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In vitro anthelmintic efficacy of ethno-medicinal plant *Annona reticulata* L. roots against Indian earthworms (*Pheretima posthuma*)

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The present study was envisaged to evaluate the ethno-medical claims of *Annona reticulata* L. roots for anthelmintic efficacy *in vitro* in comparison with the standard chemotherapeutic agent, albendazole on adult Indian earthworms (*Pheretima posthuma*). The efficacy was evaluated by monitoring gross visual motility, paralysis and mortality time and new software based tool, PASS (Prediction of Activity Spectra for Substances). Literature confirmed that roots of the plant are rich in aporphine alkaloids and acetogenins. Therefore, study includes investigation and correlation of the biological activity spectrum of the aporphine alkaloids and acetogenins using PASS with *in vitro* anthelmintic activity. *In vitro* anthelmintic trials of extract was conducted at 20, 40, 60 mg/mL. The extract was found to be effective at all concentrations but a more significant result was at concentration 60 mg/mL. This could be due to synergistic effect of both the phytoconstituents. However, exact mechanism of action need to be studied.

Keywords: Anthelmintic, *Annona reticulata*, Aporphine alkaloids, Acetogenins, PASS, Albendazole.
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Introduction

Helminthiasis is the condition associated with worm infestation. Around 2 billion people harbour these infections; in other words, worms infect more than 1⁄3 of the world's population. Three hundred million are severely ill due to worms and of those majority includes school-age children[1]. Although it is preventable and treatable still it is the major cause of death world-wide. In Africa alone, the deaths due to worms infection may be as high as 200,000 every year[2]. It is one of the major prevalent diseases in the world, particularly in the tropical countries. The factors like lack of adequate sanitary facilities and supply of pure water coupled with poverty and illiteracy responsible for the widespread nature of this disease in the developing countries[1, 3].

The treatment of these infections in the 21st century is mostly through the use of modern synthetic agents[4]. Anthelmintics are the drugs that either kill (vermicide) or expel (vermifuge) infesting helminthiasis. The gastro-intestinal helminthes lives a major challenge in treatment of helminthes diseases because they have become resistant to currently available anthelmintic drugs[4-6]. Because of the limited availability of drugs, high cost, development of resistance, chemical residue in milk and meat in host animals and toxicity problem the majority of world population depends on traditional remedies[4, 7-9].

It is estimated that more than 20,000 species of plants are used medicinally throughout the world for controlling diverse diseases[7]. Medicinal plants and fruits have been used by indigenous peoples for centuries in the treatment of a variety of problems, including infectious diseases and those caused by parasites[4].The "magical" powers of these selected species and their unique chemical profiles are responsible for vast uses of plant and their extracts became the main source for medicines[10]. The regular intake of synthetic drugs may produce dependency where there is less or no possibility of abuse in case of herbal plants. They provide pure and potentially active compounds[11]. These drugs are more potential & are used commercially as source for synthesis of synthetic drugs in pharmaceutical industries[12,13].

The plant *Annona reticulata* L. also known as Ramphal or Bullock's heart or Custard apple belongs to family Annonaceae[14]. It is a small tree with

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glabrous branches which is found in tropical region[15]. It is widely distributed throughout the India[16]. Almost all parts of plants are medicinally useful and they show various therapeutic activities as CNS depressant, anticancer, antihyperglycemic, analgesic, anti-inflammatory, antiproliferative, antiulcer and wound healing activity[14,16,17]. *In vitro* and *in vivo* antiproliferative activities were reported on *A. reticulata* root[18,19]. Three aporphine alkaloids liriodenine [ARR-01], norushinsunine [ARR-02], reticuline [ARR-03], and one acetogenin neoannonin [TAR-01] had been isolated from the roots of *A. reticulata*[20]. Traditionally the leaves of *A. reticulata* have been used for their anthelmintic property[15]. Hence, in search for therapeutically alternatives for synthetic anthelmintics the present study envisaged to evaluate the *in vitro* activity of methanolic extracts from *A. reticulata* root in comparisons with currently using anthelmintic drug albendazole.

Materials and Methods

Collection and identification of plant material

The roots of *A. reticulata* were collected in the month of January from rural areas of Beed district, in the state of Maharashtra, India and authenticated by Dr. B. D. Gachande associate professor of botany department, N. E. S. Science College, Nanded, Maharashtra, India.

