

## EVALUATION OF IN VITRO ANTHELMINTIC AND ANTIFUNGAL ACTIVITY OF DIFFERENT ORGANIC EXTRACTS OF THE SEEDS OF APHANAMIXIS POLYSTACHYA (WALL.)

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### Abstract

Dried and disease free seeds of *Aphanamixis polystachya* (Wall.) Parker were pulverized into coarse powder and subjected to different extraction systems successively with n-hexane, chloroform and methanol at room temperature. After drying and usual work up three semisolid crude extracts were obtained and stored for in vitro evaluation of anthelmintic and antifungal activity. Among the three extracts n-hexane extract showed strong anthelmintic activity against *Haemonchus contortus* collected from the female goat and its LC<sub>50</sub> value (0.10 mg/ml) was found to have more stronger activity than the standard Albendazole (LC<sub>50</sub> 0.15 mg/ml) and 100% mortality was found at the lowest concentration of 0.625 mg/ml. Chloroform extract and methanol extract showed strong anthelmintic activity (LC<sub>50</sub> values 0.156 mg/ml), very close to Albendazole. In vitro antifungal activity of the seed extracts against *Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus niger* was performed where methanol extract (500µg/disc) was found to be more dynamic than other two extracts and activity was relatively higher than that of the standard Ketoconazole (50µg/disc) against *Saccharomyces cerevisiae* and *Candida albicans*. Abundance of secondary metabolites in crude seed extracts has proclaimed *Aphanamixis polystachya* (Wall.) Parker as an influential source of anthelmintic and antifungal medicine.

**Keywords:** *Aphanamixis polystachya*, Anthelmintic, Antifungal, In-vitro study.

## Introduction

Helminthes and fungal infections are among the most common infections in men and ruminants. Gastrointestinal parasitism, especially by helminth species, impairs health by causing anorexia, flatulence, nausea, malnutrition, anemia, eosinophilia, pneumonia, tightness, stunted growth and in severe cases death. On the other hand, pathogenic fungi can also damage plants and crops, causing irreparable losses in agricultural production. In vertebrates fungal interactions are so dramatic and drastic that scientists are raising their attention to explore neoteric treatment strategies. Inadequate sanitary facilities, miserable supply of pure drinking water, afflicted food storage, unhygienic food products, accommodation system along with poverty, illiteracy and lack of awareness are some of the factors responsible for ominous break out of these diseases in the developing countries.

*Haemonchus contortus*, the causative agent of haemonchosis, is one of the most pathogenic and highly prevalent nematode parasites of small ruminants particularly in the tropics and subtropics. Haemonchosis is characterized by anemia attributable to blood loss via blood sucking activities of worms in the abomasum's [1]; causing acute disease and high mortality in all classes of livestock [2]. It is one of the top 10 constraints of sheep and goat production in East Africa [3]. These parasites cause frequent important economic losses due to the mortality in case of heavy infection. In addition, chronic infections cause weight loss, lowering of the productivity, fertility, growth, milk and meat production in ruminants [11], [13], leading to huge economic loss in livestock industry [12], [13].

*Candida albicans* is the most prevalent cause of fungal infections in people with urinary yeast infection, genital yeast infection, mucocutaneous candidiasis and oral thrush. *Aspergillus niger*, a common food contaminant, causes disease (black mold) on certain fruits and vegetables along with significant morbidity and mortality in human with pneumonia or chronic obstructive pulmonary diseases by severe infection. *Saccharomyces cerevisiae* can cause invasive infections like pyelonephritis in immune-competent patients along

with human respiratory, gastrointestinal and urinary tract disorders [4-6].

Synthetic chemical anthelmintics and antifungals, controlling haemonchosis and severe fungal infections respectively having some ungracious disadvantages of being costly, risk of environmental pollution, and increasing development of resistant populations [7], have been the spur for different research programs exploiting alternative approaches to parasite control [8] and kill or inhibit the growth or pull up the action of fungal activity. Anthelmintic and antifungal medicines derived from plants can be a solution to this world wide problem as they form safe and non-toxic agents with an altered site of action [9]. For much of our past history for ages, plant parts or entire plant extracts have been used to combat parasitism and in many parts of the world such natural products are still in use for these purposes [10]. Medicinal plants function as a potential source of medicaments for the treatment of a variety of ailments. *Aphanamixis polystachya* (Wall.) Parker, a large evergreen tree belonging to Meliaceae family, is one of the important medicinal plants growing in most of the hotter parts of India, as well as the lowlands and hill forests of Bangladesh, Malay and Ceylon, [14-16].

