acid were

also prepared in DMSO. Preliminary tests confirmed that the final concentration of DMSO used in the assays was not toxic to the nematodes.

Anthelmintic activity

Caenorhabditis elegans

Caenorhabditis elegans was maintained in sterile Petri-dishes on Nematode Growth (NG) agar (composition per litre: 3 g NaCl, 2.5 g peptone, 17 g agar, 5 mg cholesterol, 0.246 g $MgSO_4.7H_2O$, 0.147 g $CaCl_2.2H_2O$, 2.7 g KH_2PO_4, and 0.9 g K_2HPO_4) seeded with *Escherichia coli* (Brenner 1974) . The nematodes were incubated at 20 °C in the dark for 6 days prior to the anthelmintic assay which was performed using the method of Rasoanaivo & Ratsimamanga-Urverg (1993) modified by McGaw, Jäger & Van Staden (2000). Several plates were washed with 1 mℓ M9 buffer (composition per litre: 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl and 0.25 g $MgSO_4.7H_2O$) to obtain a solution of approximately 100 nematodes per 10 µℓ. Into 24-well plates, 2 mℓ of plant extracts or compounds dissolved in a small amount of DMSO and made up to the final concentration in M9 buffer were added. The final concentration of DMSO did not exceed 1 %, which was not toxic to the nematodes. Nematodes (10 µℓ) were added into the wells and plates were incubated for 2 h, and then 7 days in the dark. Non-motile or paralysed larvae were considered dead. The number of dead and viable larvae were counted after each incubation period and the percentage of dead larvae was calculated. Levamisole 5 and 10 µg/mℓ (Sigma) was used as a positive control while untreated larvae were used as negative controls.

Recovery and preparation of eggs of parasitic nematodes

Faecal pellets were collected from lambs that were infected with either *T. colubriformis* or *H. contortus*, using sterilized harnesses and collecting bags. The eggs were prepared according to a modified version (Bizimenyera *et al.* 2006) of the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for detection of resistance in nematodes of veterinary importance (Coles, Bauer, Borgesteede, Geerts, Klei, Taylor & Waller 1992).

Anthelmintic activity against third stage larvae

The eggs (1 mℓ) were added into Petri dishes in the presence of 50 µℓ of a suspension of *E. coli* (ATCC 9637) for the development of nematode larvae (Hubert & Kerboeuf 1992) and 10 µℓ (50 µg/mℓ stock solution) of amphotericin B (Sigma®) to control fungal growth. The dishes were incubated for 5 days to allow the larvae to develop to the third stage (L3). The L3 larvae were frozen at –20 °C until needed. For the anthelmintic assay, the larvae were washed off the base of the dish with M9 buffer and treated with various concentrations of compounds and extracts of *C. dentata* in wells of a 24-well plate and incubated for 48 h. Motile and non-motile larvae were counted to obtain the percentage inhibition of motility.

RESULTS AND DISCUSSION

Inhibition of motility of adult parasitic nematodes

The acetone and dichloromethane extracts of *C. dentata* inhibited motility of *T. colubriformis* at a concentration range of 0.16–2.5 mg/mℓ. The lowest lethal concentration of both the acetone and dichloromethane extracts, 0.16 mg/mℓ, led to over 60 % of larvae being paralysed. The lowest tested concentration of both extracts, 0.08 mg/mℓ, resulted in less than 10 % of non-motile larvae. The inhibition of larval motility percentages by acetone extracts was very similar to that induced by the dichloromethane extracts (Fig. 1). The average survival in untreated controls was 99 %.

The dichloromethane extract was active against *H. contortus* at a concentration range of 0.63 to 2.5 mg/mℓ. About 20 % average inhibition of motility of larvae resulted from a concentration of 0.63 mg/mℓ. The acetone extract was active at a concentration range of 0.31 to 2.5 mg/mℓ. Over 70 % of the larvae were not motile after 48 h of incubation in the presence of 0.31 mg/mℓ of the acetone extract. At this concentration (0.31 mg/mℓ) the percentage of non-motile larvae resulting from the acetone extract was significantly higher than that resulting from the dichloromethane extract. Concentrations of 2.5 mg/mℓ and 1.25 mg/mℓ of both acetone and dichloromethane resulted in 100 % inhibition of larval motility after 48 h of incubation. Concentrations of both 0.16 and 0.08 mg/mℓ of both extracts only managed to inhibit motility of less than 10 % of the larvae (Fig. 2). The average survival in untreated controls was 99 %.

