Jewel Journal of Medical Sciences (2021) 2:2: 146-155.

PRINT ISSN: 2756-4371
ONLINE ISSN:2756-4290,
https://doi.org/10.56167/jjms.2021.0202.18

Original Article

Invivo Anti-schistosomal Activity of Methanolic Extract from *Azadirachta Indica* (Neem) Seed Kernel in Experimentally Infected Wister rats.

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Abstract

Background: Schistosomiasis is one of the neglected tropical parasitic diseases of socioeconomic and public health importance and there is need to search for a potent anti-schistosomal drugs from among our rich medicinal plant population.

Objective: The objective of the study was to determine the *in vivo* anti-schistosomal activity of methanolic extract from *Azadirachta indica* seed kernel in experimentally infected Wister rats.

Methodology: Forty (40) Albino rats were randomized into eight (8) groups each of five rats to include the treatment and control groups. Thirty-five (35) Rats were infected each with 150 cercariae and treated orally with varying doses of methanolic extract of *Azadirachta indica* seed kernel at doses 400mg/kg, 600mg/kg, 800mg/kg and 1000mg/kg bodyweight of the animals at eight weeks post-infection. Praziquantel was used as positive control and infected untreated group was used as negative control. Mean worm recovery as well as percentage reduction were used indicator of drug activity relative to the infected untreated control groups.

Result: The extracts showed significant dose dependent percentage worm reduction and mean worm recovery count at various doses ranging from 21±0.80 to 5.60±0.76 and percentage worm reduction ranging from 78.3% to 94.2%. Anti-schistosomal activity of methanol extract of *Azadirachta indica* seed-kernel was confirmed to be dose dependent. The evidence of anti-schistosomal activity of the extract was supported by reduced pathological changes (induced by the infection).

Conclusion: Our research showed that methanolic extract of neem seed kernel has anti-schistosomal activity and is effective in reducing worm burden in rats after schistosomiasis infection. The plant may be considered if standardized to fight the Schistosome infection in humans.

Keywords: Azadirachta indica, Neem, Praziquantel, Schistosomiasis, Worm recovery, Worm reduction.

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Introduction

Helminthiasis, also known as helminth infection, is the most prevalent worm-borne infection in humans and other mammals, with a large global population. It can be seen in a variety of tropical and subtropical regions. According to reports, they spread the bulk of human parasite infections in poor countries[1]. Schistosomiasis is a parasitic disease caused by Schistosome trematodes (flukes) that live in the bloodstream. Schistosomiasis, also known as Bilharzia, is one of the most common parasite diseases that governments around the world ignore[2,3]. In tropical and subtropical countries, it is second only to malaria in terms of socioeconomic and public health importance[3], and it is common in poor populations lacking access to portable water and proper sanitation. In 2015, the World Health Organization (WHO) projected that 240 million individuals were afflicted with Schistosomiasis worldwide[3]. It is endemic in 78 countries, with over 90% of cases occurring in Sub-Saharan Africa, where prevalence rates in indigenous populations can surpass 50%[3]. People die at a rate of 280,000 each year[2]. In addition, it is responsible for severe morbidity, anemia, considerable development retardation as well as negative educational, and nutritional impacts not only in children but also in adults living in endemic areas[3]. Human Schistosomes are divided into three types: *Schistosoma haematobium* causing urine schistosomiasis, *Schistosoma mansoni* causing intestinal schistosomiasis, and *Schistosoma japonicum* also causing intestinal schistosomiasis.[4] Only *Schistosoma mansoni*, whose intermediate host is the freshwater snail *Biomphalaria pffeiferi*, and *Schistosoma haematobium*, whose intermediate hosts are the freshwater snails *Bulinus trancatus* and *B. (Physopsis) globosus*, infect humans in Nigeria[5].

In Africa and the Middle East, both *Schistosoma haematobium* and *Schistosoma mansoni* are found, but only *Schistosoma mansoni* is found in America[6], with *S. japonicum* found in Asia, particularly in the Philippines, Japan, and China.

