



Evaluation of Anthelmintic Activity of Different Extracts of *Azadirachta indica* Flower/Buds in Indian Earthworm

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Abstract

Helminthiasis is a parasitic infection affected widespread among variety of living creatures amongst them humans and animals are mostly affected and the cause is helminths. The strategy of the disease is very high specifically in developing countries and the basic reason is inadequate sanitary conditions and poor management practices. *Azadirachta indica* (*A. indica*) flowers/buds extracts were prepared with different solvents in increasing order of polarity (diethyl ether < ethanol < aqueous). Stock solution of the extract of *A. indica* was prepared in distilled water at the concentrations of 20, 50 and 100 mg/ml and used against test worms. The present study was aimed to evaluate the biological activity with special focus on anthelmintic activity of different extracts of *A. indica* against *Pheretima posthuma* and *Tubifex tubifex*. The time of paralysis and time of death were studied and the activity was compared with Albendazole as reference standard and distilled water was used as control. The alcohol and aqueous extract at doses 50 mg/ml and 100 mg/ml exhibited significant anthelmintic activity as evidenced by decreased paralyzing time and death time.

Keywords: Anthelmintic; Albendazole; *Azadirachta indica*; *Pheretima posthuman*

Introduction

Neem (*Azadirachta indica*) is a native plant from India, belongs to the family *Meliaceae*. It is mostly available in each and every corner of the world and can be grown in tropical and subtropical countries. As per data the plant is one of the most versatile medicinal plants in the world, the plant has both broad spectrum application in different fields like agricultural and medicinal areas. Neem has excellent medical benefits with various biological activities [1]. Neem has been popular for centuries and is used traditionally in different types of diseases. From literatures it has been observed that Neem can be used to treat multiple diseases the most peculiar thing is that almost all parts of the plant has been utilised [2]. All parts of the neem tree- like leaves, fruits, bark etc. The other parts like flowers, seeds and roots have also been used in variety of disorders like treatment of inflammation, infections, fever, skin diseases and dental disorders etc. Further as per the

scientific evidences the medicinal utilities of the plant have been described especially neem leaf. Neem leaf and its variety of constituents have been demonstrated to exhibit different disorders like immunomodulatory, antihyperglycaemic, anti-inflammatory, antifungal, antiulcer, antimalarial, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties [3]. Several studies report that neem leaves have antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, immunomodulatory, anticancer, wound healing, nephroprotective, hepatoprotective, antimalarial, antifungal, antibacterial, neuroprotective, antifertility and contraception [4,5].

Based on the literatures it has been found that the plant having variety of constituents. On phytochemical screening, it has been observed that the leaves extract of Neem contains various active compounds like steroids, alkaloids, flavonoids, saponins, terpe-

noids, glycosides, tannins, and phenolics [6]. *In vivo* studies have shown that neem leaf extract decreased sperm concentration, motility, viability, and increased sperm abnormalities. Several active compounds of neem has proven the antifertility effect [7,8]. Neem leaves extract caused changes in different level of hormones like Follicle Stimulating Hormone (FSH) and testosterone, due to the modification the plant could disrupt spermatogenesis, which may further affects the sperm quality and male fertility capacity [9]. Changes in hormones like FSH, testosterone the sperm quality are affected which is the standard criteria that serve as antifertility agents. Thus, neem leaves ethanol extract has the potential to be developed as a male contraceptive candidate.

Neem is a fast- ever growing tree basing on the physical and chemical parameters that can reach at optimum height of 15–20 metres i.e. (49–66 ft), and occasionally it may grow upto 35–40 metres (115–131 ft). It is evergreen, but in severe drought condition it may shed most or nearly all of its leaves. The branches are wide as like other trees and spreading in near areas as per the space is concerned . It has been reported that Neem oil also use to control various types of skin infections. The Bark, root, flower leaf, and fruit together cure different diseases in human like blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and pthysis [10]. Young leaves of the plant and young flowers of the plant are commonly consumed as a bitter tonic by the people for vegetable.