Preparation of extract

Roots tubers of the plant were washed in deionized water and shade dried for 10 days. The dried plant material was cut into small pieces and finely powdered (Sieve no. 85) by using dry grinder. The root powder was extracted with methanol (500 mL) by using Soxhlet apparatus. The solvent was evaporated and concentrated extract was obtained[21-23].

Preliminary phytochemical screening

Preliminary phytochemical tests for the methanolic root extract of *A. reticulata* were conducted for the detection of major chemical constituents according to the standard procedure[23,24].

Collection and authentication of worm

Indian adult earthworms (*Pheretima posthuma*) were collected from moist soil of Vishnupuri area of Nanded, Maharashtra, India and washed with normal saline solution to remove all the faecal matter. The worms were identified and authenticated by Dr. P. B. Deshmukh, zoologist from Zoology Department of N. E. S. Science College, Nanded, Maharashtra, India.

Anthelmintic activity

Anthelmintic activity of methanolic extract of *A. reticulata* roots was evaluated on Indian earthworms. The samples were prepared by dissolving 200 mg, 400 mg, and 600 mg methanolic extract of each in 1 mL DMSO and made the volume up to 10 mL with normal saline solution to prepare 20, 40, 60 mg/mL concentrations. Seven groups of Indian earthworms, each containing six earthworms approximately of equal size were used for the study. Three groups were tested with methanolic extract of different concentrations (20 mg/mL, 40 mg/mL and 60 mg/mL) and the other three groups were treated with albendazole, as a reference standard[25]. One group was treated with normal saline solution and used as control group. The groups were observed for paralysis time and death time for each earthworm and mean time was taken for all the extracts[26,27]. The paralysis time was said to occur when there is no sort of movement except when shaken vigorously and death time was recorded after ascertaining that worms neither moved when given external stimuli nor dipped in warm (50°C) water[11].

PASS computer program

The computer program PASS (Prediction of Activity Spectra for Substances) was used for prediction of anthelmintic activity of methanolic extract of *A. reticulata* root. The PASS program is used to evaluate different types of pharmacological activity for different organic molecules[28]. Prediction of this spectrum by PASS is based on SAR analysis of the training set containing more than 205,000 compounds exhibiting more than 3750 kinds of biological activities[29]. This software estimates the predicted activity spectrum as Pa, indicates the probability to be active and Pi, indicates the probability to be inactive. The probable activity values of different constituents from *A. reticulata* root for anthelmintic activity are given in the Table 1. The compounds showing more Pa value than Pi are the only constituents considered as possible for a particular pharmacological activity[28-30].

Statistical analysis

All data were expressed as the mean ± SEM. Data was subjected to one way ANOVA followed by Dunnett test. The statistical analysis conducted with Graphpad Instat Software (Version 3, USA).

Results and Discussion

Preliminary phytochemical investigation of methanolic extract revealed the presence of alkaloid, acetogenins, flavonoids, steroid and triterpenoids, glycosides, carbohydrates, proteins and tannins in *A. reticulata* root. In the present study, Indian earthworms have been used for the initial evaluation of anthelmintic activity, because of their easy availability and their anatomical and physiological appearance with the intestinal roundworm parasites of human beings[31]. Previous studies reported that alkaloids produce paralysis in earthworms[23,32]. Tannins might have anthelmintic activity by binding with free proteins in gastrointestinal tract of the earthworm and cause death[32]. It also exhibits anthelmintic activity by various mechanisms like increasing supply of digestible proteins by animals by forming protein complexes in rumen or interfering with energy generation by uncoupling oxidative phosphorylation or causes a decrease in G.I. metabolism[23]. An anthelmintic drug can either act by causing paralysis of worm or by damaging cuticle, leading to partial digestion or to rejection by immune mechanisms. They also interfere with the metabolism pathways of the worm[33,34].

The major phytoconstituents of *A. reticulata* roots is aporphine alkaloids and acetogenins, therefore, was selected to predict the biological spectrum by PASS. The PASS prediction of these compounds is summarised in Table 1.

Acetogenins are potent inhibitors of NADH ubiquinone oxido-reductase, an essential enzyme in complex-I of the electron transport system (ETS) which finally leads to oxidative phosphorylation in mitochondria. They directly act at the ubiquinone catalytic site within complex 1 and in microbial glucose dehydrogenase. Also there is inhibition of ubiquinone-linked NADH oxidase that is peculiar to the plasma membranes of cancerous cells and functions to permit cytosolic phosphorylation by restoration of NAD levels. Ultimately both mechanisms lead to lack of ATP[35-,37].