The disease continues to be a big issue in low-income nations, particularly in areas with poor sanitation, with the disease's mode of transmission limited to persons who come into contact with contaminated waters in endemic areas[3,5].

Symptoms of schistosomiasis are caused as a result of the host's reaction to the worms' eggs. Abdominal pain, diarrhea and blood in the stool are as a result of intestinal schistosomiasis. Liver enlargement is common in progressive cases and it's frequently associated with ascites and splenomegaly. The classic sign of urogenital schistosomiasis is hematuria (blood in urine). In advanced cases, fibrosis of the bladder, ureter and kidney damage sometimes do occur, with possibility of bladder cancer in the later stages[7,8]. In women, urogenital schistosomiasis may occur with genital lesions, vaginal bleeding, noodles of the vulva and pain during sexual intercourse. While in men, pathology of the seminal vesicles, prostate and other organs do occur[3,5,7,8]. This disease may also have other long-term irreversible consequences, including infertility. Other disease manifestations include eosinophilia, fever and fatigue[3]. In children, schistosomiasis can cause anaemia, stunting and a reduced

ability to learn, although the effects are usually reversible with treatment. Chronic schistosomiasis may affect people's ability to work and, in some cases, can result in death[9].

Adult worms colonize human blood vessels for years, successfully eluding the immune system while excreting hundreds to thousands of eggs daily either through the urine or stool or successfully trapped in nearby tissues[6]. Trapped eggs induce a different immune mediated granulomatous response that causes local and systemic pathological effects ranging from anemia, growth stunting, impaired cognition and decreased physical fitness to organ-specific effects such as severe hepatosplenism, peri-portal fibrosis with portal hypertension and urogenital inflammation and scarring[5,6].

The standard diagnosis for active schistosomiasis is the presence of viable eggs in urine (*Schistosoma haematobium*), faeces (*S. japonicum, S. mansoni*), or tissue biopsies[6]. However, diagnostics that are more sensitive are needed in both the field and clinics, and combined environmental and health-care management will be needed to ensure elimination of schistosomiasis as a disease[5].

Presently the control of schistosomiasis and public health measures in endemic areas consists of treatment once every 1 to 2 years with chemical anti-helminths; mostly Praziquantel[10].

Schistosomiasis treatment is based on the use of three drugs. These are Praziquantel that is effective in the treatment of all forms of schistosomiasis, Oxamniquine used to treat intestinal schistosomiasis, and Metrifonate which is effective for the treatment of urinary schistosomiasis[10,11]. Currently, the main strategy for managing the parasite in humans relies on the administration of the drug; Praziquantel (PZQ)[4]. This is the drug of choice in the treatment of schistosomiasis, mainly because it is active against most of the known *schistosome* species with little or no adverse side effects[11], the main limitation which restricts its use in many developing countries is the cost.

Although, PZQ has been documented to have least side effects in the control of schistosomiasis, using PZQ at a population level faces some limitations[2]. One of such limitations is the drug-resistant strains of the parasite that have been reported in endemic areas[4].

Therefore, the scientific community is continuously searching for some alternative drugs by screening botanical and chemical compounds for their potential activity as anti-schistosomal agents. Many reports suggest the use of medicinal plants to be a promising source for anti-schistosomal activity[12]**.** The plant kingdom has continued to play an important role in the discovery of novel and useful phytochemicals that can be utilized in modern medicine[4]. The screening of medicinal plants for their active compounds has become very important as that may serve as talented sources of data bank for antibiotic and other ailment prototypes[12].

Azadirachta indica 'Neem tree' has been known for centuries and is a member of the mahogany family, called '*Meliaceae*'. It is widely distributed in the arid tropical and sub-tropical zones of Africa, Asia, Australia and the Americas. It has been shown to have anti-fungal, anti-bacterial, anti-viral, and anti-diabetic properties. It is used also to treat various diseases and disorders ranging

from malaria to bad teeth. Almost all parts of the tree have medicinal benefits[13].