The flowers of the plant are also used, it has been reported that it is used for the treatment of fever. The flower extract of neem has been reported that it causes antioxidant property through *in vitro* free radical scavenging activity and can inhibit another mechanism like lipid peroxidation of disease like bronchogenic cancer cell line. Active compounds in the neem flowers which possesses so many activities are contain flavonoids such as rutin and quercetin. Flowers are a basic component of analgesic, curries, nectaries, soaps, stimulant [11].

Neem oil is especially used in various roles such as antipyretic, pain killer, anticholinergic, antihistaminic and as per the infection is concerned it is used as antihelminthic, antiprotozoal, antiviral, bactericidal, fungicides, insecticides, veterinary medicines, insect repellents, cosmetics, hair oils, contraceptives, lubricants, propellants, shampoos, soaps, toothpaste [12].

One more concern is the neem flowers are also known for improving digestive health and both buds as well as the flowers are used in Indian cooking. These are available in fresh, dry and powder form.

Helminths are generally restricted to tropical regions and cause enormous hazard to health and contribute to the prevalence of undernourishment, pneumonia, eosinophilia and anemia [13]. Anthelmintics are drugs used to expel parasitic worms from the body by paralyzing or killing them. It has a great importance in life of humans and life of animals in veterinary medicines. The gastrointestinal helminthes have become resistant to currently available anthelmintic drugs causing problem in treatment of helminthes diseases. Hence there is an increasing demand towards natural anthelmintics [14].

The Neem tree flowers between end of February to starting of May. In Odisha, people have used neem flower and buds for cooking as well as to treat different diseases. Basing on the background the present study has been planned to evaluate the potential role of *A. indica* flower and bud extracts as its *in vitro* anthelmintic activity. This is the first investigation of the neem flower/bud extract in different solvents for anthelmintic activity.

Materials and Methods

Plant material

Fresh *A. indica* flower and bud collected from Laxmiposi, Bari-pada, Mayurbhanj District. The plant was identified, confirmed and authenticated by taxonomist and local people of Baripada.

Preparation of extracts

The air dried plant materials was powdered and extracted successively with the different solvents. First the powered was extracted with petroleum ether in a soxhlet apparatus. After that the defatted materials of the drug was subjected to alcohol and aqueous extraction for a period of 6-7 d and filtered. All the filtrates were subjected to evaporation by using dryer like Rotary Evaporator instruments to dryness, till the thick paste remained in the evaporator. However, it was kept in a refrigerator below 4°C till the experimental study. Extract was further facilitated to identify the qualitative chemical investigation of different phytoconstituents such as Alkaloids, Flavonoids, Tannins, etc. by using different methods. Samples for in-vitro study were prepared by dissolving and suspending different extracts (Ethanol and aqueous) in the

distilled water to obtain a good stock solution. The strength of the solution is 100 mg/ml. From this prepared stock solution, different concentration of test substances are prepared the working dilutions were prepared to get concentration range of 20, 50, and 100 mg/ml.

Worm collection and authentication

Indian earthworms *Pheretima posthuma* (Annelida) were collected at Baripada, Mayurbhanj District specifically from the water logged areas near to Bus stand. They were properly washed with running tap water for the removal of the adhering dirt in the body. Aquarium worms i.e. *Tubifex tubifex* (Annelida) were collected from Bhubaneswar, Odisha.

Qualitative phytochemical analysis [15]

For qualitative phytochemical analysis standard chemical methods were performed.

- **Glycosides:** For glycosides 1 mL of freshly prepared 10% KOH was added to 1 mL of extract. The presence of compound like glycoside was confirmed by the method i.e. formation of brick red precipitates.
- **Saponins:** For saponins, frothing test was performed in which 2 ml of the extract was vigorously shaken in the test tube for 2 minutes. Presence of frothing indicated saponins.
- **Steroids:** Steroids were identified by adding 5 drops of concentrated H_2SO_4 to 1 mL of the extract in a test tube. Red coloration indicated the presence of steroids.
- **Triterpenes:** For triterpenes, 5 drops of concentrated H_2SO_4 were added to 1 mL of extract. Appearance of blue green colour indicated the presence of triterpenes.
- **Flavonoids:** Presence of flavonoids was tested by adding 1 mL of freshly prepared 5% $AlCl_3$ solution to 1 mL of extract. Yellow coloration indicated the presence of flavonoids.
- **Phenolics:** For phenolics, two drops of 5% $FeCl_3$ were added to 1 mL of the extract in a test tube. Presence of greenish precipitate indicated the presence of compound like phenolics.
- **Alkaloids:** To detect the presence of alkaloids 0.2 gm of plant extract was warmed with 2% sulphuric acid in a test tube for 2 minutes. The mixture was filtered immediately in a separate test tube and few drops of the reagent named as Dragendroff reagent, when the same were added and observed for the presence of orange red precipitates, it indicates the presence of alkaloids.