Table 1—PASS prediction score of the constituents for probable anthelmintic activity

S. No.	Chemical constituent	PASS prediction	
		Pa	Pi
	Acetogenin		
1	Neoanonin		
	Aporphine Alkaloids	0.356	0.064
2	Iriodenine	0.157	0.101
3	Norushinsunine	0.141	0.120
4	Reticuline	0.218	0.195

The aporphine alkaloids have variable spectrum of biological activity. It produces its action by blocking alpha 1- adrenoceptor which are associated with normal sleep pattern and in human narcolepsy, condition associated with disabling sleep, they are associated with partial or complete flaccid paralysis[38].The cytotoxic activity of aporphine alkaloids is because of presence of methylenedioxy ring groups that interfere with the catalytic activity of topoisomerases. They have ability to inhibit the calcium ion channels and also have effect on the ion channels of K^+, Na^+ [39,40].

The PASS prediction results were interpreted and used in a flexible manner. Only activities with Pa>Pi are considered as possible for a particular compound and if Pa>0.7, the chance to find the activity experimentally is high[29]. Although PASS predictions for acetogenins and aporphine alkaloids from methanolic extract of *A. reticulata* roots is not as per Pa>0.7 but still it exhibits significant potent anthelmintic activity as that of standard drug albendazole which might be due to combine effect of these compounds.

Normal control saline solution treated *Pheretima posthuma* remained active with whole body movements (Plate 1). Albendazole treated control group Indian earthworms become slender, shrunken, paralyzed and then finally died after 3.14 min at 60 mg/mL concentration (Plate 2c). In case of 40 mg/mL concentration, the earthworms showed

Plate 1- Normal saline treated Indian earthworms remained active with whole body movements. Paralysis or mortality was not observed

paralysis and died after 4.14 min of exposure (Plate 2b) whereas the earthworms shrunken and remains motile only some of body parts and died after 5.53 min at 20 mg/mL concentration (Plate 2a). The Indian earthworms showed change in body movement and paralyzed before death when treated with albendazole. Table 2 shows results of saline, albendazole and extract treatment on Indian earthworm.

When the Indian earthworms were exposed to methanolic extract of *A. reticulata* roots at 60 mg/mL concentration, decrease in the movement is observed and became paralyzed at 1.02 min (Plate 3c). The worms become immobile and death was observed after 2.14 min which is more significant than standard. The strong paralysis attack followed by death was observed. At concentration 40 mg/mL, the Indian earthworms became paralyzed within 1.48 min but slight movement was observed and death time was found to be 3.45 min (Plate 3b) whereas death time was found to be 4.34 min with slow paralysis attack at concentration 20 mg/mL (Plate 3a). The effective dose dependent reduction in motility and killing of Indian earthworms was observed for methanolic extract of *A. reticulata* roots. The significant reduction in motility and paralysis is observed in the group treated with

Table 2—*In vitro* effect of different concentration of methanolic extract of *A. reticulata* roots and albendazole on survival of Indian earthworms

Control/ Albendazole/ Extract	Concentration of Albendazole/ Extract (mg/mL)	Time taken in minutes	
		Paralysis	Death
Control	Saline	-	-
Albendazole	20	3.34 ± 0.130	5.53 ± 0.008
	40	1.37 ± 0.059**	4.14 ± 0.008**
	60	1.04 ± 0.012**	3.14 ± 0.007**
Extract	20	2.18 ± 0.009**	4.34 ± 0.015**
	40	1.48 ± 0.0.040**	3.45 ± 0.014**
	60	1.02 ± 0.006**	2.14 ± 0.014**

P> 0.05, **P* < 0.05, ***P* < 0.01 values are in ± SEM, n=6, when compared with Albendazole and results were analyzed by one way ANOVA followed by Dunnttes multiple comparison test. Values of *P* < 0.05 were considered as statistically significant.

