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EVALUATION OF ANTHELMINTIC EFFICACY OF *CITRUS AURANTIFOLIA* (Christm) FRUIT JUICE AGAINST *HELIGMOSOMOIDES BAKERI*

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ABSTRACT
This study was carried out to evaluate the anthelmintic activity of *Citrus aurantifolia* (Christm) fruit juice locally used as an anthelmintic. The anthelmintic activity of the fruit juice on eggs, first stage larvae (L_1) and adults of *Heligmosomoides bakeri* was examined by *in vitro* and *in vivo* tests. The fruit juice was prepared to obtain seven increasing concentrations. Distilled water and albendazole were used as negative and positive control groups, respectively. At the concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.5 and 1.0 mg/ml the fruit juice inhibited the hatching of eggs by 2.5, 10.5, 11.5, 13.5, 30.0, 90.25 and 100 %, and killed the larvae by 40.5, 37.5, 47.5, 55.0, 92.5 and 100.0 %, respectively. The plant caused a significant ($P<0.05$) deparasitization rate of 80.5 % of the adult worms at the dose of 800 mg/kg per os in mice. The data revealed dose-dependent anthelmintic activity, thus justifying its use in the traditional medicine.

KEY WORDS: Key Words: *Citrus aurantifolia* fruit, Egg hatch inhibition, larvicidal, Deparasitization, albendazole.

INTRODUCTION

The use of medicinal plants to treat helminthosis has been for ages especially in Africa and other developing countries of the world. This practice was pronounced due to cultural believes of the people and the ready availability of the plants (Brandt *et al*., 1995). However, the advent of synthetic drugs has undermined the use of medicinal plants for the management of diseases. The use of synthetic drugs however, are bedeviled by high cost, drug residue in animal products, adverse drug effects, environmental pollution, rapid development of resistance of helminth parasites to the drugs and slow development and licensing on new drugs (Saeed *et al*., 2007). These have led to the need to screen medicinal plants for their anthelmintic potentials since plants based drugs are cheap and relatively safe. Helminthosis affects humans and livestocks all over the world (Githiori *et al*., 2004) especially in the tropics and subtropics (Perry *et al*., 2002). In humans, the dominating helminth parasites are *Trichuris tichura, Necator americanus, Ancylostoma duodenale, Ascaris lumbricoides*. These parasites are believed to have infected about 3 billion people around the world (Wabo *et al*., 2013). In animals, a blood sucking helminth of economic importance is *Haemonchus contortus.* The blood loss often results to anaemia, loss of condition and death of the host animals especially in heavy infection (Miller and Horohov, 2006). This helminth infects both domestic animals and wild ruminants and has caused significant loss to small ruminants producers worldwide especially in areas where extensive grazing is practiced (Waller and Chadrawathani, 2005). Economically, the losses caused by helminthosis include: reduction in feed intake resulting in low weight gain, lowered fertility, decrease in milk production and death of the animal (Waller, 1997).

Citrus aurantifolia (*C. aurantifolia*) is a small shrubby tree, about 5 m tall. It is an evergreen and ever bearing tree that is densely and irregularly branched and possesses short and stiff spines (thorns). The fruits are globose to ovoid berry of about 3-6 cm in diameter and sometimes have apical papilla. It is yellow when ripe but usually picked green commercially. The fruit juice is acidic and fragrant, sour as lemon juice but more aromatic. It is usually valued for its unique flavor compared to other limes (Sethpakdee, 1992). The plant is used in traditional medicine as an antiseptic, antiviral, antifungal, astringent, diuretic, mosquito bite repellent, for the treatment of stomach ailments, constipation, headache, arthritis, colds, coughs, sore throats and used as appetite stimulant (Morton, 1987; Nallely *et al*., 2012). This present study aimed at evaluating the *in vitro* and *in vivo* anthelmintic efficacy of the fruit juice of *C. aurantifolia* against the ova, first stage larvae and adult of *Heligmosomoides bakeri* (a murine adapted trichostrongylid commonly used as model for anthelmintic screening).