Statistical application

The experiments were carried out in triplicate. All the results are reported as mean \pm standard deviation (SD).

Anthelmintic activity

The anthelmintic assay was carried out as per the method of Ajayieoba., *et al.* with minor modifications. The assay was performed *in vitro* using adult earthworm (*Pheretima posthuma* and *Tubifex tubifex*) owing to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings for preliminary evaluation anthelmintic activity [16].

The 20 ml formulations containing different concentrations of each extract (20, 50 and 100 mg/ml in distilled water) were prepared in a concentration gradient manner. In each container five numbers of worms (same type) were placed. The parameters like Time for paralysis was noted when no movement of any sort could be observed except the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water at 50°C. Albendazole (25 mg/ml) was used as reference standard while distilled water as the control.

Results and Discussion

The Neem (*Azadirachta Indica*) is a tropical fast growing tree. In India, this is called as 'pharmacy of the village'. Each part of the tree such as leaves, bark, fruits, flowers have been used for centuries in India for purposes like pharmaceuticals, cosmetics, agriculture, medicinal cures, insect repellents etc. From India the tree and the knowledge about its many uses and benefits has spread. The preliminary phytochemical analysis of different plant extracts evidenced the presence of multiple components in the extracts. The results revealed the presence of flavonoids, tannins, steroids, saponins, terpenes, and phenolic compounds in Table 1.

The results of anthelmintic activity of all the plant samples are summarized in Table 2 and 3. It is evident that ethanol and aqueous extracts of *Azadirachta Indica* exhibited anthelmintic activity in dose-dependent manner giving shortest time of paralysis and death with 50 mg/ml and 100 mg/ml concentration. The ethanol extract at 100 mg/ml caused paralysis of 12.8 min and time of death of 20.9 min., while aqueous extract revealed paralysis at two different doses i.e. 50 mg/ml and 100 mg/ml. In 100mg/ml

Table 1: Preliminary qualitative phytochemical analysis of the *Azadirachta indica* flower/buds extracts.

For various extracts of <i>A. indica</i>								
Test for	Tanins	Saponins	Fats and Oils	Alkaloids	Flavonoids	Coumarin	Terpenoids	Steroids
Type of extract								
Diethyl ether	+	-	+	-	-	-	-	-
Chloroform	+	-	-	+	-	+	-	-
Ethanol	+	+	-	+	+	+	-	+
Aqueous	+	+	-	+	+	+	-	+

(+) shows the presence of constituents, (-) shows the absence of constituents.

Table 2: Effect of different extracts of *Azadirachta indica* on anthelmintic activity on *Pheretima Posthuma*.

Group	Treatment	Concentration (mg/ml)	Time taken for paralysis (P) and death (D) of worms in minutes	
			P	D
I	Vehicle	DW	-	-
II	Albendazole	25	11.8 ± 2.52 ^c	17.1 ± 3.24 ^c
III	PEAI	20	3.8 ± 3.46	27.83 ± 2.13
IV		50	5.3 ± 3.48	22.9 ± 4.78
		100	6.6 ± 2.3	20.9 ± 5.34
V	MEAI	20	4.5 ± 1.6	26.7 ± 6.12
		50	7.9 ± 1.8	23.9 ± 3.4
VI		100	12.8 ± 3.1 ^c	20.9 ± 4.14 ^c
VII	AQAI	20	5.9 ± 2.2	24.5 ± 4.25
		50	10.6 ± 3.7 ^c	20.9 ± 5.6 ^c
VIII		100	11.4 ± 5.6 ^c	16.3 ± 3.8 ^c

Values are expressed in MEAN ± S.E.M of six animals. One Way ANOVA followed by Dunnet’s t-test. (F-value denotes statistical significance at *p < 0.05, **p < 0.01) (t-value denotes statistical significance at ^ap < 0.05, ^bp < 0.01 and ^cp < 0.001 respectively, in comparison to group-II).

