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Research Article

ANTHELMINTIC ACTIVITY OF EXTRACTS OF CITRUS AURANTIFOLIA (CHRISTM) FRUIT PEELS AGAINST EXPERIMENTAL HELIGMOSOMOIDES BAKERI IN MICE

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ABSTRACT

Citrus aurantifolia (Rutaceae) is commonly known as Lime orange. The plant is widely used in African and Asian traditional medicine. This present study was aimed at evaluating the *in vivo* anthelmintic activity of the extracts of *C. aurantifolia* fruit peel against *Heligmosomoides bakeri* (nematode) as a model. The crude methanol extract (CME) was partitioned using solvents of different polarities into ethylacetate (EE), butanol (BE) and aqueous methanol extract (AME). The median lethal doses (LD50) of the extracts were determined in mice. Seventy mice infected with *H. bakeri* were randomly allocated into 14 groups of 5 mice each and were treated with CME, AME, EE and BE at doses of 250, 500 and 1000 mg/kg. All treatments were administered orally on the 16^{th} , 17^{th} and 18^{th} day post infection. Anthelmintic activity was assessed by comparing the worms recovered from the treated groups with the non-treated control group. At the dose of 1000 mg/kg, BE, CME, EE and AME caused a deparasitization rate of 79, 75, 75 and 51 %, respectively. CME, BE and EE produced a significant (p<0.05) deparasitization rate. This study demonstrated that *Citrus aurantifolia* fruit peels possess anthelmintic activity that might be caused by one or more of the secondary metabolites contained in the plant.

Keywords: Citrus aurantifolia fruit peels, Heligmosomoides bakeri, Anthelmintics, Plant extracts, Acute toxicity.

1. INTRODUCTION

Parasitic diseases have been of great medical concern over the years as they are considered to cause severe economic losses to livestock production [1]. Among the parasitic diseases, helminthosis poses a major constraint to livestock production globally especially in the tropics and subtropics [2]. In the developed world, the major impact of helminthosis results probably from the cost of controlling the disease, while in the developing countries like Nigeria, the disease causes direct production losses in livestock [3]. The production losses result from severe morbidity and mortality in livestock caused by the disease. Helminthosis is severe in the tropics especially in Nigeria during the wet season when the infection rate is high up to 100 % in cattle but the infection persist throughout the year in small ruminants [4]. These high infection rates prevent livestock from attaining the maximum performance rate [5].

In Nigeria, the major control strategies adopted against helminthic infections is chemotherapy. However, the treatment of helminth diseases using modern drugs is accompanied by widespread toxicity and resistance development by the parasites against the drug.

Citrus aurantifolia (*C. aurantifolia*) is a polymembranous plant cultivated in many countries of the world [6]. The plant

has been used traditionally to treat several diseases such as cough, sore throat, malaria, dysentery, colic and bacterial, fungal, viral and parasitic infections [7]. Citrus fruit peels have long been a source of herbal products especially in Chinese medicine [8]. Large amounts of citrus processing waste are left after the processing of citrus fruits for juice and jam production [9]. The use of citrus peels extracts for the control of helminth parasites will not only provides a cheap alternatives to chemotherapy but also reduces impact on environment [10].

This present study aimed at evaluating the *in vivo* anthelmintic efficacy of the extract of *C. aurantifolia* fruit peels in mice experimentally infected with *Heligmosomoides bakeri* (a murine adapted trichostrongylid) commonly used as model for anthelmintic screening [11].

2. MATERIALS AND METHODS

2.1. Experimental Animals

Apparently healthy albino mice (*Mus musculus*) of both sexes aged between 8 to 10 weeks were bred in the Animal House, Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, were used in this study. The number of animals used was approved by the university ethical committee. The mice were allowed to acclimatize to laboratory conditions for two weeks. The mice were maintained on standard feed. Water was 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 consists of five mice which were identified by marks on their tails using permanent markers of different colours. The experiment was done in accordance with the animal use ethics of the institution.

2.2. Plant Collection, Identification and Processing

C. aurantifolia fruits were collected from cultivated plants in Kogi State in the month of January, 2014. Samples of the fruits, leaves and flowers of the plant were identified at the Herbarium, Department of the Biological Sciences, Ahmadu Bello University, Zaria by a taxonomist (U.S. Gallah). A sample of the plant was deposited and given a voucher specimen number of 990. The fruits were washed, manually peeled and dried under shade to prevent heat destruction. The dried fruit peels were made into powder using wooden mortar and pestle. The powdered plant material was extracted by maceration with methanol in the ratio of 1:3 w/v in a separatory funnel as described by Handa et al. [12]. Thereafter, the extract was evaporated to dryness over water bath at 50-60 °C. Seventy grams of the CME was dissolved in 700 ml of distilled water and serially partitioned with ethylacetate and butanol at the ratio of 1:1 v/v using a separatory funnel. After evaporating the solvents, the portions were subsequently referred to as crude methanol extract (CME), ethylacetate extract (EE), butanol extract (BE) and aqueous methanol extract (AME) and were tested for anthelmintic activities in mice experimentally infected with *H*. bakeri.