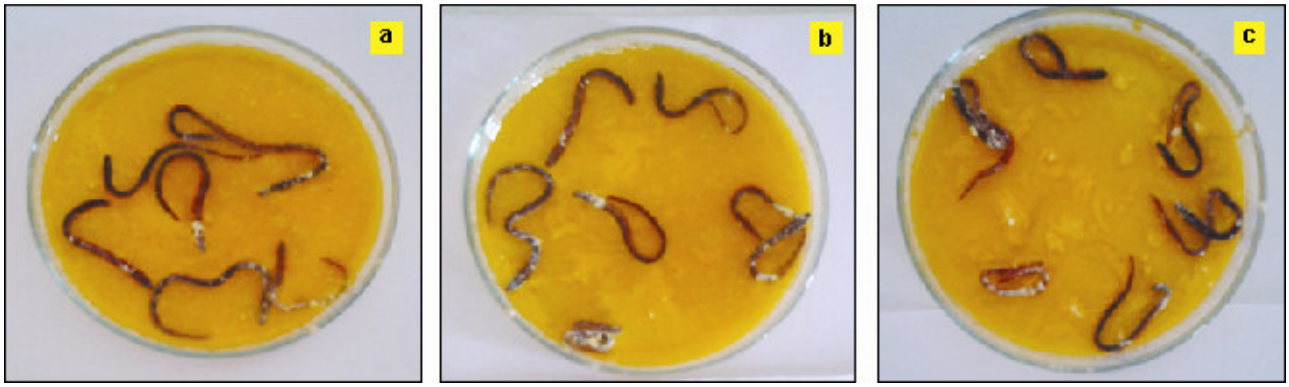

Plate 2- Effect of albendazole on motility, paralysis and mortality time for 20, 40 mg/mL (a, b) and 60 mg/mL (c) concentration

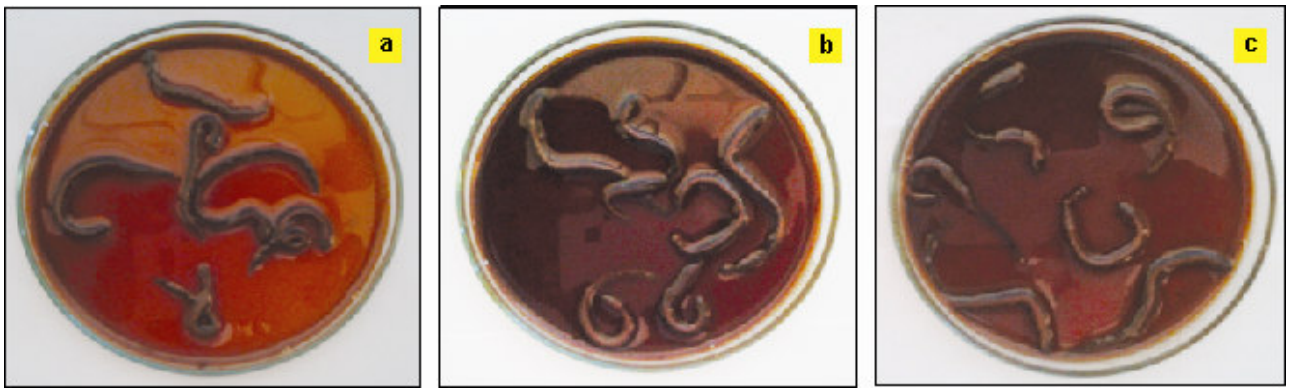

Plate 3- Effect of methanolic extract of *Annona reticulata* roots on motility, paralysis and mortality time for 20, 40 mg/mL (a, b) and 60 mg/mL (c) concentration

60 mg/mL concentration of methanolic extract of *A. reticulata* roots.

Thus it is evident from the results that the methanolic extracts of *A. reticulata* roots at different concentration possesses dose dependent anthelmintic activity but significant effectiveness is at 60 mg/mL. The active anthelmintic compounds of *A. reticulata* roots are not known however, it is reported to contain high amount of aporphine alkaloids and acetogenins. Acetogenins interfere with ATP production and electron transport system whereas aporphine alkaloids produces partial or complete flaccid paralysis and interfere with ion channels of Ca^{2+}, K^{+}, Na^{+}[Ref. 35-37]. When compared with albendazole, the result showed by the extract was very potent that might be because of combined effect of phytochemicals. This combined synergistic effect of phytochemicals was responsible for paralysis and mortality of Indian earthworms.

Conclusion

The phytochemicals of *A. reticulata* roots may act as anthelmintic therefore, the mode of action of these compounds need to be studied.

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