

Alpinia Nigra (Family Zingiberaceae): An Anthelmintic Medicinal Plant of North-East India

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Abstract Traditionally used medicinal plants have been a source of relief in the control of different types of diseases throughout the globe. Economically backward people, who resides in remote areas of developing countries, rely heavily on indigenous way of get rid of parasitic infections using folk medicine. *Alpinia nigra* is one such medicinal plant, widely used in different parts of North-East India to cure intestinal helminth infection. Essential oils of different species of *Alpinia* contain flavonoids, terpenoids and kavalactones which have been in used to cure inflammation, hypertension, bacterial and helminth infection. In this review, the crude alcoholic extract of *A. nigra* that causes destruction and degeneration of surface architecture of tegument, inhibit energy metabolism related enzymes and also enzyme responsible for neuromuscular co-ordination are discussed.

Keywords Parasitic infection, folk medicine, *Alpinia nigra*, tegument, energy metabolism, neuromuscular

1. Introduction

Indigenous system of herbal therapy is becoming an increasingly attractive approach to control parasitic infection, particularly in developing countries. Among parasites, helminth and helminthic infections (helminthiasis) remain to be one of the major health problems affecting billions of people all over the world[1,2]. They are the most common infectious agents of human beings that contribute in the wide spread occurrence of undernourishment, anaemia, eosinophilia and pneumonia[3]. They are also responsible for considerable economic losses to the livestock industry of marginal farmers, particularly of developing countries[4-6]. People living in tropical and sub-tropical countries with low per capita income, poor hygienic conditions suffers most because of the presence of favourable conditions for the proliferation of the parasite[7] and, also for the propagation of intermediate hosts that are an essential link in the life cycle of the parasite[8].

Over the last few decades the most common method of controlling helminthiasis all over the world include the use of commercial anthelmintic drugs of various types. WHO has listed several essential anthelmintic drugs which are safe to use, available in single dose and of low cost, e.g., mebendazole, albendazole, pyrantel pamoate and levamisole, which are found to be effective against soil-transmitted helminths like *Ancylostoma duodenale*, *Ascaris*

lumbricoides, praziquantel for schistosomes and foodborne trematodes and both ivermectin and diethylcarbamazine (DEC) for curing filariasis[9,10]. However, despite of having developed health care facilities, sophisticated instrumentations and advancement in chemotherapy, there is still lacking of proper and effective tools to deal with helminthic infections. Review of literature revealed that out of 1,556 new chemical entities marketed between 1975 and 2004, only few drugs like albendazole, oxamniquine, praziquantel and ivermectin were developed to treat helminthiasis and found effective[11,12]. Therefore, there is always been a need to find new anthelmintic drugs because current drugs do not control all parasitic infections well. Moreover, high treatment frequency, single-drug regiment or frequent use of the same anthelmintic has led to the development of resistance among helminth population[13,14]. Similarly, its negative, undesired effects as well as limited availability to the rural areas further restricted the effective control of helminthiasis[15-18], causing new threat to human society. Because of this limited availability and in-effectiveness of commercial drugs towards controlling helminthiasis, scientists are now looking for new drugs based on traditional knowledge and traditionally used medicinal plants as an alternative remedies[19-22].

Contribution of plants to fight against various diseases dates back several centuries, and has been documented by the ancient Chinese, Indian and North African Civilisations[23-25]. Though the advancement of synthetic medicines, to certain extend, has lifted the health care and livelihood of people, yet the use and importance of plants and its botanicals for the same has never been neglected and a large number of plants are screened for their efficacy against

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various helminthic infections[26-30]. Several such studies based on traditional medicinal knowledge were done in Indian sub-continent to test the putative anthelmintic activity of different plants. *Alpinia nigra* is one such plant, shoot of the plant along with a part of rhizome is used by the indigenous tribal people of Tripura, India, as vegetable, whereas aqueous juice of shoot of the plant is consumed to get rid of intestinal helminth infection[31-33].

The north-eastern region located between 87°32'E to 97°52'E latitude and 21°34'N to 29°50'N latitude is known for its genetic resources all over the world and is the Biogeographical Gateway to India and finds place in part of two-biodiversity hotspots in India[34]. Use of plant and plant-parts, like leaves, bark, root etc. in order to get rid of various diseases is a kind of common practice to the people of this region, especially the ethnic groups. Out of the estimated 800 species consumed as food plants in India, about 300 species of plants are found in Eastern Himalaya alone[35]. Despite of its biological diversity, the region remains disgracefully data-poor for a wide variety of plant taxa. However, the existing traditional knowledge based on plant and animal resource remains a positive indicator for researcher.