MATERIALS & METHODS

Experimental Animals

Apparently healthy albino mice (*Mus musculus*) of both sexes within the ages of 10 to 12 weeks were bred in the animal house, Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria. They were allowed to adapt to laboratory conditions for two weeks. The

mice were maintained on commercial chick growers mash (Vital feed®). Water and feed were provided *ad libitum*. Wood shavings were used as bedding and were changed every two days. The mice were grouped and kept in cages. Each group consisted of three mice which were identified by marks on their tails using permanent markers of different colours.

Plant Collection, Identification, Extraction and Partioning

Fresh samples of *C. aurantifolia* fruits were obtained from the premises of the Ahmadu Bello University, Zaria, Nigeria in 2014. Taxonomic identification was established by a botanist with the Herberium section of the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria. A voucher specimen was deposited there and labeled Specimen number 990. The fruits were properly washed and sliced into two halves each, which was then squeezed gently into a clean container. The resulting lime juice was filtered through a Whatman number 1 paper, and residual pulp and seeds were discarded. The lime juice were immediately prepared into different concentrations using distilled water and were all tested for anthelmintic activities.

Source of *H. bakeri* and *H. bakeri* infected mice

Infective third stage larvae (L_3) of *H. bakeri* and mice infected with the parasite were obtained from the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

Recovery of *H. bakeri* eggs

Three grams of freshly passed out faeces from artificially infected mice (*Mus musculus)* was collected using a tea spoon into a centrifuge tube and homogenized using a pestle in 12 ml of saturated sodium chloride solution. The solution was filtered through a tea sieve and the filtrate centrifuged at 5 *g* for 5 minutes. The supernatant was poured into a beaker and 100 ml of distilled water was added to it. The supernatant added to the distilled water was again centrifuged at 5 *g* for 5 minutes. Using a 10 ml syringe, the supernatant was aspirated and discarded and the sediment re-centrifuged for 5 minutes at 5 *g* after adding more water. The sediment obtained after aspirating the supernatant with a syringe was examined under the light microscope at ×10 objective for the presence of *H. bakeri* eggs. The recovered eggs were used for the *in vitro* egg hatch test or cultured for 48 hours to obtain the first stage larvae of *H. bakeri* used for larvicidal test.

Evaluation of ovicidal and larvicidal activity of the extracts

The *in vitro* anthelmintic studies involved the evaluation of ovicidal (Egg hatch inhibition test) and larvicidal activities of the plant on the eggs and first stage larvae of *H. bakeri*. Different concentrations (0.01, 0.02, 0.03, 0.04, 0.05, 0.5 and 1.0 mg/ml) of the fruit juice were prepared by dissolving it in distilled water. One ml of each concentration was incubated with the larvae and eggs of *H. bakeri* contained in 1 ml solution in a Petri dish and incubated at room temperature for 24 and 48 hours, respectively. Albendazole (1 ml in each Petri dish) of the same concentrations above and distilled water were used as treated and untreated controls, respectively. The plates were covered to prevent evaporation. The tests were done in triplicate. For the ovicidal test, the content of each well of the Petri dish was pipetted and placed on a glass slide and examined microscopically at ×10 magnification. All the unhatched eggs as well as the L_1 in each well were counted and recorded.

The percentage inhibition of egg hatching was calculated using the formula described by Wabo *et al*. (2010).

$$\text{Hatchinh rate (\%)} = \frac{Number\ of\ L1\ larvae}{Number\ of\ egg\ cultured} x100$$

For the larvicidal test, the content of each Petri dish was stirred and pipetted unto a clean glass slide and then examined under the microscope at ×4 magnifications to count the number of larvae that were dead or alive. A larva was considered alive if it moved any part of its body or migrated from one point to another; but if the larva showed no observable motion after 5-10 seconds interval, it was considered dead.

The percent mortality (Mc %) was determined using Abott's formula for corrected mortality (Wabo *et al*., 2005).

$$\text{Mc (\%)} = \frac{Mce - Mt}{100 - Mt} x100$$

Where Mce is the mortality obtained during the test and Mt the mortality registered in the untreated control.

