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ANTHELMINTIC ACTIVITY OF CITRUS SINENSIS (L) OSBECK FRUIT JUICE AGAINST HELIGMOSOMOIDES BAKERI

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Abstract

The anthelmintic activity of Citrus sinensis fruit juice on eggs, first stage larvae (L1) and adults of Heligmosomoides bakeri was examined by in vitro and in vivo tests. Freshly harvested C. sinensis fruit was washed and sliced into two halves each, which were squeezed gently into a clean container to obtain the juice after filteration through a Whatsman paper. 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 11.4±2.77, 11.90±3.61, 14.03±2.72, 18.67±2.0, 24.93±2.06, 89.60±1.80 and 100±0.0 %, and killed the larvae by 34.70±5.74, 48.35±1.65, 65.97±6.28, 78.55±1.45, 87.85±2.15 and 97.77±2.23 %, respectively. The plant caused a significant ($P<0.05$) deparasitization rate of 74.6 % of the adult worms at the dose of 800 mg/kg per os in mice. This study demonstrates that Citrus sinensis fruit juice possess anthelmintic activity.

Keywords: Anthlmintic, Citrus sinensis, Fruit juice, Albendazoles, Heligmosomoides bakeri.

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INTRODUCTION

There is an increasing interest in citrus fruits consumption across the world because of their rich sources of vitamin C, folate, dietary fibre, and minerals as well as many phytochemicals, including flavonoids, amino acids, triterpenes, phenolic acids and carotenoids (Rajarajan *et al.,* 2009). Citrus species are also valued for their ethnomedical uses as they are used to treat cough, sore throats, constipation, headache, cold and stomach ailments (Aslin, 2014). Frequent consumption of Citrus fruit juice have been associated with lower risk of stroke and cancers such as colorectal, esophageal, gastric and stomach cancers (Patil, 2009). More so, citrus possesses antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, laxative and astringent properties (Milind and Dev, 2012). The major Citrus species grown all over the world is *Citrus sinesis* (sweet orange). It is an evergreen flowering tree generally growing to 9–10 m in height (Orwa *et al.,* 2009). It accounts for about 70% of the total citrus output (Milind and Dev, 2012). In view of its ready availability and rich photochemicals, attention has been drawn to its pharmacological evaluation. The importance of this is to provide alternative source of drugs for the currently used synthetic drugs that are constrained by high cost, toxicity, adverse effect, residue in animal products and resistance (Enejoh *et al.,* 2015). Several plants have been screened against parasitic diseases especially helminthosis. Helminth infections are usually chronic and debilitating in nature in humans. In animals, the disease manifest clinically as inappetance, lethargy, dullness, loss of general body condition, rough hair coat, pallor of mucous membrane, depression and anaemia (Okewole and Oduye, 2001). The disease results in impairment of behavior, reduced growth rates, low fertility, low birth weight, reduced animal products such as milk, wool, meat, hide and skin (Waller, 1997). This present study aimed at evaluating the *in vitro* and *in vivo* anthelmintic efficacy of the fruit juice of *C. sinensis* against the ova, first stage larvae and adult of *Heligmosomoides bakeri* (a murine adapted trichostrongylid commonly used as model for anthelmintic screening).

MATERIALS AND METHODS

Plant Collection, Identification and Preparations

Fresh samples of *C. sinensis* fruits were obtained from Zaria, Kaduna State, Nigeria in the second quarter of 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. The fruits were properly washed and sliced into two halves each, which were squeezed gently into a clean container. The fruit juice obtained was filtered through a What man number 1filter paper, and residual pulp and seeds were discarded. The juice were immediately prepared into different concentrations using distilled water and were all tested for anthelmintic activities.

Experimental Animals

Fifteen 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.

Recovery of *H. bakeri* eggs

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, Ahmadu Bello University, Zaria. The parasites were maintained in mice kept in the animal house of the Department of Veterinary Parasitology and Entomology. The eggs of the parasites were obtained according to the method described by Enejoh et al., 2015a. 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 method described by Enejoh et al., 2014 was used for 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).

$$\text{Hatching rate (\%)} = \frac{\text{Number of } L_1 \text{ larvae}}{\text{Number of eggs cultured}} \times 100$$

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 10-20 seconds interval, it was considered dead. The percent mortality (Mc %) was determined using Abott's formula for corrected mortality (Wabo *et al*., 2005).

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

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).

***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. sinensis* 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 method described by Enejoh et al. (2015b) was employed for the worm count. The percentage deparasitization was calculated using the formula described by Suleiman *et al.* (2005):

$$\frac{N - n}{N} \times 100$$

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 ovicidal test showed that the fruit juice of *C. senensis* 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 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. However, 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. 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. sinensis* fruit juice and albendazole, respectively.

