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In vitro anthelmintic activity of the stem-bark of *Combretum molle* R. Br. x. G. Don (Combretaceae) against *Haemonchus contortus*

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Livestock production is hampered to a great extent by helminth parasites throughout the world. Plant derived drugs have been used for ages against these parasites; however, there is limited scientific knowledge on their efficacy against helminths. In this study, established techniques were used to detect anthelmintic activity of extract and fractions of *Combretum molle* R. Br. x. G. Don (Combretaceae) using a bioassay-guided purification approach. Two kilograms of powdered stem bark of *C. molle* were extracted with 10 L of absolute methanol. The crude methanol extract was dissolved in water and partitioned successively in petroleum ether, chloroform and n-butanol to obtain the petroleum ether (PE), chloroform (CP), n-butanol (BP) and aqueous methanol (AMF) fractions. The AMF was further subjected to liquid column chromatography (LCC) to obtain 12 different fractions that were pooled together into 3 major fractions based on the similarity of their relative front (Rf) values. Fractions I, II, and III obtained during the column chromatographic separation and the AMF were tested for activity against the eggs of *Haemonchus contortus* using the egg hatch inhibition assay (EHIA). Column fractions I, II and AMF demonstrated activity that is comparable to levamisole (standard anthelmintic), and inhibited helminth egg hatching by 91.7, 91 and 92%, respectively *in vitro*. The refined fraction of *C. molle* and the separated components of the fraction showed promising effect against *H. contortus* eggs and could serve as potential anthelmintics.

Key words: *Combretum molle*, anthelmintic activity, *Haemonchus contortus*, medicinal plant.

INTRODUCTION

Some of the limitations associated with using synthetic conventional anthelmintic include amongst others, the development of resistance by the parasites (Waller, 1997; Geerts and Gryseels, 2000), chemical residues and toxicity (Gasbarre et al., 2001), increased cost, non-adaptability of drugs and non-availability in rural areas (Jabbar et al., 2007). These factors have therefore necessitated the search towards effective anthelmintic

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drugs, especially from plant sources. Plants contain valuable chemical compounds that could offer alternative means to the problems of modern synthetic drugs (Abdul, 1990).

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, dysmenorrhoea and infertility in women, leprosy, syphilis, microbial infections and general body weakness (Hutchings et al., 1996). *Combretum molle* R. Br. x. G. Don (Combretaceae) is reported to be used for treatment of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) related infections (Bessong et al., 2005) and malaria (Abebe and Ayehu, 1993). It is also reported to possess antibacterial (Eloff, 1998, 1999; Khan et al., 2000), antimycobacterial (Asres et al., 2001) and antifungal effects (Pegel and Rogers, 1985).

In a related study, the leaf and stem bark extracts of *C. molle* were also reported to have anthelmintic activities (Ademola and Eloff, 2010; Simon et al., 2008). This study was therefore aimed at determining the anthelmintic activity of the crude extract and fractions of *C. molle*.

MATERIALS AND METHODS

Plant collection

Ten kilogrammes of the stem-bark of *C. molle* were collected in the field around Zaria (Kaduna State), Gwagwalada (Federal Capital Territory) and New-Bussa (Niger State), all in the savannah zone of northern Nigeria in the months of March and April (dry season). The plant was identified and authenticated by Mallam Musa at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. It was deposited with a voucher specimen number of 2797 (Simon et al., 2008). Plants collected in the dry season contain high concentration of bioactive constituents (Ademola and Eloff, 2010).

Plant preparation, extraction and processing

The plant part was air-dried, pulverized into powder using mortar and pestle, and sieved. Two kilogrammes of the powdered plant was extracted with 10 L of absolute methanol (Sigma Aldrich 32213) in Soxhlet apparatus (Quick fit corning Ltd; Stafford, England) to obtain the crude methanol extract (CME). The CME was concentrated to dryness *in vacuo* using a rotary evaporator coupled to a thermo-regulator. Furthermore, 316 g of the CME was partitioned between petroleum ether and water, then chloroform and water, and finally between n-butanol and water using 250 ml of each solvent (Brain and Turner, 1975). The aqueous methanol fraction (AMF) showed significant and high (>90% inhibitory effect) biological activity during the preliminary anthelmintic trial and was therefore subjected to column chromatography (Brain and Turner, 1975). Column (75 cm by 2.5 cm) chromatography of the adenosine monophosphate (AMP, 1.5 g) was done using chloroform: methanol step gradient followed by 20% water in methanol elution. Initially, 100% chloroform was used and then reduced to 0% chloroform by the addition of 10% methanol in succession. This was followed by an isocratic elusion using 20% water in methanol. Twenty five fractions were collected during each elution. Based on their similar thin layer chromatography (TLC) profile on elution with n-butanol, glacial acetic and water (8:1:1) as mobile phase; fractions with similar Rf values were combined and concentrated to solid forms *in vacuo*.