Experimental infection of mice

The mice were infected orally with about 150 L_3 *H. bakeri* contained in 0.4 ml of distilled water using a blunted tip 18 G needle mounted on a 1 ml syringe. Fourteen days post infection, droppings from the infected mice were obtained by placing the mice in clean plastic cages for 10 - 20 minutes and feces produced by the mice were collected into a labeled container and examined quantitatively for the presence of *H. bakeri* eggs using the simple flotation method to establish infection (Soulsby, 1982; Ngongeh and Fakae, 2011).

***In vivo* Anthelmintic Screening of the Extracts**

Fifteen mice infected with *H. bakeri* were randomly allocated into 5 groups of 3 mice each. Groups 1-3 were treated with *C. aurantifolia* fruit juice at the dose of 800, 400 and 200 mg/kg, respectively. Group 4 and 5 were treated with albendazole (10 mg/kg) and distilled water (5 ml/kg), respectively and served as treated and untreated controls, respectively. All treatments were administered orally on the 16th, 17th and 18th day post-infection.

Post mortem worm counts

At the end of the treatment (19 days post-infection), all mice were deprived of food but not water for 24 hours so as to empty the gastrointestinal tract and ease the worm counting process. The mice were euthanized in chloroform chamber and the gastrointestinal tract removed immediately. The adult worms were recovered and counted as described by Ngongeh and Fakae (2011). The percentage deparasitization

was calculated using the formula described by Suleiman *et al*. (2005):

$$\frac{N-n}{N}x100$$

Where "N" = mean number of worms found in untreated control mice.
"n" = mean number of worms found in treated mice.
Percentage deparasitization of 70 and above was considered significant in this experiment.

Data Analysis

Results obtained were expressed as mean ± standard error of mean (±SEM). Analysis of variance (ANOVA) using GraphPad Prism Version 5.0 was used to compare the anthelmintic effects of the fruit juice of *C. aurantifolia* to albendazole and the non-treated (distilled water) group. The mean in different group was compared using Tukey Post hoc test. Value of P< 0.05 was considered significant. The 50 % inhibitory concentration (IC_{50}) and the 50 % larvicidal concentration (LC_{50}) were also determined from a log concentration-response curve.

RESULTS

Egg hatch inhibition

The fruit juice of *C. aurantifolia* significantly ($p < 0.05$) inhibited the hatching of eggs of *H. bakeri* in a concentration-dependent manner (Figure 1). Unhatched eggs were considered as dead due to the effect of the juice. At the concentrations of 0.5 -1.0 mg/ml, the egg hatch inhibition rates produced by the juice were not statistically different (P>0.05) from the effect produced by albendazole at the same concentrations. However, at concentrations range of 0.01 to 0.05 mg/ml, the egg hatch inhibition of albendazole was significantly (P<0.05) different from that of the plant. The transformation of mortality rates into probits and concentrations into log decimals gave a linear relation. The equation of the straight line was used to calculate the 50 % inhibitory concentrations (IC_{50}). The value of IC_{50} was 0.43 and 0.39 mg/ml for *C. aurantifolia* fruit juice and albendazole, respectively.

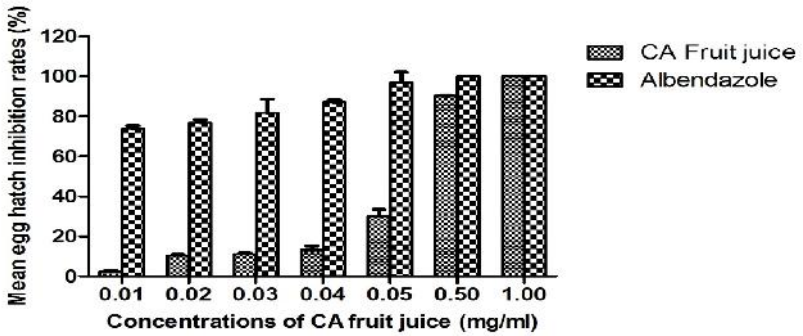

FIGURE 1. Inhibitory effects of different concentrations (0.01 – 1.0 mg/ml) of fruit juice of *C. aurantifolia* (CA) and albendazole on the egg hatching of *H. bakeri* after 48 hours of incubation. Values are mean (± SEM)