Two compounds isolated from *C. dentata* leaves, namely, betulinic acid and lupeol were tested to evaluate their ability to paralyse *T. colubriformis* and *H. contortus* larvae. Unfortunately, ursolic acid was not isolated in sufficient quantities to test for

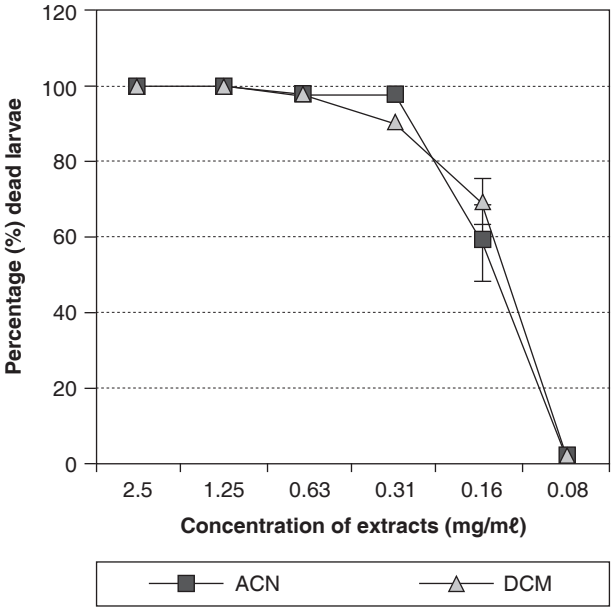

FIG. 1 Percentage of dead *T. colubriformis* larvae after 48 h of incubation in the presence of varying concentrations of the acetone (ACN) and dichloromethane (DCM) extracts of *C. dentata* leaves

The results are means (with standard deviations) of two independent experiments performed in triplicate. The estimated LC_{50} value was 0.15 mg/mℓ for both extracts

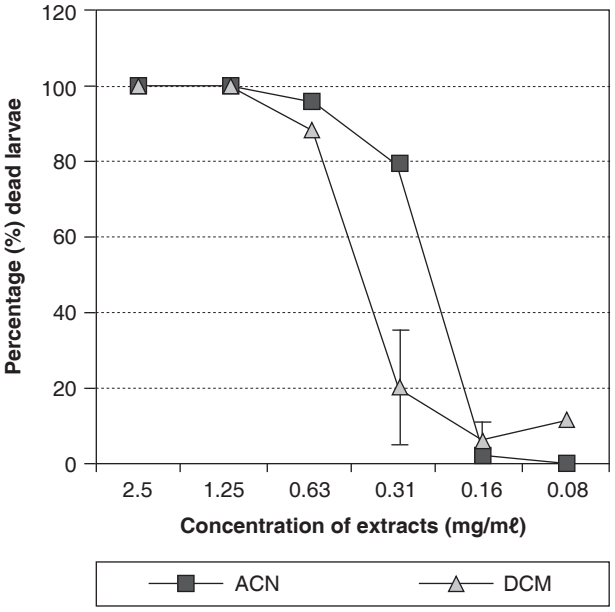

FIG. 2 Percentage of dead *H. contortus* larvae after 48 h of incubation in the presence of varying concentrations of the acetone (ACN) and dichloromethane (DCM) extracts of *C. dentata* leaves

The results are means (with standard deviations) of two independent experiments performed in triplicate. The estimated LC_{50} values were 0.2 mg/mℓ for acetone and 0.45 mg/mℓ for DCM extract