2.3. Phytochemical test

The CME, EE and BE were subjected to phytochemical tests using standard techniques described by Tiwari et al. [13] to detect the presence of metabolites.

2.4. Acute toxicity studies

Acute toxicity studies to determine the median lethal dose (LD_{50}) were conducted using the method described by Lorke [14] and modified by Jegede et al. [15]. Briefly, the mice were divided into 5 groups of 3 mice each. The aqueous solution of the crude methanol extract was administered through the oral route. The mice in groups 1, 2, 3 and 4 were given the extracts orally at the dose of 10, 100, 1000 and 5000 mg/kg, respectively. The fifth group received distilled water at 5 ml/kg (maximum volume administrable to rats/mice at a time) [16] and served as the control. The mice were observed for 72 hours for signs of toxicity. The animals that survived were further monitored for two weeks. Thereafter, the mice were autopsied.

2.5. Parasite

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

2.6. Experimental infection of mice

Seventy mice were infected orally with about 200 L_3 of *H. bakeri* contained in 0.2 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 20 minutes and faeces 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.

2.7. In vivo Anthelmintic Screening of the Extracts

The *in vivo* anthelmintic trials of the extracts of *C. aurantifolia* fruit peels were conducted as described by Enejoh et al. [17]. Seventy mice infected with *H. bakeri* were randomly allocated into 14 groups of 5 mice each. Groups 1-3, 4-6, 7-9, 10-12 were treated with crude methanol extract (CME), aqueous methanol extract (AME), ethylacetate extract (EE) and butanol extract (BE), respectively. Each extract was tested at doses of 250, 500, 1000 mg/kg. Group 13 and 14 were treated with albendazole (7.5 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.

2.8. 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 Ngongeh and Fakae [18] with little modifications was employed for the worm count. The entire length of the small intestine of each mouse was removed and cut open longitudinally with a pair of fine surgical scissors. The adult worms were recovered by suspending the small intestine in normal saline. The solution was incubated at room temperature (25°C) for 20 hours to enable the migration of the worms into the solution. The solution was centrifuged and the supernatant discarded. The sediment containing the worms was poured into a Petri dish and then observed under a stereomicroscope. The adult worms seen were counted and recorded. Furthermore, the intestines removed from the normal saline earlier were placed in 10 % formalin for one hour and then observed under the stereomicroscope for counting of adult worms that did not migrate into the normal saline.

The percentage deparasitization was calculated using the formula described by Suleiman et al. [19]:

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

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.

2.9. Data Analysis

Results obtained were expressed as mean \pm standard deviation (\pm SD). Analysis of variance (ANOVA) using GraphPad Prism Version 5.0 was used to compare the anthelmintic effects of the different extracts of *C. aurantifolia* fruit peels 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.

3. RESULTS

3.1. Extraction Yield of Plant Material

The yield and colour of the crude extract and different portions obtained from the pulverized *C. aurantifolia* are presented on Table 1.

Table 1: Yield and colour of Semi-solid portions of C.aurantifoliafruitpeelsextractextractaftersolventpartitioning

Extracts	Yield	Percentage	Colour
	(gm)	yield	
Crude methanol	110	13*	Brown
Ethylacetate	4.7	6.7	Brown
Butanol	17.5	25	Brown
Aqueous methanol	30.7	43.9	Light brown

*Percentage yield of crude methanol extract from the pulverized C. aurantifolia fruit peels

3.2. Phytochemical screening

The preliminary phytochemical screening of *C. aurantifolia* fruit peel extracts showed that CME, EA and BE contained carbohydrate, glycosides, steroids, flavonoids, phenol, alkaloids, saponins, triterpenes and condensed tannins. However, AME contain all the listed phytoconstituents except alkaloids.

3.3. Acute Toxicity Study

The crude methanol extract of *C. aurantifolia* did not produce death in treated animals up to a dose of 5000 mg/kg. Gross and histopathological examination of animals that died and those euthanized at the end of the study did not showed any organ pathology at any of the treated doses.