Larvicidal activity of *C. aurantifolia* fruit peel extracts

The fruit juice of *C. aurantifolia* also had significant ($p< 0.01$) larvicidal activity against L_1 of *H. bakeri*. The effect is concentration-dependent (Figure 2). At the concentrations of 0.05 -1.0 mg/ml, the larvicidal effects produced by the juice were not statistically different (P>0.05) from the effect produced by albendazole. However, at lower concentrations (0.01 - 0.04 mg/ml), the larvicidal effect of albendazole was significantly (P<0.05) different from the that of the plant.. The LC_{50} was 0.41 and 0.37 mg/ml for *C. aurantifolia* and albendazole, respectively.

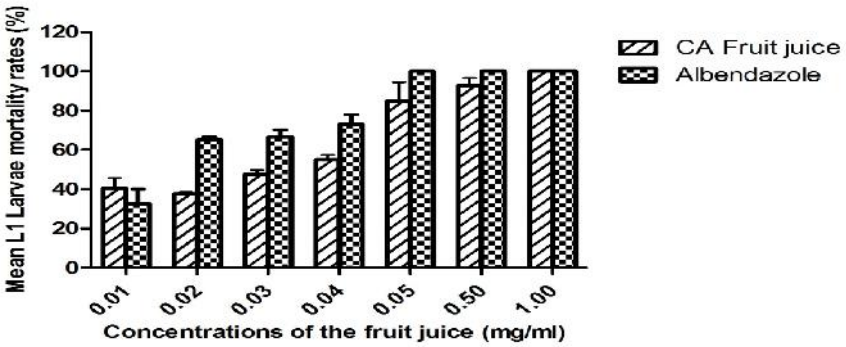

FIGURE 2. Mean mortality effects of different concentrations (0.01 – 1 mg/ml) of the fruit juice of *C. aurantifolia* (CA) and albendazole on the L_1 Larvae of *H. bakeri* after 24 hours of incubation at 25°C. Values are mean (± SEM).

Post-mortem worm counts

The mean worm counts and the deparasitization rates are shown in Table 1. The anthelmintic effect produced by *C. aurantifolia* at the dose of 800 mg/kg was not significantly (P>0.05) different from that of albendazole. Though the anthelmintic activity of this plant was dose-dependent, the anthelmintic effect produced by *C. aurantifolia* at the dose of 400 and 200 mg/kg were not significant since the deparasitization rates were less than 70 %, also their effect were significantly (P<0.05) lowered than that of albendazole.

TABLE 1. Mean Worm Counts (±SEM) in mice treated with varying doses of *C. aurantifolia* fruit juice 16-days post infection with 150 L_3 of *H. bakeri*

Dose of *C. aurantifolia* Juice (mg/kg)	Mean worm counts (±SD: n=3)	Deparasitization rates (%)
800	8.33±1.86	80.5
400	14.5±2.50	65.9
200	19.5±3.50	54.1
ABZ (10 mg/kg)	2.5±0.5	94.4
DW (5 ml/kg)	42.5±0.5	