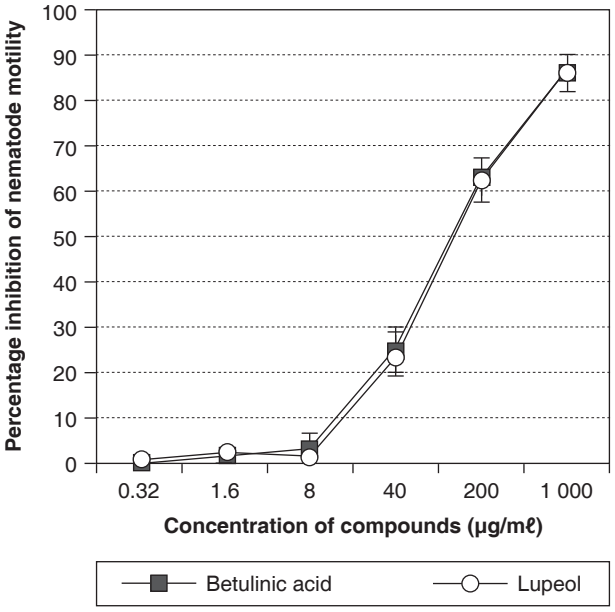

FIG. 3 Percentage of dead *T. colubriformis* larvae after 48 h of incubation in the presence of varying concentrations of the lupeol and betulinic acid

The results are means and standard deviations of two independent triplicate experiments. The estimated LC_{50} value was 80 µg/mℓ for both lupeol and betulinic acid

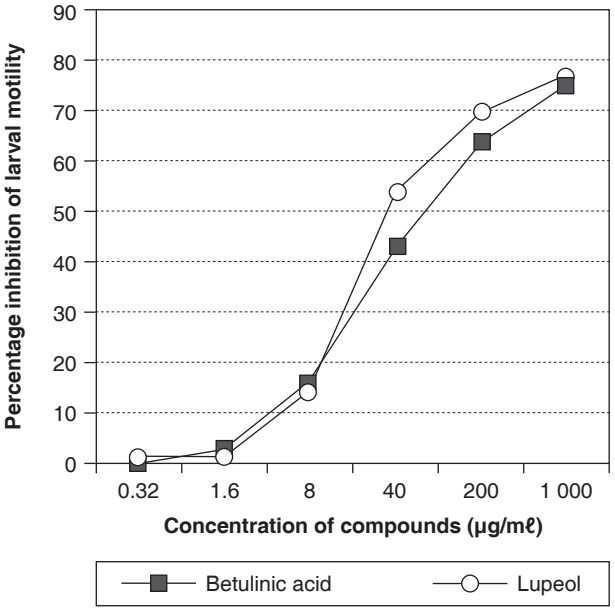

FIG. 4 Percentage of dead *H. contortus* larvae after 48 h of incubation in the presence of varying concentrations of the lupeol and betulinic acid

The results are means and standard deviations of two independent triplicate experiments. The estimated LC_{50} values were 20 µg/mℓ for lupeol and 50 µg/mℓ for betulinic acid

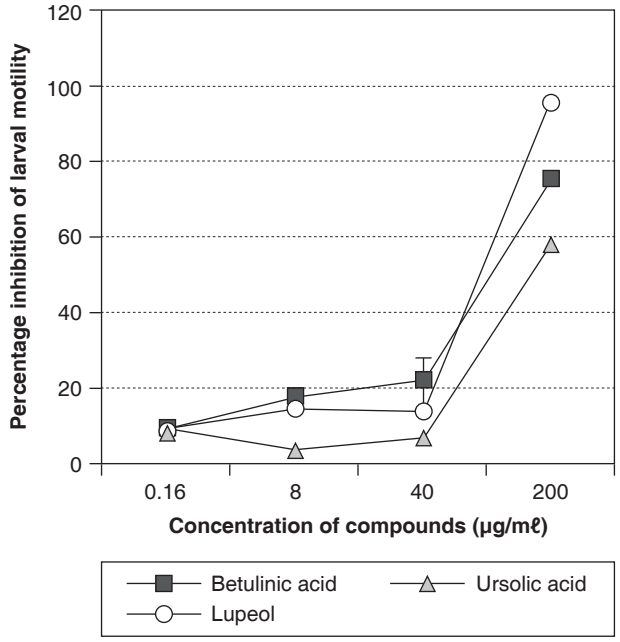

FIG. 5 Inhibition of motility of free-living nematode (percentage of immotile worms), *C. elegans* with compounds isolated from *Curtisia dentata* leaves

