Evaluation of anthelmintic activity of isolated constituents from Combretum molle extract in an in-vitro egg hatch inhibition assay

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Abstract

A strategy to detect anthelmintic constituents in Combretum molle using a bioassay-guided purification approach was tested. Two kilograms of \textit{C. molle} stem bark powder was extracted with 10 L of absolute methanol by soxhlet extraction. The crude methanol extract was partitioned successively in petroleum ether, chloroform and n-butanol to obtain the aqueous methanol portion; this in turn was purified by bio-activity guided fractionation in liquid column chromatography (LCC) and thin layer chromatography (TLC). The active constituents (tagged as constituent A, B and C) obtained from this purification processes were investigated to determine their relative bio-activities against gastrointestinal nematodes of sheep by means of an egg hatch inhibition assay (EHIA). The isolated constituents demonstrated anthelmintic efficacies of 91.7\%, 91.0\% and 51.8\% for A, B and C respectively. The efficacy of constituents A and B were comparable to that of levamisole (97.3\%). The ultraviolet (UV) absorption and spectral data (\(\lambda_{max}\), nm) for constituents A, B and C were 211.00 nm, 227 nm and 229.00 nm respectively indicating iridoid glycosides to be the most likely isolated constituents from the extract.

Keywords: Anthelmintic, \textit{Combretum molle}, gastrointestinal nematodes, iridoid glycoside, levamisole.

Introduction

The obvious limitations of synthetic conventional anthelmintic, such as development of resistance by parasite, high cost and several side effects; have geared the search for other sources of effective drugs especially from plant sources. These naturally produced plant drugs, therefore offer alternative that can overcome the problems of modern synthetic drugs (Susan and Steve, 2003). \textit{Combretum molle} ‘R.Br Ex G.Don’ (also called Soft-leaved Combretum, Velvet bush willow) is a tree with large, straighter pole than most species of Combretum, distinguished by its rough bark and dense crown. It occurs throughout Tropical Africa and in the Arabian Peninsula in areas where woodlands and wooded grasslands predominate, often forming pure stands on hillsides (Keay, 1989). Traditional health practitioners in many parts of Africa usually employ the leaves and barks of Combretum species as remedies for a variety of both human and animal ailments, including abdominal discomfort, body pains, respiratory disorders, colds and fevers, ear and eye ailments, schistosomiasis, hookworms, dysmenorrhea and infertility in women, leprosy, syphilis, microbial infections and general body weakness (Hutchings et al., 1996). It is also reported to be used for the treatment of HIV/AIDS related infections (Bessong et al., 2005), malaria, (Abebe and Ayehu, 1993) and possess antibacterial (Eloff, 1998; Eloff, 1999; Khan et al., 2000), antmycobacterial (Asres et al., 2001a) and antifungal effects (Pegel and Rogers, 1985). The leaf and stem bark extract of \textit{C. molle} were also reported to have anthelmintic effect on nematodes (Simon et al., 2008; Ademola and Eloff, 2010). This study therefore aims at carrying out chemical investigations of \textit{C. molle} for its active constituents as well as determining the anthelmintic activity of isolated pure chemical constituents.

Materials and methods

\textit{Plant collection and preparation for extraction}

Ten kilogram of \textit{Combretum molle} was collected in the field around New-Bussa in Niger State in the months of March and April (2009) to ensure high concentration of the bioactive constituents (Ademola and Eloff, 2010). The plant was identified at the herbarium in the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria; and a voucher number 2797 was deposited in the herbarium.

The plant sample was air-dried, pulverized into powder using mortar and pestle and sieved. Five kilogram of the sieved powder was extracted with 10 L of absolute methanol (Sigma Aldrich 32213) in Soxhlet apparatus (Quick fit corning Ltd; Stafford, England) according to the manual of Youn et al. (2003) and Onyeyigli et al. (2001). The crude methanol extract (CME) was concentrated to dryness in a vacuum using a rotary evaporator coupled to a thermo-regulator and a vacuum pump (Hordegen et al., 2003).
Following the procedure of Brain and Turner, (1975); 316 g of the dried CME was suspended in 500 mL of water and then partitioned serially in 750 mL each of petroleum ether (Sigma Aldrich 32213), chloroform (Sigma Aldrich 32213) and N-butanol (Sigma Aldrich 32213), to obtain the AMP which was used in the study based on the recommendations of Simon et al. (2008).