Anthelmintic trial

Helminth eggs

Female adult *H. contortus* worms were collected from Zaria Abattoir and identified in Helminthology Laboratory of the Department of Parasitology and Entomology, Ahmadu Bello University, Zaria. The parasite was crushed in mortar with pestle and about 60 ml water was added to the crushed worms and filtered in a 100 mesh sieve (100 × 150 µm). The assay was done using the modified McMaster method as described by Sloss et al. (1994). The filtrate was placed in three 20 ml centrifuge tubes and centrifuged at 2000 g for 15 min. The supernatant was decanted and the volume of the sediment was adjusted to 20 ml in a graduated test tube. Using a hypodermic needle, 0.3 ml of the sediment was placed on a McMaster slide (Weber Scientific International, England®) and the eggs were counted under light microscope. The number of eggs per ml was estimated.

In vitro egg hatch inhibition assay

The *in vitro* egg hatch assay was done using the method of Coles et al. (1992). Briefly, 100 freshly harvested helminth eggs contained in 0.2 ml of water were placed in each well of a 48-well flat-bottom microtitre plate. For each replicate, 60 µg of each isolated bioactive constituent was dissolved in 60 µl 4% dimethyl sulphoxide (DMSO) and made up to 300 µl with distilled water, and then distributed equally into six wells, such that each well contained 10 µg of the constituents (Stephen et al., 1996). Similarly, the untreated and the treated control wells contain 60 µl of 4% DMSO and 50 µl levamisole (1 µg/ml) per well, respectively. The eggs were incubated for 48 h at 27°C in an incubator. After 48 h, 10% Lugol's iodine solution was added to stop the eggs from hatching and all the unhatched eggs and the first-stage larvae (L_1) were counted using a microscope with inverted lens. Percentage inhibition of helminth egg hatching was calculated using the formula of Cavier (1973):

$$(N - n) / N \times 100\%$$

Where N = mean number of unhatched eggs in untreated control wells, n = mean number of unhatched eggs in treated wells.

Statistical analysis

Data obtained were expressed as mean ± standard error of the mean (mean ± SEM) and were subjected to one way analysis of variance (ANOVA) followed by Dunnett's comparison post-hoc test using GraphPad prism version 4.0, 2003 (from GraphPad software, San Diego, California, USA) to compare the level of significance between the groups. A statistical probability of $P < 0.05$ was considered significant.