After treatment with compounds the nematodes were incubated for 2 h before counting motionless larvae. The LC_{50} values were estimated at 120 µg/mℓ for lupeol, 140 µg/mℓ for betulinic acid and 180 µg/mℓ for ursolic acid

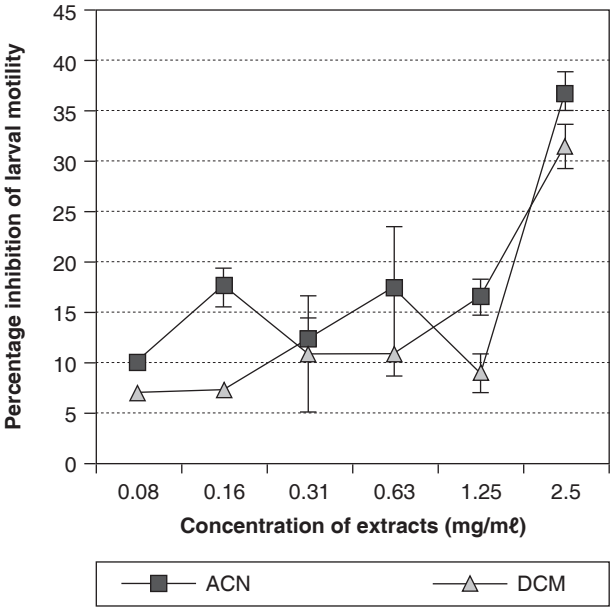

FIG. 6 Inhibition of motility of free-living nematode, *C. elegans* with acetone and dichloromethane of *Curtisia dentata* leaves

After treatment with plant extracts the nematodes were incubated for 2 h before counting motionless larvae

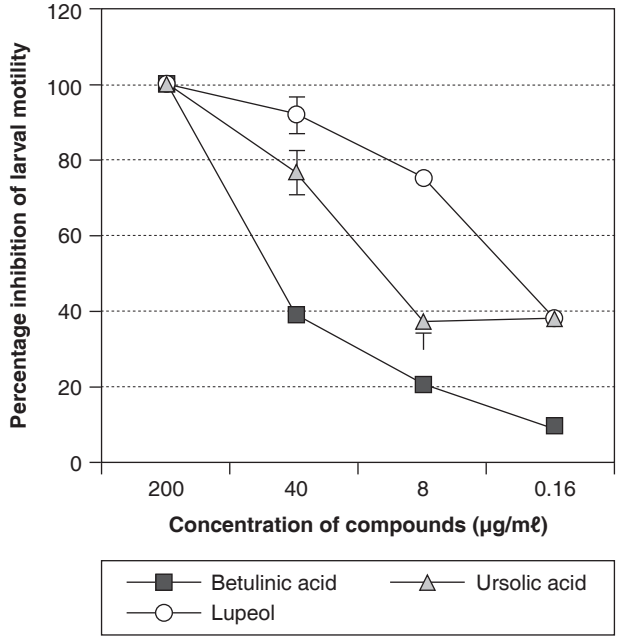

FIG. 7 Inhibition of motility of free-living nematode, *C. elegans* with isolated compounds from leaves of *Curtisia dentata*

After treatment with the pure compounds, the nematodes were incubated for 7 d before counting motionless and motile larvae. The LC_{50} values 2 µg/mℓ for lupeol, 70 µg/mℓ for betulinic acid and 12 µg/mℓ for ursolic acid