Separation of bioactive constituents through LCC

Using the wet packing technique of Agrawal and Paridhavi (2007), 50 g of silica gel G 60-200 microns (Interchin, France) was suspended in 250 mL of chloroform (the mobile phase) in a beaker and poured into the glass column (30 cm long with an internal diameter of 2 cm). The silica/chloroform suspension was run continuously through the column until the column settled uniformly, care being taken to avoid air bubbles and cracks. One gram of AMP was mixed with 1 g of sand (Courtin and Warner Ltd. Lewes, Sussex, England) and packed in cotton wool and then pushed into the column until it was in contact with the silica gel. Using the gradient elution technique of Agrawal and Paridhavi (2007), 30 mL each of the various eluting solvents were run through the packed column one after the other and 25 mL of the fractions were collected in sample bottles at the end of running each eluting solvent (Table 1).

Monitoring of column fraction TLC

The fractions obtained from the column (fractions 1-9) were monitored on TLC in N-butanol, glacial acetic acid and water (8:1:1) and similar fractions were pooled together based on the retention factor (Rf) values of the TLC plate.

Isolation of constituents on TLC

Aluminium sheets and glass-backed TLC plates (20 x 20 cm; Merck, silica gel 60 F254) were used for the isolation of compounds. The plates were divided into sizes of 10 cm x 1.5 cm. A light pencil line was drawn 1 cm from the bottom and top edge of the chromatographic plate. 600 mg of the pooled column fraction (i.e. fractions 5 to 9; Table 1) was dissolved in 60 mL of absolute methanol and placed as preparatory on the line drawn at the bottom of 40 TLC plates using a 10 µL capillary tube which delivers approximately 10 µg onto the plate and subsequently placed in the eluting solvent (30 mL of n-butanol, glacial acetic acid, water; ratio of 8:1:1) in a TLC tank which was filled to a depth of 0.5 cm.

The solvent was allowed to migrate upward on the prepared TLC plate (so as to separate the individual compounds in the extract based on their polarities) until the pencil line drawn across the top edge (which serve as the solvent front) was reached. The plates were then removed from the chamber and air-dried. After drying, a portion of the plate (1 x 0.5 cm) was cut off using a glass cutter and sprayed with a detecting reagent (5% anisaldehyde, methanol, 10% concentrated sulphuric acid; ratio of 90:10: 10) in order to visualize the constituents on the eluted plate after heating for 3 min at 110°C in an oven (Fischer Scientific, China). The plates were also visualize under camac universal TL-600 ultraviolet (UV) light at 360 nm and 254 nm and the fluorescin (360 nm) or quenching (254 nm). Compounds were marked and the spot (layers) were outlined with a light pencil.

Concentration of compounds isolated on TLC

The detected compounds on the plate when visualized under the UV spectra (camac universal TL-600 spectrophotometer) were separately with a pointed needle and then individually scrapped into different 250 mL beakers based on bands (colour) of separation. The silica/constituent mixture scrapped; were dissolved in 100 mL of 70% methanol, shaken and poured into 10 mL centrifuge tubes and centrifuged at 5000 rpm (SG 1.2 ) (HermLe. GmbH & Co. FR. Germany) for 5 min. The supernatant which contains the active constituent was decanted into a 150 mL beaker and allowed to air-dry at room temperature living behind the compound. The compound was therefore obtained as a white crystal.

Identification of constituents using mass spectroscopy

Mass spectroscopy analysis was carried out on an ultraviolet (UV) Visible Double Beem Spectrophotometer (Hellious Zeta. Thermo Scientific U.K) fitted with a fused silica HP-5MS capillary column (30 m x 0.25 mm; film thickness 0.25 µM). The temperature in the oven was set at 3°C/min for maximum of 60°C. Helium gas was used as a carrier, and delivered at a flow rate of 2 mL/min. The spectrophotometer was coupled to a Hewlett-Packard 6890 mass selective detector with MS operating parameters specifications of 70 eV ionization voltage; 200°C ion source temperature; labda max and wave length. 0.1 mg of the isolated constituent was dissolved in 5 mL of absolute methanol.