Considering the vast potentiality of plants as sources of anthelmintic and antifungal drugs due to the availability of gigantic secondary metabolites with significant bioactivities, the present study was undertaken to screen the in vitro anthelmintic and antifungal activity of different organic extracts (n-hexane, chloroform, and methanol) of the seeds of *Aphanamixis polystachya* (Wall.) Parker.

## Methods

Collection and Identification of plant.

The plant is a woody tree found to grow wild in the rain forest of South east Asia including India, Sree Lanka and Bangladesh.

Healthy, disease free seeds of *Aphanamixis polystachya* (wall.) Parker were collected during summer the month of May, 2014 from Rajshahi, Bangladesh. The plant was authenticated by Botany Department, University of Rajshahi and preserved in Phyto-Pharmacology and Natural Product Research Laboratory, Department of Pharmacy, University of Rajshahi-6205, Bangladesh.

#### Preparation of plant extracts

The seeds were washed with fresh water to remove dirty materials and sun dried for several days. The dried seeds were thrashed into coarse powder by suitable laboratory grinding machine. About 300 gm of powdered seeds were macerated with 600ml n-hexane in a reagent bottle for 7days accompanying occasional shaking and stirring. The whole mixture was then underwent a coarse filtration by clean, white cotton, followed by a filtration through Whatmann filter paper. The filtrate (n-hexane extract) was then evaporated through rotary evaporator using 120 rpm at 50°C for 25 minutes followed by desiccation to get the yellow colored, semisolid, aromatic, bitter n-hexane extract (APHE) with the yield value 8.67%. The residue was further subjected to successive solvent extraction using chloroform and methanol. The resulting semisolid, aromatic, bitter, dark brown chloroform extract (APCE) and brown colored methanol extract (APME) were obtained using the same procedure with the yield values 7.33% and 5.33% respectively. These extracts were then stored in beaker with aluminum foil coverings in a dry place and used for subsequent in vitro anthelmintic and antifungal activity screening.

#### Experimental procedure:

##### Anthelmintic activity

The experiment was done on adult *Haemonchus contortus* by the method illustrated by Abdi Mohammed et al., [17].

##### Collection of parasites

Adult *Haemonchus contortus* were collected from abomasums of the infected female goat. Immediately after slaughtering of female goat, the abomasums were collected and transported to laboratory. The collected parasites were washed and kept in freshly prepared phosphate buffer saline (PBS).

##### Study Protocol

Four groups of approximately equal size parasites consisting of ten parasites in each group were used for the present study.

Group-1: Methanol extract (APME) of different concentrations (5, 2.5, 1.25, 0.625, 0.3125, 0.1562 and 0.07812 mg/ml).

Group-2: Chloroform extract (APCE) of different concentrations (5, 2.5, 1.25, 0.625, 0.3125, 0.1562 and 0.07812 mg/ml).

Group-3: Hexane extract (APHE) of different concentrations (5, 2.5, 1.25, 0.625, 0.3125, 0.1562 and 0.07812 mg/ml).

Group-4: Positive control (Albendazole) of different concentrations (0.125, 0.25, 0.5, 1 and 2 mg/ml).

After 24 hours, the extract was washed away and the parasites were re-suspended in PBS for 30 minutes for possible recovery of the parasite motility. Finally, the number of motile (alive) and immotile (dead) parasites were counted and recorded for each concentration.

From these data, the percentage of mortality of parasites was calculated for each concentration of the sample. The median lethal concentration (LC<sub>50</sub>) of the test samples was obtained by a plot of percentage of the killed parasites against the concentration of the sample. A mortality index was calculated as the total number of dead parasites divided by the total number of parasites per petridish as well.

#### Antifungal activity

Gradual health worsening due to fungal diseases is a threat for modern generation globally. Here, the antifungal assay was performed by disc diffusion technique [18-20], which ascertained a qualitative or semi qualitative measurement of the sensitivity or resistance of fungi to the plant extracts.

#### Test organisms used for antifungal activity

Determination of antifungal activity was performed in contrast to *Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus niger*. Pure cultures of these organisms were collected from Microbiology Laboratory, Department of Pharmacy, University of Rajshahi, Rajshahi-6205.