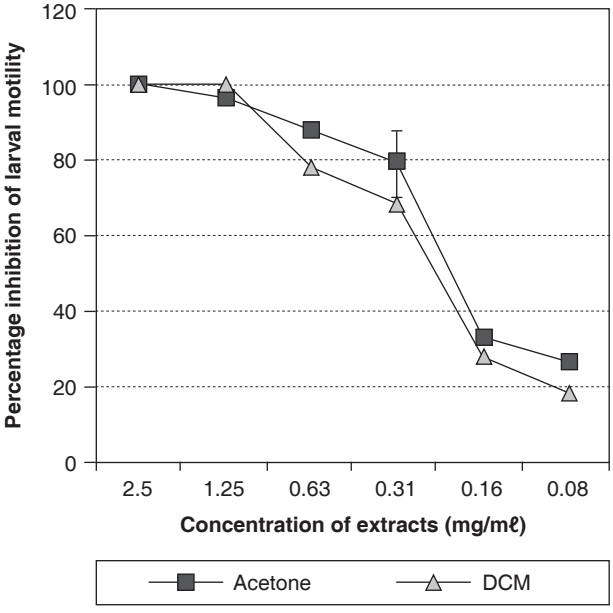

FIG. 8 Inhibition of motility of free-living nematode, *C. elegans* with the acetone and dichloromethane extracts of the leaves of *Curtisia dentata*.

After treatment with the extracts, the nematodes were incubated for 7 d before counting of motionless and motile larvae. The calculated LC_{50} value was 0.2 mg/mℓ for both fractions

anti-parasitic activity. Lupeol and betulinic acid were tested at a concentration range of 0.32–1 000 µg/mℓ. Both compounds inhibited larval motility only at high concentrations (Fig. 3 and 4). Low concentrations did not result in appreciable numbers of motionless larvae. The average survival in untreated control of both species was 99 %. Lupeol and betulinic acid are poorly soluble in aqueous media. Upon mixing with water during dilution, precipitation resulted in a milky suspension, possibly making these compounds unavailable to act on the test nematode larvae. Modifications of their structures may result in more soluble derivatives of these lupane triterpenes.

The extracts showed more activity against the larvae than the isolated compounds. It would seem that, apart from lupeol and betulinic acid, other components in the extract contribute to the activity against larval motility. This emanates from the fact that betulinic acid and lupeol were, by themselves, not very active against the tested parasitic nematodes. The compounds failed to exert 100 % inhibition of larval motility at all concentrations tested.

The compounds and extracts did not affect egg hatching of *H. contortus* and *T. colubriformis* (results not shown). In the controls the number of unhatched eggs was comparable to eggs treated with plant extracts and compounds. It was observed that at high concentrations (16.7, 8.3, 4.17 and 2.1 mg/mℓ) of plant extracts no eggs or larvae were present, suggesting that lysis occurred. In concentrations where the numbers of motile larvae were low the eggs still hatched prior to paralysis.

Inhibition of motility of free-living nematodes

In vitro anthelmintic activity assay methods using *C. elegans* as the test organism provide a cheap, simple and rapid system in which broad-spectrum anthelmintic activity of plant extracts and pure compounds can be evaluated, keeping in mind the limitations of using a free-living nematode as a model for anti-parasitic activity (Eloff & McGaw 2006). Furthermore, it has been reported that most registered deworming drugs have demonstrated activity against *C. elegans,* supporting the use of *C. elegans* as a model for testing compounds and plant extracts against parasitic nematodes (Simpkin & Coles 1981).

Three compounds isolated from the dichloromethane extract of *C. dentata,* namely, lupeol, betulinic acid and ursolic acid, together with the acetone and dichloromethane extracts were assessed for ability to inhibit the motility of the free-living nematode, *C. elegans.* After 2 h of incubation, the highest concentrations of isolated compounds (200 µg/mℓ) inhibited up to 90 % of larval motility. Concentrations ranging from 0.16 to 40 µg/mℓ resulted in about 20 % inhibition of larval motility after 2 h of incubation at 25 °C (Fig. 5). The highest concentrations of extracts (2.5 mg/mℓ) resulted in about 35 % inhibition of larval motility whereas lower concentrations ranging from 1.25 to 0.08 mg/mℓ resulted in less than 20 % inhibition of larval motility (Fig. 6). In the untreated control 4 % motionless larvae were observed after 2 h of incubation. In the levamisole-treated (10 µg/mℓ) controls average inhibition of larval motility was 25 %.