Table 3: Effect of different extracts of *Azadirachta indica* on anthelmintic activity on *Tubifex* worms.

Group	Treatment	Concentration (mg/ml)	Time taken for paralysis (P) and death (D) of worms in minutes	
			P	D
I	Vehicle	DW	-	-
II	Albendazole	25	17.2 ± 3.6 ^c	27.1 ± 4.1 ^c
III	PEAI	20	84.8 ± 7.8	212.6 ± 8.4
IV		50	71.7 ± 6.8	202.8 ± 10.2
		100	62.6 ± 7.2	134.6 ± 8.6
V	MEAI	20	64.9 ± 5.6	126.8 ± 6.12
		50	44.2 ± 4.2	88.9 ± 8.2
VI		100	22.6 ± 4.1 ^c	40.6 ± 5.3 ^c
VII	AQAI	20	45.4 ± 4.2	115.4 ± 9.2
		50	32.9 ± 2.8 ^c	46.9 ± 4.2 ^c
VIII		100	20.5 ± 3.6 ^c	32.3 ± 4.1 ^c

Values are expressed in MEAN ± S.E.M of six animals. One Way ANOVA followed by Dunnet’s t-test. (F-value denotes statistical significance at *p < 0.05, **p < 0.01) (t-value denotes statistical significance at ^ap < 0.05, ^bp < 0.01 and ^cp < 0.001 respectively, in comparison to group-II).

the worm paralyzed at of 11.4 min and time of death of 16.3 min against the earthworm *Pheretima posthuma*. All the neem extracts showed the anthelmintic activity as per different parameters. The peculiar thing is that the extract posses the mortality rate at the same concentration during the six hours time period. This difference in activity is attributed to difference in type and quantity of different phytochemicals present in each plant extract.

In the present study the crude methanol and aqueous extracts were further partitioned using solvents of various polarity and the fractions were tested for anthelmintic activity.

In table 3, it has been observed that both alcohol and aqueous extracts at 100 mg/ml, played significant roles among all the extracts of *A. indica* against *Tubifex* worms.

The results can be considered significant since the extracts are crude samples with a number of compounds and can be a source of phytochemicals with anthelmintic activity comparable to standard drugs used. Although the rate of death of worms after each hour was different for different doses, at the end of six hour time period the rate of death of worms was same. The effect of extracts on the death of the worms, according to the result may be indicated as aqueous > alcohol extracts.

A number of studies are available for anthelmintic activity of tannins, alkaloids and flavonoids [13]. The presence of these phytochemicals may be responsible for the observed anthelmintic activity of plant extracts in present study. Tannins have been shown to interfere with coupled oxidative phosphorylation thus blocking ATP synthesis in these parasites. Tannins may also bind to the cuticle of the helminth's body surface making it immobile causing the parasite to become paralysed leading to its death [17]. The difference in activity of different plant extracts may also be due the difference in total phenolic and flavonoid contents in these extracts. Since the polar fractions contained a high phenolic and flavonoid content than the non-polar fractions this may explain the observed difference in the anthelmintic activity of these extracts.

Conclusion

Plants are one of the most important sources of medicines. The role of medicinal plants in promoting the ability of human health to cope with the unpleasant and difficult situations is well documented from ancient times till date all over the world. Medicinal

plants are rich in secondary metabolites which are potential sources of drugs and of therapeutic importance. There is increasing interest in the use of plant extracts as therapeutic agents. The study has shown that ethanol and aqueous extracts of *A. Indica* flower have significantly determined anthelmintic activity. But aqueous extracts of *A. Indica* shown most significant anthelmintic activity as compare to the ethanol extracts. And aqueous extract are shown the significant activity as compared to Albendazole. Further studies are in process to identify the possible Phytoconstituents responsible for anthelmintic activity.

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