#### Preparation of medium

To prepare required volume of nutrient agar medium, 28 gm of the prepared medium was

dissolved in 1000 ml distilled water. It was then heated in water bath to dissolve the agar until a transparent solution was appeared.

#### Preparation of subculture

20 ml and 5 ml of prepared media were dispensed in a number of clean test tubes to prepare plates and slants respectively. The tubes were then plugged with cotton and sterilized in an autoclave at a temperature of 121°C and pressure of 15 lbs. /sq. inch for 15 minutes. With the help of an inoculation loop, the test organisms were transferred from the pure culture to the agar slants in a laminar airflow unit. The inoculated slants were then incubated at 37.5 °C for 18-24 hours to assure the growth of test organisms.

#### Preparation of test plates

The test organisms were transferred from the subculture to the test tube containing 20 ml autoclaved medium with the help of an inoculating loop in an aseptic area. The fungal suspension was immediately transferred to the sterile petri dishes in an aseptic area and was rotated several times, first clockwise and then anti-clockwise to assure homogeneous distribution of the test organisms. After that the medium was cooled at room temperature, and then it was stored in a refrigerator at 4°C.

#### Preparation of discs and test samples

**Sample discs:** Sterilized filter paper discs having 5 mm in diameter were prepared with the help of punch machine and were used as Sample discs.

**Standard discs:** Ketoconazole (50 µg/disc) was used as a reference standard for screening antifungal activity.

#### Blank discs:

These were used as negative control to ensure that the solvent and the filter paper were not active themselves.

#### Preparation of test samples

Test samples were prepared by dissolving 10 mg of methanol (APME), chloroform (APCE) and n-hexane (APHE) extract in 1 ml of methanol, chloroform and n-hexane solvent respectively. So the concentration of stock solution was 10µg/µl. To each disc 50µl of stock solution was added which results in the formation of a disc containing 500 µg of extract.

#### Placement of the discs and measurement

By means of a pair of sterile forceps, the sample impregnated discs were placed gently on the solidified agar plates seeded with the test organisms in order to ensure proper contact with the medium. The plates were then kept in a refrigerator at 4°C for 24 hours to ensure proper diffusion. Finally, the plates were incubated at 37.5°C for 24 hours and the antifungal activity of the test agents was determined by measuring the diameter of the zones of inhibition in mm with a transparent scale and compared with the standard disc.

## Results

#### Anthelmintic activity

All the extracts had inhibitory effect on the survival of *Haemonchus contortus* in a dose dependent manner. The percentage of mortality after 24 hours exposure of *Haemonchus contortus* to three different extracts: methanol (APME), n-hexane (APHE) and chloroform (APCE) of *Aphanamixis polystachya* (Wall.) Parker seeds and standard Albendazole are shown in the Table-1; Table-2 and Fig-1. A comparative measurement of LC<sub>50</sub> values (mg/ml) among different extracts of *Aphanamixis polystachya* (Wall.) parker seeds along with positive control Albendazole is also shown in Fig-2.

#### Antifungal activity

All the extracts showed potential antifungal effects against *Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus niger* at 500µg/disc. A refulgent comparison among inhibitory areas of fungal growth on agar plates demonstrating diameter of zone of inhibition (mm) of three

different extracts: methanol (APME), n-hexane (APHE) and chloroform (APCE) of *Aphanamixis polystachya* (wall.) Parker seeds along with standard ketoconazole is shown in the Table-3 and Fig-4.

## Discussion

### Anthelmintic activity

The use of natural compounds has the potential to be a complementary control option which may reduce the reliance on drug treatment and slow the development of resistance parasites. Since time immemorial, several experiments of natural plant extracts as de-wormers for humans and livestock have long been practiced, however scientific validation of these practices and identification of active compounds have been lacking [21-23]. Anthelmintic effects of plants are normally ascribed to secondary metabolites such as essential oils [24], flavonoids, alkaloids, terpenoids [25] or polyphenols such as proanthocyanidins [26], also known as condensed tannins. Tannins are non-nitrogenous plant constituents and have an astringent action on mucous membranes as they precipitate protein from the cells of mucous membranes and exert a protective action. Some synthetic phenolic anthelmintics e.g. niclosamide, oxyclozanide, bithionol etc., are reported to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation [27]. Moreover, direct anthelmintic effects of purified condensed tannins have been confirmed in in vitro assays against, amongst others, *Haemonchus contortus* [28], *Ostertagia ostertagi* [29] and *Ascaris suum* [30] as tannins can bind to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite and cause death [31] [32]. Tannin containing plants increase the supply and absorption of digestible protein by animals [33]. This is achieved by the formation of protein complexes in the rumen by tannins, which later dissociate at low pH in the abomasum to release more protein for metabolism in the small intestines of ruminant animals [34]. Piperazine citrate, being a heterocyclic ring possessing alkaloid, blocks the intake of acetylcholine from the host organism. Likewise some plant extracts contain simplexin as a