2. Botanical Description of *Alpinia nigra* (Gaertn.) Burtt

2.1. Taxonomy

Kingdom: Plantae
 Order: Zingiberales
 Family: Zingiberaceae
 Subfamily: Alpinioideae
 Tribe: Alpinieae
 Genus: *Alpinia*
 Species: *nigra*

2.2. Synonyms

Zingiber nigrum Gaertner, Fruct. Sem. Pl. 1: 35. 1788
Alpinia allughas (Retzius) Roscoe
Heritiera allughas Retzius
Languas allughas (Retzius) Burkill

2.3. Morphology

Alpinia nigra belonging to the family Zingiberaceae, is an herbaceous plant, grows well on riverside and can also grow on moist land. The underground stem is rhizome and aerial stem is pseudo-stem which consists of leaf sheath, approximately 3.08 meters in height. Leaves are simple, alternate, oblong, entire and acute at base and apex of the plant with very short petiole and very long leaf sheath with small ligule. The plant has penicle type of inflorescence. The infloret have instant involucre bract and irregular direction. One infloret is composed of four florets. Flower is perfect, irregular symmetry, 3 sepals and 3 lobes, 3 petals and 3 lobes

white-green in colour, petals encloses the young flower. Stamen is one, fertile, no spur on anther. The 5 sterile stamens are flat and separated at the apex, call labellum, showy and white-pink, petaloid staminode. Pistil is one, inferior ovary, style is between the anthers. Stigma is over the anthers with three carpels, axile placentation. Fruit is berry having many seeds, pericarp is thin and green when it is young and becomes black and brittle when it gets old. The pollen of *A. nigra* is monad, inaperture spheroidal, small size, isopolar, radial symmetry and sculpturing is echinate[36].



Figure 1. *Alpinia nigra*, (a) Complete Plant with Flower and (b) Shoot Part Of *A. nigra*

2.4. Geographical Distribution

Alpinia nigra (Gaertn.) Burtt (Zingiberaceae) is distributed primarily in Yunnan and Hainan Province of China, Thailand, Bhutan, Sri Lanka and India[37]. North-east region of India is one of the richest and diverse regions for Zingiberaceae where 88 different species of plant belonging to 19 genera are reported[38]. *A. nigra* occurs in marshy slope between low hillocks in different parts of Assam and Tripura, two states of north-east India.

3. Folk Uses

The genus *Alpinia* is well known for its immense medicinal value and has been used as a part of the human diet in several parts of the Globe (De Araujo Pinho *et al.*, 2005, Hsu *et al.*, 1987, Jantan *et al.*, 2005)[39-41]. Apart from its different folk medicinal uses like anti-rheumatic oilment, anti-inflammatory, anti-bronchitis, anti-bacterial, antifungal, anti-diabetes, anticancer and as tonic, the different species are also in use as essential spice and food flavouring product in different parts of Asia[42-55].

In Tripura, India, natives consume aqueous juice of shoot of *A. nigra* to cure intestinal parasitic infection. The plant is also a favourite vegetable diet, however, in some parts it is

also used as food flavouring product by the indigenous people of Tripura. Similarly, the aqueous extract of shoot and rhizome of *Alpinia nigra* (common name "Tora") has been used in Assam for curing health problems like bone weakness, irregular menstruation, jaundice and gastric ulcers[56].

4. Phytochemicals From *Alpinia nigra*

A large number of chemicals have been isolated and studied from the genus *Alpinia*; however, limited literature is available on *A. nigra* and its phytochemicals. In China, Qiao et al.[57] studied the seed clusters of *A. nigra* and isolated two bioactive flavone glycosides, astragalins and kaempferol-3-O-glucuronide from the plant. Out of the two chemicals, kaempferol-3-O-glucuronide was found to be a dominant compound in the seed clusters distributed primarily in the pulp. In addition to this, two major volatile oils, β -Pinene and α -pinene have also been isolated from the fruits and rhizomes, of *A. nigra*[58].