DISCUSSION

The results of the *in vitro* and *in vivo* anthelmintic study revealed that *C. aurantifolia* fruit juice significantly inhibited the hatching of *H. bakeri* eggs and killed the larvae of the helminth as well as reduced the worm burdens in mice in a concentration and dose-dependent manner. The mode of action of *C. aurantifolia* fruit juice could be similar to that of albendazole, judging from the manner of concentrations–dependent anthelmintic activities, but more work need to be done for rational conclusions. The ovicidal and larvicidal effect produced by the plant and albendazole might be caused by the diffusion of the anthelmintic drugs through the external surfaces such as eggshells and the cuticles of larvae or the diffusion of the drug through the intestinal cells (Alvarez *et al.*, 2001). The mechanism of action of albendazole on adult worms has been established (Riviere and Papich, 2009) but that of Citrus fruit juice is still unknown. It is possible that the phytochemicals contained in the plant entered into the adult worm through transcuticular absorption to cause paralysis and death of the worm (Dobson *et al.*, 1986) or the secondary metabolites (e.g. tannins) bind to available proteins and deprived the worms of nutrient leading to their death (Athanasiadou *et al.*, 2001). The anthelmintic property of plants is dependent on numerous substances that are found in them. These could be alkaloids, sugars, saponins, aromatic oils, resins and other medicinally useful chemicals (Suleiman *et al*., 2005; Kollins *et al*., 2012). *C. aurantifolia* have been shown to contain phytochemicals like carbohydrate, glycosides, phenols, tannins, saponins, alkaloids, steroids, triterpenes and flavonoids; some of which might be responsible for the plants' anthelmintic activities (Okwu, 2008; Pathan *et al*., 2012; Nallely *et al*.,2012; Enejoh, 2014). The *in vitro* and *in vivo* activity of albendazole was higher in this study. The IC_{50} and LC_{50} values for albendazole were 0. 39 mg/ml and 0.37 mg/ml for egg hatch inhibition and larvicidal test, respectively. The higher efficacy of albendazole in this study could be due to the fact that albendazole is a pure active substance, while the fruit juice contains several chemical compounds, among them were the active ingredient with ovicidal and larvicidal actions. In general, the extract of a plant has small concentrations of active compounds with a great number of promising properties (Ademola and Ellof, 2011). From this study, there was a variation between the *in vitro* and *in vivo* anthelmintic activity of *C. aurantifolia* fruit juice. *In vivo* tests no, doubt give more reliable data, but they require greater amount of compound, large number of animals and much time (Iqbal *et al*., 2004). In this study, the *in vitro* anthelmintic test of the fruit juice showed activity as high as 100 % at the concentration of 1.0 mg/ml and above, whereas, the *in vivo* anthelmintic test produced a deparasitization rate of 80.5 % at the dose of 800 mg/kg. The differences between *in vitro* and *in vivo* efficacies could be attributed to the gastrointestinal tract environment as well as the physiology of the animals which may alter the helminth's response to treatment. In addition, oral drug administration is limited by many factors such as: first pass effect, drug forming complexes with food, destruction of drug by gastric acid or enzymes and the effect of gastric pH on the drug. These might have limited the availability and efficacy of the juice *in vivo.* However, in the *in vitro* study, the juice had direct contact with the parasites resulting in the high efficacy. It is worthy of note that several parts (*e.g.* leaves, bark, peels, seed, essential oils, fruit sack and fruit juice) of citrus species have been screened for anthelmintic activities previously and they showed positive anthelmintic activities, but most of the studies were based on *in vitro* experiments (Sonali *et al*., 2011; Bairagi *et al*., 2011; Abdelqader *et al*., 2012). There is no doubt that some *Citrus species* had efficacy against helminthes but there is the need to step up from *in vitro* studies. We therefore recommend that further *in vivo* studies involving the target helminth parasites for specific animals should be conducted to evaluate the efficacy of this plant on these animals. Also, there is need to isolate and characterize the active principles from extracts of *C. aurantifolia* fruit juice and establish their mechanisms of action. This could provide a safe and effective anthelmintic drug.

CONCLUSION

The results of this present study showed that *C. aurantifolia* fruit juice possessed anthelmintic activities both *in vitro* and *in vivo* and thus justify the traditional claims.

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LIST OF METRIC SYSTEM AND SYMBOLS

>	Greater than
%	Percent
˚C	Degree centigrade
<	Less than
kg	Kilogram
L_1	First stage larvae
L_3	Third stage larvae
mg	Milligram
ml	Millilitres
P	Probability