chief alkaloid which may possess the same type of pharmacological action and expels the worms by peristaltic movement [35], of intestine. The present works proved the usage of this plant extracts in treatment of *Haemonchocis* in our nation.

In this study, anthelmintic assay was performed on adult Bangladeshi parasites, *Haemonchus contortus* using various extracts of *Aphanamixis polystachya* (Wall.) Parker seeds in a dose dependent manner. Colorimetric method determined that methanol, chloroform and hexane extracts of *Aphanamixis polystachya* (Wall.) Parker seeds have significant amount of proanthocyanidins or condensed tannins [36]. Preliminary phytochemical screening also revealed that crude seed extracts also contain polyphenol derivatives, [37], [38], alkaloid [39], triterpenoid [40], which provide possible mode of anthelmintic action against *Haemonchus contortus*.

From the experiment, it is evident that APME (methanol), APCE (chloroform) and APHE (n-hexane) produced mortality of adult *Haemonchus contortus* significantly to the level 100% at a concentration of 2.5 mg/ml, 5 mg/ml and 0.625 mg/ml (Table-1; and Fig-1) respectively. on the other hand, Albendazole, a broad spectrum anthelmintic, decreases in microtubules in the intestinal cells, absorptive function, and uptake of glucose by the adult and larval forms of the parasites thereby depletes glycogen storage and insufficient energy supply for the production of adenosine triphosphate by inhibiting microtubules polymerization after binding to the colchicine sensitive site of  $\beta$ -tubulin [31], brought about 100% parasite mortality at a concentration of 0.5 mg/ml within 24 hours (Table-2). The APME and APCE showed LC<sub>50</sub> at 0.156 (mg/ml) whereas the APHE exposed a LC<sub>50</sub> value at 0.1 mg/ml and standard Albendazole at 0.15 mg/ml (Fig-2). The mortality index after 24 hours exposure of *Haemonchus contortus* to different concentrations (mg/ml) of APME, APCE and APHE was 1 at a concentration of 2 mg/ml, 5mg/ml and 0.625mg/ml respectively whereas standard Albendazole showed mortality index 1 at a concentration of 0.5mg/ml.

The result of the present study indicates that all the three extracts of *Aphanamixis polystachya* (Wall.) Parker seeds tested for the anthelmintic activity possess a momentous anthelmintic effect as

compared to the standard Albendazole. Here, significant variation in the concentrations among different extracts of *Aphanamixis polystachya* (wall.) Parker seeds was observed. The APHE (n-hexane) gave the highest yield in comparison with other extracts and standard Albendazole. Moreover, all the extracts in the current study exhibited 100% mortality at a concentration of 5mg/ml. Plant materials evaluated in this study can be identified to serve as anthelmintic agents.

#### Antifungal activity

The antifungal activity of different extracts of *Aphanamixis polystachya* (Wall.) Parker seeds against the three pathogenic fungi expressed the scientific cogency of the plant being used traditionally as an antifungal drug. The inhibition of fungal strains by the extracts may be attributed to the presence of soluble phenolic and polyphenolic compounds in the extract which significantly confuse of permeability barrier of membrane structures [41], [42]. Saponins, a structurally diverse class of secondary metabolite, comprise a non-polar core and a polar glycosyl group or groups, which give the molecules amphipathic characteristics. Conventionally, saponins are classified as either triterpenoid or steroidal, with a subclass of steroidal alkaloids (steroidal glycoalkaloids), depending on the structure of the hydrophobic core [43]. Within plants, saponins components are believed to have evolved to provide defense against phytopathogenic fungi as they have potent antifungal activity, are generally localised to epidermal layers of plant tissues, and have been demonstrated to have a defense role in several pathogenic interactions [44]. The amphipathic nature of saponins suggests that saponins can permeabilise fungal membranes. The proposed mode of action is that the hydrophobic core inserts into the outer membrane, forming a complex with ergosterol (unique features of fungal membrane). Subsequent interaction between the polar glycosidic side chains leads to aggregation, pore formation and loss of membrane integrity [45]. The ability to permeabilise membranes has been demonstrated in vitro on model membranes and in vivo in a study that used *Saccharomyces cerevisiae* to explore the anti-fungal activity of the steroidal