### Table 1. Eluting solvents used in the liquid column chromatography.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Solvents</th>
<th>Ratio</th>
<th>Volume ran through column (mL)</th>
<th>Volume collected from column (mL)</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chloroform</td>
<td>Pure</td>
<td>30</td>
<td>25</td>
<td>Intermediate</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform/MEOH</td>
<td>9:1</td>
<td>30</td>
<td>25</td>
<td>Inter/polar</td>
</tr>
<tr>
<td>3</td>
<td>-do-</td>
<td>4:1</td>
<td>30</td>
<td>25</td>
<td>-do-</td>
</tr>
<tr>
<td>4</td>
<td>-do-</td>
<td>7:3</td>
<td>30</td>
<td>25</td>
<td>-do-</td>
</tr>
<tr>
<td>5</td>
<td>-do-</td>
<td>3:2</td>
<td>30</td>
<td>25</td>
<td>-do-</td>
</tr>
<tr>
<td>6</td>
<td>-do-</td>
<td>5:4</td>
<td>30</td>
<td>25</td>
<td>-do-</td>
</tr>
<tr>
<td>7</td>
<td>-do-</td>
<td>1:1</td>
<td>30</td>
<td>25</td>
<td>-do-</td>
</tr>
<tr>
<td>8</td>
<td>Methanol</td>
<td>Absolute</td>
<td>30</td>
<td>25</td>
<td>Polar</td>
</tr>
<tr>
<td>9</td>
<td>H₂O in MEOH</td>
<td>20% H₂O</td>
<td>30</td>
<td>25</td>
<td>Neural in polar</td>
</tr>
</tbody>
</table>
Two mL of this mixture was placed in the capillary column, inserted into the hp-chromatograph and ran to obtain the labda max and the wave length (Sebnem et al., 2003).

Collection of Haemonchus contortus eggs for in-vitro assay
Female adult *H. contortus* were collected from the abomasums of sheep slaughtered at the Zaria Abattoir and identified in the Helminthology Laboratory Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria, aided by standard descriptions of Taylor et al. (2007) and microscopy. The parasites were crushed in mortar with pestle to obtain the helminth eggs. Thereafter, 60 mL of water was added to the crushed worms, and filtered through a 100 mesh sieve (150 µM). The filtrate was placed in three 20 mL centrifuge tubes and centrifuged at 1,000 rpm (SG 1.2) for 15 min. The supernatant was decanted and the volume of the sediment was adjusted such that 0.2 mL contained 100 eggs. The suspension was placed/well in 36 wells of a 48-well flat-bottom microtitre plate (Coaster, Cambridge. MA) for egg count under light microscope. The total number of eggs per µL was determined. The counting of *H. contortus* eggs for the in-vitro egg hatch assay was done using the modified Mcmaster method described by Sloss et al. (1994).

In-vitro egg hatch inhibition assay
The in-vitro egg hatch assay was done using the method described by Coles et al. (1992). The volume of the eggs sediment was adjusted such that 0.2 mL contained 100 fresh eggs of *H. contortus* and then 0.2 mL of the egg suspension was placed/well in 36 wells of a 48-well flat-bottom microtitre plate (Coaster, Cambridge. MA) containing the isolated bioactive constituents from the TLC spots (as visualized under UV light and spot under the detecting agent) obtained from the pooled column fraction of the fractionated aqueous methanol portion. The bioactive constituents evaluated were from the constituents A (top spot on the TLC Plate), constituents B (middle spot on the TLC Plate) and constituents C (bottom spot on the TLC Plate). One milligram (1 mg) of the bioactive constituent was dissolved in 1 mL of distilled water to give 1 µg in 1 µL.