After 7 days of incubation the highest concentrations of all tested compounds resulted in 100 % inhibition of larval motility. Lupeol was the most active of the isolated compounds. A concentration of 40 µg/mℓ of lupeol produced over 80 % inhibition of larval motility while betulinic acid led to 40 % inhibition. Ursolic acid, though less active than lupeol, produced higher inhibition of motility than betulinic acid at most concentrations tested (Fig. 7). All the compounds led to 100 % paralysis of larvae at 200 µg/mℓ. Enwerem *et al.* (2001) reported that 500 µg/mℓ of betulinic acid caused 100 % paralysis of *C. elegans* larvae after 7 days in treatment. In the untreated controls approximately 85 % motility was observed. In 5 and 10 µg/mℓ levamisole-treated controls 65 % and 48 % motility were observed respectively.

Both the acetone and dichloromethane extracts of *C. dentata* leaves were similar in their inhibition of larval motility after 7 days of incubation, displaying a concentration-dependent effect. Concentrations ranging from 2.5 to 0.63 mg/mℓ of each extract led to over 80 % inhibition of larval motility after 7 days of incubation. At 0.31 mg/mℓ, both extracts led to over 60 % inhibition of larval motility while lower concentrations were less effective (Fig. 8).

Several extracts of different medicinal plants show anthelmintic activity (Raj, 1975; Enwerem *et al.* 2001; Ademola, Fagbemi & Idowu 2004). Hounzangbe-Adote *et al.* (2005) tested the *in vitro* effects of four tropical plants for activity against parasitic nematodes. They reported that concentrations of between 300 and 2 400 µg/mℓ of ethanolic extracts of *Zanthoxylum zanthoxyloides, Carica papaya, Morinda lucida* and *Newbouldia laevis* were active against *H. contortus*. Bizimenyera *et al.* (2006) reported that extracts of *Peltophorum africanum* had *in vitro* activity against egg hatching and larval development of *T. colubriformis.* The ED_{50} values reported were 0.619 mg/mℓ for leaf extract, 0.383 mg/mℓ for bark extract and 0.280 mg/mℓ for extract of the roots (Bizimenyera *et al.* 2006). In this study *in*

vitro activity of extracts of *C. dentata* leaves was demonstrated against parasitic and free-living nematodes in a concentration-dependent manner. These results indicate that the leaves of *C. dentata* may be useful to treat helminthosis in South African folk medicine.

Despite the indication that some plant extracts are active against several nematodes, the exact mechanism remains unclear. Some compounds such as palasonin, the active principle of *Butea frondosa* inhibit glucose uptake and accelerate glycogen depletion in target nematodes (Kumar, Mishra, Tandan & Tripathi 1995). The mechanism of action of many anthelmintic plant extracts may involve inhibition of energy metabolism (Dahanukar, Kulkarni & Rege 2000).

In this study, betulinic acid induced paralysis of the tested nematodes, *C. elegans*, *H. contortus* and *T. colubriformis* at concentrations between 1 000 and 200 µg/mℓ. Against *C. elegans*, betulinic acid induced 100 % paralysis at a concentration of 200 µg/mℓ. These results confirm the study by Enwerem *et al*. (2001) in which it was reported that betulinic acid isolated from *Berlina grandiflora* had strong activity against *C. elegans* at concentrations of 100 and 500 µg/mℓ. The mechanism involved in the activity of betulinic acid against parasitic and free-living nematodes is yet to be described. Furthermore, its activity against parasitic nematodes *in vivo* and *in vitro* has not been previously demonstrated. As far as the available literature is concerned, this appears to be the first report of the effects of betulinic acid against the parasitic nematodes, *T. colubriformis* and *H. contortus*. Investigations of the *in vivo* activity of both the extracts of *C. dentata* and the isolated compounds may conclusively indicate their potential as anthelmintics. There was no obvious correlation between anthelmintic activity and anti-*Candida* activity of both extracts and isolated compounds. The calculated R^2 value was 0.0132.

The use of free-living nematodes may provide information on the activity of potential anthelmintics against parasitic nematodes. Extracts of various medicinal plant species may provide solutions to ill-health of small ruminants caused by parasitic nematodes in poor communities of southern Africa.

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