Material and Methods

Plant Collection, Preparation and Extraction

Azadirachta indica seeds were collected and authenticated at the herbarium unit of Botany Department, Gombe State University with a voucher number GSUH 30. It was prepared by washing, drying under the shade and gently crushed to remove the kernel. The kernels were then ground into fine powder and extracted using methanol.

Collection, Screening of Snails and Shedding of Cercaria

Snails collected from Dadin Kowa were bred and maintained as described by Mandsen[14]. They were identified as either *Bulinus physopsis globosis* or *Biompleria pferfferi* using appropriate keys described by Kristensen[15].

The snails were maintained in the aquaria and were screened for infectivity by cercaria by use of a strong light bulb that emitted 160watts of energy for 2 hours. The infected snails were separated from the non-infected ones by their ability to shed cercaria. Snails found to be infected were further exposed to the light to enhance shedding of cercaria. Longiforcate cercaria of *Schistosomes* were identified as the cercarial type shedded by these snails. The shedding process was done by transferring the infected snails into 10ml beaker containing original water from the snail's pond with at least five (5) snails per beaker and exposed to lamp of 100watts capacity for between 5-30minutes to enhance full shedding of cercaria. The cercarial suspensions were pooled together into a plastic bucket. A dropping pipette was used to take a portion each of the cercarial suspension and counted in Petri dishes under a dissecting microscope. A volume containing estimated 150 cercariae for challenge to each rat was counted and put into separate water in a bucket and exposed to sun before the infection process.

Definitive Host of Schistosomiasis

Healthy albino rats (40) free of ailments were used as definitive hosts of schistosomiasis. They were housed at temperature of about 25ºC and fed on rat's pellets and water. Their faecal matter and urine were examined through the routine method for eggs of parasite. Thirty-five (35) healthy albino rats were infected separately each with about 150 cercaria obtained from wild infected snails in water contained in a bucket by using modified tail immersion method. They were kept for 8weeks in labelled cages and observed on a daily basis for any sign of infection. During that period the changes in urine colour, weight, fur (skin), and faecal samples were observed and recorded. Faecal samples collected were observed using both floatation and sedimentation methods for eggs recovery. After 6 weeks post-infection, two of the infected rats were sacrificed to ascertain the presence of infection.

At the end of 8 weeks post-infection, infected rats were grouped into seven groups of five rats each. The groups I-IV were treated with 400, 600, 800 and 1000mg/kg body weight of Neem extract respectively. Group V were given 600mg/kg of PZQ tablets and group VI were left untreated to serve as negative control. The negative control received only water and their normal feed. Maximum tolerated dose of the extract was determined as described by

Basil[16] using 5 rats (group VII) while group VIII were the uninfected control. During and after treatment, the animals' physical behaviours, urine colour and faecal samples were observed and recorded. Treated animals were allowed a resting period of 3 weeks after which they were mercifully sacrificed. The organs were removed after anesthesia with chloroform and identified using method described by Manton[17] for both gross pathology and histopathology changes. Worms were recovered from the blood vessels of urinary bladder and the intestine by dissection method described by Olivier[18]. Worms recovered were compared between the different groups and results obtained were recorded.

Statistical Analysis and Presentation of Findings

ANOVA using the SPSS version 20 was used in the evaluation of results for the Mean worm reduction between the different groups of animals with a P-value less than 0.5 considered significant. Tables were used in the presentation of results for all findings.

Results

The results of physical observation for infected rats showed the successful establishment of *Schistosoma* infection. This was noticed by dark yellowish to brown coloured urine observed as from the 5th week post-infection. Some of the infected rats passed watery stools from the 6th week post-infection, with loss of fur in all the infected rats. Due to infection manifestation, the animals appeared dull, weak, and some lost their appetite, indicating successful establishment of infection. Animals treated with Praziquantel (PZQ) and Methanolic extract of the Neem (MNE) showed improvement as the watery stools passed out by the infected animals stopped on the 2nd day of treatment and by the 3rd day, normal coloured urine was passed and hair regeneration was also seen. Animals treated with PZQ and MNE showed an improvement in the general conditions of their bodies as they became active and were feeding well. This signified a successful treatment of Schistosome infection with PZQ and MNE.