3.4. Post-mortem worm counts

The mean worm counts and the deparasitization rates are shown on Table 2. There was no significant (p>0.05) difference in the worm counts of mice treated with CME, AME, EE and BE at 250, 500 and 1000 mg/kg. However, deparasitization produced by albendazol (treated control) was significantly (P<0.05) different from all treated groups.

Table 2: Mean Worm Counts (\pm SD) in mice treated with varying doses of *C. aurantifolia* fruit peel extracts 16-days post-infection with 200 L₃ of *H.* bakeri

Treatments	Doses	Mean worm	Percentage (%)
	(mg/kg)	counts	deparasitization
	0 0	(±SD: n=5)	-
CME	250	15.2 ± 7.36^{d}	62
	500	11.6 ± 3.78^{b}	71
	1000	10.0 ± 2.45^{b}	75
	250	$26.2 \pm 4.15^{\circ}$	35
AME	500	$22.6\pm6.27^{\rm c}$	44
	1000	$19.8 \pm 3.83^{\circ}$	51
BE	250	14.8 ± 4.72^{d}	63
	500	12.4 ± 2.30^{b}	69
	1000	$8.6 \pm 3.85^{\mathrm{b}}$	79
EE	250	11.6 ± 3.21^{b}	71
	500	11.0 ± 4.85^{b}	73
	1000	10.2 ± 3.96^{b}	75
ALB	10	1.5 ± 1.29^{a}	96
DW	5ml/kg	$40.0 \pm 4.35^{\circ}$	0

Means with different superscript letter (a, b, c, d, e) differ significantly (p<0.05) from one another.

CME = *crude methanol extract of C. aurantifolia fruit peels.*

AME = aqueous methanol extract of C. aurantifolia fruit peels.

BE = butanol extract of C. aurantifolia fruit peels.

EE = ethylacetate extract of C. aurantifolia fruit peels.

ALB = albendazoleDW = distilled water.

4. DISCUSSION

The study showed that the extracts of *C. aurantifolia* fruit peels were relatively non-toxic to mice when given per os at doses upto 5000 mg/kg. This showed to that the plant is relatively safe [20]. Similarly, Gross and histopathological examination of treated mice revealed no observable lesions on different organs of the treated mice. This result which showed that the extracts were non-toxic agrees with findings by Chunlaratthanaphorn et al. [21] who reported the non-toxic effect of water extract of *C. aurantifolia* root in both acute and subchronic toxicity studies in rats. Similarly, subchronic toxicity study on a related species; *Citrus aurantium* indicated a very low toxic effect of the plant in mice [22]. However, acute toxicity studies on the fruit juice of *C. aurantifolia* showed that the fruit juice is toxic to rats when given at doses above 3.5 g/kg [23]. In vivo anthelmintic study showed that CME, BE and EE possesse significant anthelmintic activity as they were able to cause deparasitization rates of 70 % and above especially at the dose of 500 to 1000 mg/kg. AME did not show a significant (p>0.05) deparasitization rate compared to CME, BE and EE. This is similar to the findings of Abdeqader et al. [10] where the aqueous extract of *Citrus* peel had the least anthelmintic activity.

Phytochemical tests revealed the presence of alkaloids, glycosides, tannins and flavonoids in the extracts of C. aurantifolia. Tannins are polyphenolic compounds and have similar chemical properties with some synthetic phenolic anthelmintics like niclosamide, oxyclosanide and bithionol which were shown to interfere with the energy generation in helminths by uncoupling oxidative phosphorylation. It is possible that tannins contained in the extracts of this plant produced similar results. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite and cause their death [24]. Alkaloids produced anthelmintic effect by acting on the central nervous system of the worms and causing their paralysis [25]. Limonene is a compound isolated from the Citrus fruit peels. Limonene has been shown to have anthelmintic activity against Haemonchus contortus in ruminants. The mode of action of limonene is unknown; however investigations suggested that it has inhibitory effects on nematode growth and also interfere with parasite enzymes [26]. The anthelmintic activity produced by the extracts could be due to one or more of the phytoconstituent present. It has been shown that the combine presence of the chemical constituent may enhance their pharmacological action [27, 28]. CME, BE and EE contained similar phytochemicals, perhaps that is why their anthelmintic efficacies were not significantly different from one another.

It can be concluded from this study that *C. aurantifolia* fruit peels has promising potential as an anthelmintic agent. A full investigation into the anthelminthic activity of the plant in target animals is therefore recommended. Moreover, there is the need to conduct detailed toxicological studies of the extract in both laboratory and target animal species to justify the clinical investigation of the plant extracts.

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