Using a 10 µL capillary tube with the capacity to deliver of 10 µg, 60 µg suspension of each bioactive constituent was dissolved in 60 µL of 4% dimethyl sulphoxide (DMSO) to give 120 µL (v/v) in a 1 mL test tube after which 180 µL of distilled water was added to give a final volume of 300 µL (Stephen et al., 1996). This was then incubated in this mixture for 48 h at 27°C in an incubator (Shermond, ILCA, N1742, England). All the eggs contained 10 µg of the constituents. Each microtitre plate also contained 6 wells of a placebo control (i.e. containing 10 µL of 4% DMSO/well in 40 µL of distilled water) and a positive control contained 50 µL levamisole at 10 µg/well (Stephen et al., 1996). The eggs were mixed in this mixture for 48 h at 27°C in an incubator (Shermond, ILCA, N1742, England). After 48 h, a drop of 10 % lugol’s iodine solution was added to arrest eggs from further hatching (Stephen et al., 1996). The eggs were incubated in this mixture for 48 h at 27°C in an incubator (Shermond, ILCA, N1742, England). All the eggs and the first-stage larvae (L1) that had hatched within 48 h were counted. Data was expressed as percentage unhatched eggs using the formula; N-n/N x 100% (Cavier, 1973), Where N= number of unhatched eggs in placebo control, and n the number of unhatched eggs in bioactive and or positive control wells.

Statistical analysis
Data obtained were expressed as mean ± standard error of the mean (SEM) and subjected to analysis of variance (ANOVA) and Dunnett comparison test to compare the means of the egg hatch inhibition among the treatment groups and controls, using a software package for GraphPad prism (version 4.0, 2003). A statistical probability of P<0.05 was the criterion for significance.

Table 2. Column fractionation of aqueous methanol portion.

<table>
<thead>
<tr>
<th>Solvent composition</th>
<th>Column fraction</th>
<th>Quantity of fractions after conc. (mg)</th>
<th>Percentage yield (%)</th>
<th>Response to solvent system</th>
<th>Retention factor (Rf) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure chloroform a</td>
<td>5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform/Methanol (9:1) b</td>
<td>22</td>
<td>2.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform/Methanol (4:1) c</td>
<td>35</td>
<td>3.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform/Methanol (7:3) d</td>
<td>58</td>
<td>5.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform/Methanol (3:2) e</td>
<td>80</td>
<td>8.0</td>
<td>+</td>
<td>0.470</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform/Methanol (5:4) f</td>
<td>122</td>
<td>12.2</td>
<td>+</td>
<td>0.465</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform/Methanol (1:1) g</td>
<td>186</td>
<td>18.6</td>
<td>+</td>
<td>0.471</td>
<td>-</td>
</tr>
<tr>
<td>pure Methanol h</td>
<td>220</td>
<td>22.0</td>
<td>+</td>
<td>0.468</td>
<td>-</td>
</tr>
<tr>
<td>20% Water in Methanol i</td>
<td>192</td>
<td>19.2</td>
<td>+</td>
<td>0.464</td>
<td>-</td>
</tr>
</tbody>
</table>

Plate 1. TLC showing the location of the active constituents A, B and C.
Results

Extraction of plant material and partitioning of crude extracts
Following extraction of 2 kg of stem bark of *C. molle* with 70% absolute methanol as solvent, a yield of 413 g (21%) was obtained. Using petroleum ether, chloroform and N-butanol as partitioning solvents, the respective yields following partitioning of 316 g crude methanolic extract were 3 g (0.94%), 11 g (3.48%), 112 g (35.3%) respectively, leaving 191g (60.12%) as the aqueous methanol portion.

Column fractionation of aqueous methanol extract
At the end of running the column, the column fractions were pooled together to obtain two fractions (i.e fraction A and B). The fractions B (e, f, g, h and i) had similar retention factors (RF) of 0.470, 0.465, 0.471, 0.468 and 0.464 respectively. Fraction A (which comprises of fractions a, b, c and d) did not respond to TLC (Table 2).

Isolation and concentration of compound on TLC
Three spots (A, B, C) were located on TLC after spotting 600 mg of the pooled column fraction, the individual spots were scrapped, dissolved in 100 mL of 70% methanol, centrifuged and the supernatants concentrated to dryness to give a respective yields of active constituents A (120 mg), B (60 mg) and C (80 mg).