glycoalkaloid saponin, [46]. That study also showed, the algycone of steroidal glycoalkaloid saponin, did not permeabilise membranes, in fact inhibits ergosterol biosynthesis is capable of inducing programmed cell death in the fungus. Furthermore, a number of studies have proposed that potato steroidal glycoalkaloids (saponins), a-chaconine and a-solanine, have identified a range of toxic effects that are distinct from membrane permeabilising activity [47], e.g. Alkaloids intercalate into cell and/or DNA, Flavonoids binds form complex with cell and inactivate enzymes.

The The study exposed that all the extracts: APME, APCE, APHE had antifungal activity and the APME had the most significant antifungal activity against all tested fungi. The zone of inhibition of APME was found to be 40 mm, 11 mm, 39 mm against *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae* at 500 µg/disc respectively. The zone of inhibition of APCE was 7 mm, 8 mm and 11 mm against *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae* at 500 µg/disc respectively whereas APHE exposed zone of inhibition 8 mm, 7 mm and 10 mm against *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae* at 500 µg/disc respectively. Standard chemical Ketokonazole, an available imidazole antifungal medication, disturbs biosynthesis pathway of ergosterol from lanosterol by inhibiting the enzyme cytochrome P450 14-alpha-demethylase (P45014DM) [48], exhibited zone of inhibition 33 mm, 20 mm, and 20 mm against *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae* at 50 µg/disc respectively (Table-3; and Fig-3). Blank discs did not show any zone of inhibition against the pathogenic fungi demonstrating that the solvents and the filter papers were not active themselves as antifungal agents. The APME yielded the highest norms of antifungal activity in comparison with other extracts and standard Ketokonazole. Over and above, the plant extracts used in this study were showed potential antifungal activities due to the presence of gallic and ellagic acid polyphenol derivatives, [37], [38], alkaloid [39], triterpenoid [40], flavonoid [49], Saponin [49], glycoside [50], in crude extracts, which are supposed to provide possible antifungal action through either membrane disruption or cell complex formation or enzyme inactivation for

different organic extracts (methanol, Chloroform, n-Hexane) of *Aphanamixis polystachya* (Wall.) Parker seeds. Therefore, it is worth to mention that the methanol extract (APME) and the n-hexane extract (APHE) of *Aphanamixis polystachya* (Wall.) Parker seeds may be an immense source of antifungal and anthelmintic agent for future which may exert desired and alternative modes of action at low doses. This is only a preliminary study and furthermore investigations are required to explore the bioactive molecules which will ensure the veritable reasons of extracts' activities as well as their mechanism of action with a minimum mischievous effect.

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**Table-1:** Percentage of mortality after 24 hours exposure of *Haemonchus contortus* to different concentrations of APME, APCE & APHE.