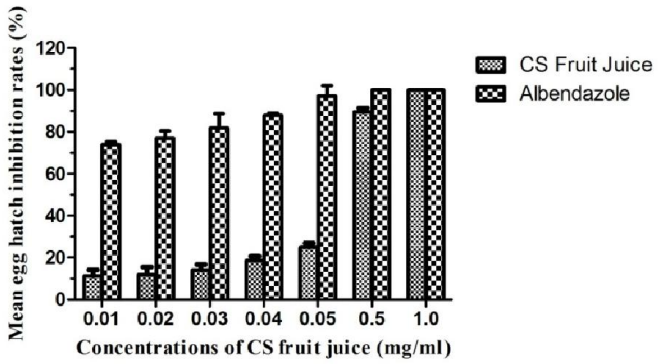

Figure 1. Inhibitory effects of different concentrations (0.05 – 1.0 mg/ml) of fruit juice of *C. sinensis* (CS) and albendazole on the egg hatching of *H. bakeri* after 48 hours of incubation. Values are mean (± SEM)

Larvicidal activity of *C. aurantifolia* fruit peel extracts

The fruit juice of *C. sinensis* also had significant ($p< 0.05$) concentration-dependent larvicidal activity against L_1 of *H. bakeri* (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 unlike what was observed at a lower concentrations (0.01 - 0.04 mg/ml) where the larvicidal effect of albendazole was significantly (P<0.05) different from that of the plant. The LC_{50} was 0.41 and 0.37 mg/ml for *C. aurantifolia* and albendazole, respectively.

Post-mortem worm counts

The mean worm counts and the deparasitization rates are shown in Table 1. The anthelmintic effect produced by *C. sinensis* at the dose of 800 mg/kg was not significantly (P>0.05) different from that of albendazole.

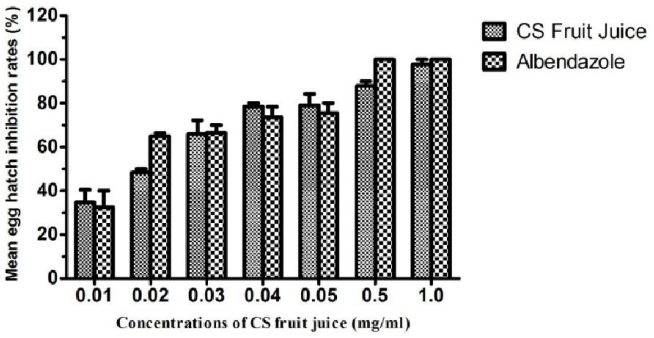

Figure 2.Effects of different concentrations (0.05 – 1 mg/ml) of the fruit juice of *C. sinensis* (CS) and albendazole on the L_1 Larvae of *H. bakeri* after 24 hours of incubation at 25° C. Values are mean (± SEM)

The anthelmintic effect produced by *C. sinensis* 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. sinensis* fruit juice 16-days post-infection with 150 L_3 of *H. bakeri*

Dose of *C. sinensis* Juice (mg/kg)	Mean worm counts (±SEM: n=3)	Deparasitization rates (%)
800	10.33±2.5	74.6
400	14.5±2.5	64.2
200	21.0±0.5	48.2
ABZ (10 mg/kg)	3.4±0.5	91.6±0.5
DW (5 ml/kg)	40.5±2.5	

DISCUSSION

There is a growing interest in the use of medicinal plants as anthelmintics in many parts of the world especially in the developing countries (Hammond et al., 1997). This is because plant based drugs are cheap and relatively safe. In this study, the results of the *in vitro* and *in vivo* anthelmintic study revealed that *C. sinensis* 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.

Higher concentrations of 1 mg/ml produced an anthelmintic efficacy *in vitro* that is not significantly (p>0.05) different from that of albendazole (standard drug). Usually, an increase in the concentration of drug often produce an increased efficacy until maximum concentration is obtained above which the drug will produce no efficacy. The reduction in worm burden caused by the fruit juice of this plant was also dose dependent. At 800 mg/kg, the anthelmintic activity of the plant was significant (i.e. above 70 %). *In vivo* anthelmintic studies are important in order to determine the efficacy of a potential drug on the actual subject. However, current trends tend to discourage such research as ethical issues with respect to animal rights are gaining ground. It is pertinent to note that *in vitro* anthelmintic study may have a remarkable result but may not be or may be less efficacious when tested *in vivo* on

animals as seen from this research. One of the major factors affecting the efficacy of plant extracts in *in vivo* anthelmintic studies is the route of administration. Most plant extracts screened against helminth parasites *in vivo* were administered orally. When medications are administered orally, the bioavailability generally decreases either due to incomplete absorption or first pass effect. This however, varies from animal to animals (Riviere and Papich, 2009).

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). It is also 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., 2007). *C. sinensis* 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). 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 ingredients with ovicidal and larvicidal action (Ademola and Ellof, 2011).

Conclusion

From the above results, we concluded that the fruit juice of *C. sinensis* possessed anthelmintic activities both *in vitro* and *in vivo* and thus, justify the traditional claims.

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