At the end of the 6th week post-infection and treatment, faecal samples were taken from the rats and prepared using the routine methods of floatation and sedimentation. Microscopic examination of these samples showed that the treated animals were negative of the Schistosome parasites. Maximum tolerated dose of MNE was determined by using rats of the same age and weight in each of the treated groups. No death was recorded in the concentrations used. However, drowsiness was observed in some rats while others especially those treated with concentration of 1000mg/kg body weight showed some pathological changes

At the end of the 8th week post-infection, worms were recovered from both the urinary bladder, intestine and from the abdominal organs (the kidney, lungs, liver, and spleen). This showed a mixed infection with both species of *Schistosome.* They were counted and the results are expressed as mean ± standard error (S.E) as shown in Table 1. Tapeworms were also recovered from the intestine of the infected rats. However, the tapeworm burden also decreased with increase in concentration of MNE, whereas percentage worm reduction increased with increase in concentration.

Table 1: Mean worm recovery and percentage worm load reduction in different concentrations of MNE and PZQ

Group	Concentration (mg/kg)	Worm load Count (Mean ± S.E.M)	%Worm Reduction after treatment
I	400	21.00 ± 0.80	78.30
II	800	15.60 ± 0.85	83.90
III	1600	9.20 ± 0.87	90.50
IV	3200	5.60 ± 0.96	94.20
V	PZQ	3.20 ± 0.95	96.70

The results from gross pathology (Table 2) and histopathology showed that MNE has less pathological effects on the internal organs as evident in the uninfected treated control group (Group VIII). The animals treated with the highest concentration of MNE had no internal organs inflammation with none having moderate to severe granulomas.

Table 2: Percentage gross Pathology of livers of rats treated with different concentrations of MNE and PZQ

	Granulomas (%)				Adhesion (%)		Inflammation (%)	
	None	Few	Moderate	Severe	AP	AA	I	NI
I	0	20	40	40	80	20	80	20
II	0	40	60	0	60	40	60	40
III	0	60	40	0	20	80	40	60
IV	20	60	20	0	20	80	20	80
V	60	40	0	0	20	80	20	80
VI	0	0	40	60	80	20	80	0
VII	80	20	0	0	0	100	0	100

Key: *UTC = Uninfected Treated Control, IUC = Infected Untreated Control, AA = Adhesions Absent, AP = Adhesions Present, I = Inflamed and NI = None Inflamed*

The probit mortality of different concentrations of MNE and PZQ was determined to calculate the percentage of organisms killed at certain concentration.
Results of probit mortality (Table 3) showed group V has the highest percentage worm mortality while group VI has the lowest percentage mortality. Groups treated with different concentrations of MNE has percentages ranging from 86.0 to 96.0

Table 3: Probit values of different concentrations of MNE and PZQ

Group	Mean no. of cercaria inoculated	Mean no. of worms recovered	Log10 conc	% Mortality	Probits
I	150	21	2.6021	86.0	6.08
II	150	16	2.7781	89.0	6.22
III	150	11	2.9031	92.0	6.40
IV	150	6	3.0000	96.0	6.75
V	150	3	2.7781	98.0	7.05
VI	150	97	-	53.0	5.08

Lethal concentrations of *Schistosoma* infected albino rats with methanol extracts of *A. Indica* seed kernel were estimated (Table 4) to know the concentration that would kill certain percentages (50, 95, and 99) of exposed animals. The probit mortality percentages of the test animals increase with increase in concentration indicating that the extract activity is dose dependent. The values of 17.21, 147.40 and 849.05 are the concentrations that will respectively kill 50, 90 and 99 percent of *schistosome* parasites in the test animals.

Table 4: Result for Lethal Concentrations (LC) of Schistosoma infected albino rats with methanol extracts of *A. Indica* seed kernel.