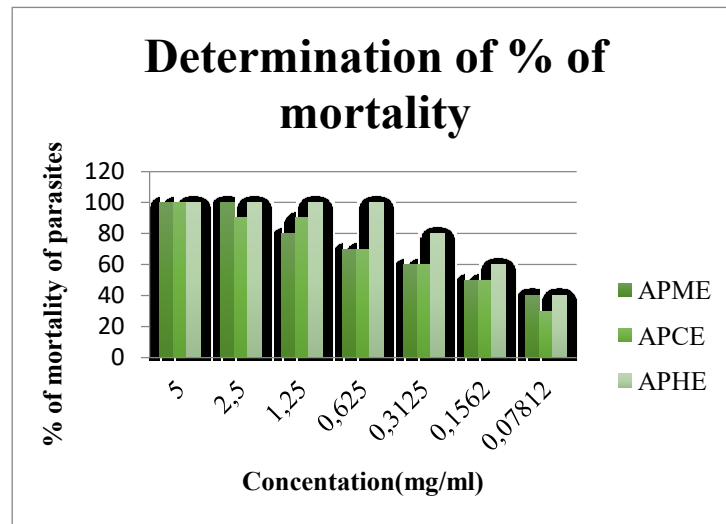
Sample	No. of Sample	Concentration (mg/ml)	Total parasites	Live	Dead	Mortality index	% Mortality	LC <sub>50</sub> (mg/ml)
APME	1	5	10	0	10	1	100	0.156
	2	2.5	10	0	10	1	100	
	3	1.25	10	2	8	0.8	80	
	4	0.625	10	3	7	0.7	70	
	5	0.3125	10	4	6	0.6	60	
	6	0.1562	10	5	5	0.5	50	
	7	0.07812	10	6	4	0.4	40	
APCE	1	5	10	0	10	1	100	0.156
	2	2.5	10	1	9	0.9	90	
	3	1.25	10	1	9	0.9	90	
	4	0.625	10	3	7	0.7	70	
	5	0.3125	10	4	6	0.6	60	
	6	0.1562	10	5	5	0.5	50	
	7	0.07812	10	7	3	0.3	30	
APHE	1	5	10	0	10	1	100	0.10
	2	2.5	10	0	10	1	100	
	3	1.25	10	0	10	1	100	
	4	0.625	10	0	10	1	100	
	5	0.3125	10	2	8	0.8	80	
	6	0.1562	10	4	6	0.6	60	
	7	0.07812	10	6	4	0.4	40	

APME = methanol extract of *Aphanamixis polystachya* (wall.) Parker seeds APCE = chloroform extract of *Aphanamixis polystachya* (wall.) Parker seeds APHE = n-hexane extract of *Aphanamixis polystachya* (wall.) Parker seeds.

**Table-3:** *In vitro* antifungal activity of different extractives of *Aphanamixis polystachya* (wall.) Parker seeds.

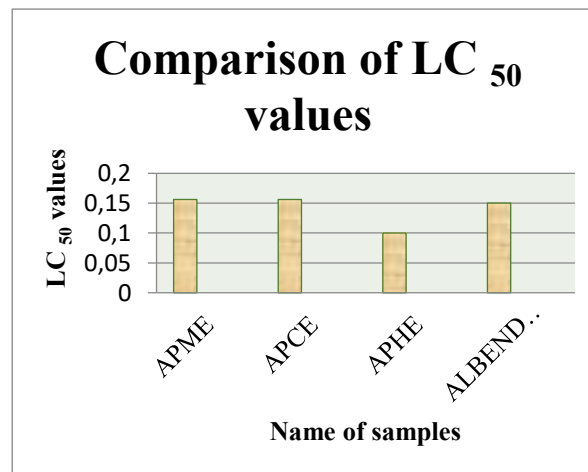
Name of fungi	Diameter of the zone of inhibition(mm)			
	APME (500µg/disc)	APCE (500µg/disc)	APHE (500µg/disc)	Ketoconazole (50µg/disc)
<i>Candida albicans</i>	40	7	8	33
<i>Aspergillus niger</i>	11	8	7	20
<i>Saccharomyces cerevisiae</i>	39	11	10	20

APME = methanol extract of *Aphanamixis polystachya* (wall.) Parker seeds, APCE = chloroform extract of *Aphanamixis polystachya* (wall.) Parker seeds, APHE = n-hexane extract of *Aphanamixis polystachya* (wall.) Parker.

**Fig-1:** Determination of % of mortality at different concentrations of the extracts.**Table-2:** Percentage of mortality after 24 hours exposure of *Haemonchus contortus* to different concentrations of Albendazole.

Albendazole	Concentration (mg/ml)	Total parasites	Live	Dead	Mortality index	% Mortality	LC <sub>50</sub> (mg/ml)
	2	10	0	10	1	100	0.15
	1	10	0	10	1	100	
	0.5	10	0	10	1	100	
	0.25	10	3	7	0.7	70	
	0.125	10	8	2	0.2	20	

APME = methanol extract of *Aphanamixis polystachya* (wall.) Parker seeds, APCE = chloroform extract of *Aphanamixis polystachya* (wall.) Parker seeds, APHE = n-hexane extract of *Aphanamixis polystachya* (wall.) Parker seeds.

**Fig-2:** Comparison of LC<sub>50</sub> values of different extracts of *Aphanamixis polystachya* (wall.) Parker seeds and Albendazole.

**Fig-3:** Determination of antifungal activity of different extracts of *Aphanamixis polystachya* (wall.) Parker seeds at 500µg/disc.