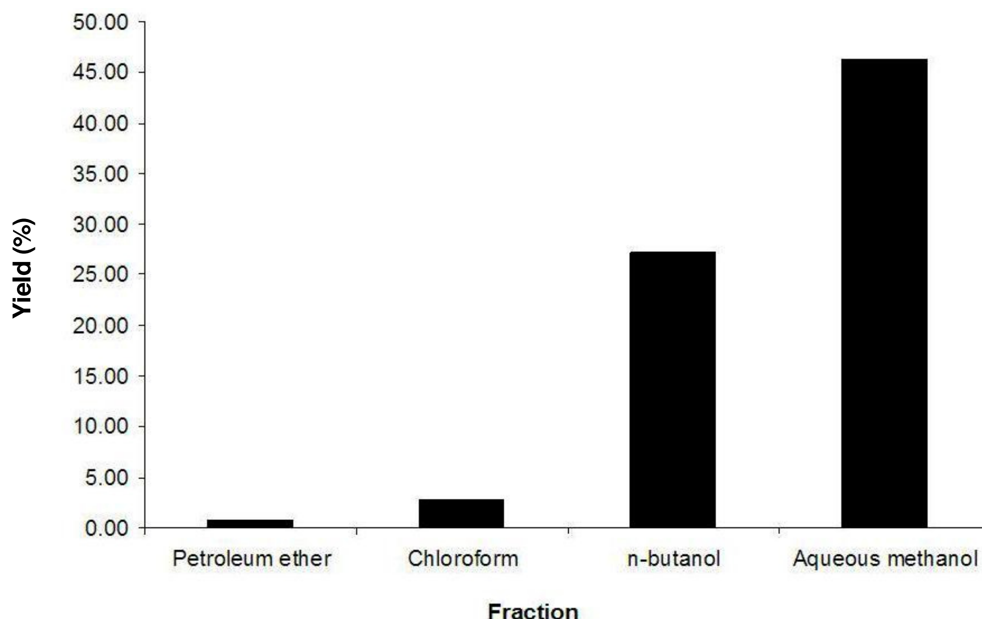

Figure 1. Percentage yield of different fractions obtained after solvent-solvent partitioning of crude methanol extract of *Combretum molle*.

Table 1. The effect of compounds and column fraction of *Combretum molle* on *H. contortus* egg hatchability.

Test substance (µg/ml)	Mean number of hatched eggs after treatment ± SEM	Egg hatch inhibition (%)
Column fraction I	8.33±1.4[a]	91.7
Column fraction II	9.0±1.6[a]	91
Column fraction III	48.17±3.0[b]	51.8
Aqueous methanol fraction	8.0±1.7[a]	92
Levamisole	2.67±1.0[a]	97.3
4% DMSO in water	95.67±1.2[c]	4.3

Means with the same subscript letter did not differ significantly (P > 0.05).

RESULTS

Yield of extracts and fractions

The crude methanol extract (CME) gave a yield of 413 g (20.65%), which appeared light brown. The yield obtained from individual fractions together with the residual AMF after partitioning of the CME is shown on Figure 1. The petroleum ether, chloroform and n-butanol extracts are whitish, yellowish and reddish brown in colour, respectively. There was 76% recovery of the extract following partitioning. Column chromatographic fractionation of 1.5 g of AMF gave 9 fractions that were pooled together into 3 major fractions that is, fractions I, II and III based on the similarities of their Rf values. Fractions I, II and III gave a yield of 120, 250 and 550 mg, respectively.

In vitro egg hatch inhibition assay

Fractions I, II and III inhibited the hatching of *H. contortus* eggs by 91.7, 91.0 and 51.8%, respectively, whereas the AMF produced 92.0% inhibition. Levamisole (positive control drug) produced 97% inhibition, while water (negative control) produced 3.5% inhibition (Table 1). Statistically, there were no significant differences ($P > 0.05$) between the mean egg count obtained from groups treated with fractions I, II, III, AMF and levamisole. However, there was a high significant difference ($P < 0.01$) between the mean egg counts from groups treated with fraction III, levamisole and all other groups in this study. Similarly, there was a significant ($P < 0.05$) difference between the water treated group (negative control) and the group that was treated with fraction III (Table 1).

DISCUSSION

Following the partitioning of CME of *C. molle*, about 76.8% of the extract was recovered. This indicates a loss of 23.2% of the total mass of the CME during the process of solvent-solvent extraction. In some cases, a pellicle is formed between different phases and the pellicle could have been discarded along with the solvent. Similar effect was reported by Suleiman et al. (2012) and accounted for the observed loss of plant material during fractionation process.

Column fractions I, II and the aqueous methanol portion of *C. molle* extract inhibited the hatching of *H. contortus* eggs by 91.7, 91.0 and 92%, respectively in an *in vitro* egg hatch assay. This compares very well to egg hatch inhibition produced by levamisole (97%). Attempts to grade the anthelmintic activity of different substances have been made. One such grading according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) said that for a substance to be an effective anthelmintic, its activity must be 90% or above (Coles et al., 1992). The higher anthelmintic activity of 92% produced by AMF could possibly be attributed to the synergistic action of the different constituents. Plant fractions or column fractions from plants are mixtures of different compounds. These compounds can act in concert to produce a much higher activity than the individual compounds. Synergism had been demonstrated in some plant extracts (Aqil et al., 2005).

Saponins were shown to be present in the extract of *C. molle* (Ademola and Eloff, 2010). Saponins destabilize membranes and increase cell permeability by combining with membrane-associated sterols (Gee and Johnson, 1988). Perhaps that could explain the anthelmintic effect of the plant.

Conclusion

Based on the results presented in this work, *C. molle* offers an opportunity for further research aimed at getting a new effective alternative for treating haemonchosis of ruminants. However, detailed toxicity and *in vivo* pharmacological trials are required to justify its clinical use.

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