Lethal Concentration	Log10 Conc.	Lethal dose estimate
LC50	5.00	17.21
LC90	6.28	147.40
LC99	7.33	849.05

Discussion

Worm count (worm burden recovered and percentage worm reduction) is the most direct way of determining anti-schistosomal efficacy of a drug. In this experiment, worms were recovered from all the infected rats which indicated a successful establishment of *schistosome* in these rats. Worms recovered from the urinary bladder and that of the intestine is an indication of mixed infection with both species of *schistosome.* This could be attributed to the snails been used as source of infection were those from the wild and may be infected with both the species.

Tapeworms recovered from the experimental animals whose burden decreased with increase in concentration of the extract suggest that the methanolic extract of Neem seed kernel is also effective against tapeworms.

The low worm recovery and high reduction in percentage worm recovery from rats treated with MNE indicated that the extract has anti-schistosomal activity (Table1) as evident by significant difference between the means and percentage worm recovery from treated groups and the untreated control group. The efficacy of the extract differs between different concentrations as seen by considerable increase in percentage worm reduction with increase in concentration of the plant. The results from our study are similar with the work of Musili *et al*.[19] who demonstrated that (*A. indica)* has appreciable anti-schistosomal activity. They also a reported dose-dependent % worm reduction at different doses of *A. indica* and *Ekebergia capensis* in rats infected with *Schistosoma*. A dose dependent percentage worm reduction in mice infected with *schistosome* and treated with doses of *A. indica* and *Mirazid* was reported in a study by Taher *et al*. [20] who found that ingestion of Mirazid and *A. indica* plant extracts orally or their combination to infected mice was effective in reducing worm burden and egg count when compared with infected untreated mice, indicating their effective anti-schistosomal action. Another study reported a 100% mortality of worms in an *in-vitro* assessment of effect of *A. indica* leaf extract on resistant *Staphylococcus aureus* biofilm formation and *Schistosoma mansoni*[21]. It was also reported that a high worm burden reduction using *Maytenus senegalensis, Terminalia glaucescens* and *Colocassia antiquorum* extracts in rats[22]. Crude aqueous extract of ginger against *Schistosoma mansoni* was reported to have a schistomicidal activity[23]. The findings of this study is also in agreement with Muema *et al*[12] who reported decrease in worm densities with increase in concentration of extract of 3 plants species (*Malus domestica, Allium cepa,* and *Citrus limon*). Dong *et al*[24] determined the anti-schistosomal activity of *Artemisini*

derivative of PZQ in rats and reported high % of worm reduction rate in different stages of worms. There was a high reduction in worm burden and anti-schistosomal activity using aqueous ginger extract against *Schistosoma mansoni* in rats infected with cercariae[25].

The results of this study however, contradicted results from Adamu *et al*. [26] who reported little or no anti-schistosomal properties of extracts of *Jatropha curcas (L)* on *Schistosoma mansoni* infection in mice.

From the results, the extract reduced some of the pathological changes induced by Schistosomes as seen in the different groups treated with the extract (Groups I to IV) which had lesser changes as compared to uninfected control (Group VIII). These results is in tandem with the work of Hamza *et al*[27] who did a histopathological studies on mice experimentally infected with *Schistosoma mansoni* and treated by *A. indica* (Neem) and *Mirazid* (MZ) and found a marked decrease in liver fibrosis in groups of mice. Also some pathological changes observed on some laboratory animals treated with some local plants extracts showed that the therapeutic nature of the extracts and PZQ reduced the pathological conditions of infected animals. The mortality rate of the worms closely fitted the Probit distribution which showed a significant increase in the mortality rate following exposure to different concentrations of the extract.

Conclusion

It can be concluded that methanolic extract of neem seed kernel has anti-schistosomal activity and is effective in reducing worm burden in rats after exposure as is evidenced from percentage worm reductions with increase in the concentration of the plant extract. This showed a promising result which may be considered if standardized to help fight the Schistosome infection in humans.

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