Identification of isolated constituents using UV and mass spectrum
Constituents A, B and C were obtained as amorphous powders. The UV absorption spectrum exhibited maxima (λmax, nm) in methanol of 229 nm (A), 227 nm (B) and 211 nm (C) (Fig. 1, 2 and 3).

In-vitro egg hatch inhibition assay
Constituents A, B and C inhibited *H. contortus* egg hatching by 91.7%, 91% and 51.8% respectively, whereas PCF (i.e. the pooled column fractions that was used for TLC that gave rise to the three different constituents A, B and C) produced 92% inhibition. The control treatment levamisole (positive control) produced 97% inhibition of hatch whereas water (negative control) produced only 3.5% inhibition of egg hatch (i.e. about 96.5% of the eggs incubated were able to hatch) (Table 3 and Fig. 5).

Statistically, there was no significant differences (P>0.05) between the mean percentage inhibition caused by constituents A, B and PCF and levamisole. However, there was a highly significant difference (P<0.01) between the means of constituent C and levemisole. When compared to the mean egg hatch inhibition caused by water (95.67), the mean inhibition caused by constituent A (8.33), B (9.0), PCF (8.0) as well as levamisole (2.67) were each much significantly (P<0.01) higher. Also, the mean inhibition caused by constituent C (48.17) showed significantly (P<0.05) higher egg hatch inhibition when compared with water (negative control) (Table 3).

Discussion
The isolated constituents A and B from the pooled column fraction of the aqueous methanol extract demonstrated anthelmintic activity comparable to those of levamisole a conventional anthelmintic, and were able to produced 91.7% and 91% egg hatch inhibition *in-vitro* respectively. This is in line with the recommendation of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles *et al*., 1992) of 90% and above as did the levamisole (97.3%). Constituent C gave a much lower percentage egg hatch inhibition (51.8%) compared to constituents A, B as well as with levamisole.
However, the pooled column fraction (PCF), from where constituents A, B and C were obtained, gave a much higher percentage egg hatch inhibition than either A or B. This suggests that the constituents when combined in a single dose against helminthes would produce a better synergy than administering either one of constituent alone. Based on the UV absorption spectral data (λmax; nm) range (211-C, 227-B and 229.00-A nm), iridoid glycosides was the most likely isolated constituent from the column fraction of the extract.

In similar studies, iridoid glycosides with the same spectral data were isolated from *Globularia dumulosa* and *G. davisiana* by Hasan et al. (2003) and Calis et al. (2002a) respectively. An iridoid glycosides isolated from *Veronica pectinata var. glandulosa* and *Nepeta septenarenata Eremb* showed spectral data of lambda max of 210 nm, 299 nm and 331 nm (Sebnem et al., 2003) and lambda max of 200 nm (aucubin), 220 nm (ajugol), 224 nm (ajugoside) (Heba and Abd, 2010) which is consistent to that isolated from the plant under study. This compound has been shown to be responsible for inducing tonic contraction on the muscle of the *C. elegans* causing their expulsion from rats GIT in an experimental infection (Kim et al., 1992; Hong, 2000). Iridoid glycosides isolated from *Nyctanthes arborotristis* demonstrated anthelmintic activity against nematodes as reported by Sureka Shruti et al., (2009), Kozhiparambil et al. (1985) and Stupnner et al. (1993). However, in another separate study conducted by Gustine et al. (1998), aucubin an iridoid glycoside isolated from the leaves of plantain did not show anthelmintic activity against nematodes in cattle. This is to say that not all iridoid glycosides have anthelmintic activity.

**Conclusion**

Based on the results presented in this work, *C. molle* offers an opportunity for a new anthelmintic compound. This plant may offer an alternative source for the control of gastro-intestinal nematodes of sheep and goats. However, further spectroscopic studies on the active principles and the development of quality assurance protocols involving the use of reference substance of plant origin for this extract is recommended.

**Acknowledgements**

The authors thank the University of Abuja, Nigeria for granting the main author, a staff development leave. The authors also like to thank all the technical staff of the Department of Veterinary Parasitology and Entomology for their assistance.

**References**