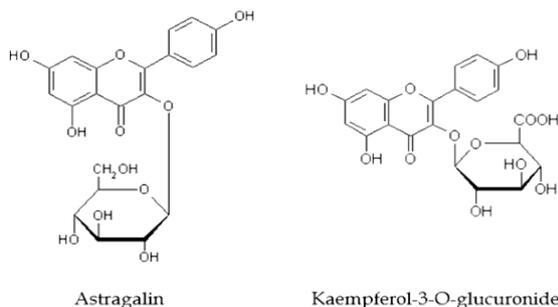


Figure 2. Two Bioactive Flavone Glycosides From *Alpinia nigra* Seed Clusters

5. Anthelmintic Efficacy of *Alpinia nigra*

5.1. Mortality and Motility Study

Studies on mortality and motility conducted on intestinal giant fluke, *Fasciolopsis buski* to see the anthelmintic efficacy of *A. nigra* crude-extract and reference drug, praziquantel (PZQ) revealed that on exposure to different concentrations of plant extract as well as PZQ, the parasites became immobile, followed by flaccid paralysis and finally death. The effects of different concentrations of plant extract and the drug were shown in Table 1. Flukes maintained in phosphate buffered saline (PBS, pH 7.4) were taken as control. It can be seen from the table 1 that crude extract of *A. nigra* at its highest dose (20mg/ml) showed more or less similar extent of efficacy when compared with PZQ at 2.5mg/ml PBS. Control parasite showed more than 21h of physical activity. A dose dependant efficacy was observed as higher concentration of crude extract leads to paralysis and death at shorter period of time. A similar kind of dose dependent anthelmintic activities were also observed in cestode and nematode parasites when treated with extract of *Flemingia vestita*, *Accacia oxyphylla*, *Milletia pachycarpa*, *Potentilla fulgens* and *Lysimachia ramosa*[59-61].

Table 1. *In Vitro* Effect Of Alcoholic Shoot-Extract Of *A. nigra* and Reference Drug, Praziquantel on *F. buski*. Values Are Taken As \pm SE, Number Of Replicates, N = 4

Incubation medium	Time taken in h		
	Doses (mg/ml PBS)	Paralysis	Death
<i>A. nigra</i>	20	2.14 \pm 0.48	3.94 \pm 0.06
	10	3.90 \pm 0.10	5.78 \pm 0.17
	5	5.40 \pm 0.08	8.86 \pm 0.11
	2	9.50 \pm 0.30	11.35 \pm 0.37
PZQ	2.5	2.11 \pm 0.26	2.75 \pm 0.42
Control (PBS)	-	-	21.05 \pm 0.22

5.2. Histochemical studies

Trematode have an external body surface covered with an unusual structure termed as tegument. It protects the parasites from the adverse conditions inside the host body and also plays many important functions like evasion of host immune system, absorption of certain nutrients, excretion of metabolites, control of motility and osmotic gradients. Structurally, the tegument consists of cytoplasmic syncytium, basal lamina followed by sub-tegumental cells[62]. The presence of several vital enzymes viz. acid phosphatase (AcPase), alkaline phosphatase (AlkPase), adenosine triphosphatase (ATPase), 5'-nucleotidase (5'-Nu) has been reported in the tegument, and in various organs/tissues of many flat worms[63-66]. All these enzymes which are involved in various metabolic processes are also believed to be involved in absorption and/or digestion in the parasite[67,68]. Investigations on all these key tegumental enzymes and glycolytic enzymes of *F. buski* revealed to be reduced when treated with *A. nigra* plant extract and the drug.

The alcoholic crude extract of *A. nigra* and the reference drug (praziquantel) were found to reduce the activities of AcPase, AlkPase and ATPase (Fig. 3-5). In control parasites, the staining intensities of all the enzymes were found to be higher compared to the treated ones. The tegumental layer, subtegumental zone as well as the body musculature was found to be deeply staining. Out of all the tegumental enzymes studied, highest staining intensities were observed in tegumental and sub-tegumental layer. AcPase and AlkPase showed deepest staining compared to the ATPase. The AcPase activity of the somatic musculature and intestine were diminished, while mild visible staining was observed in the tegument and sub-tegument regions of the plant extract-treated fluke (Fig. 3b). Almost all the regions of the praziquantel-treated flukes showed totally diminished staining activity for AcPase (Fig. 3c). Reduction in the activity of AlkPase was also observed in the tegument, sub-tegument, somatic musculature and intestine of the crude plant extract- and praziquantel-treated flukes (Fig. 4). The ATPase activity was also found to be reduced in *Alpinia*- and praziquantel-treated parasites (Fig. 5) as compared to the controls. In control fluke ATPase activity could be noticed throughout the body, while on exposure to plant extract and drugs, reduction in the staining activity were noticeable. Both in the plant extract and drug treated parasites (Fig. 5b, c), highest reduction in stain intensity has been seen in the dorsal side compared the ventral side of the fluke.

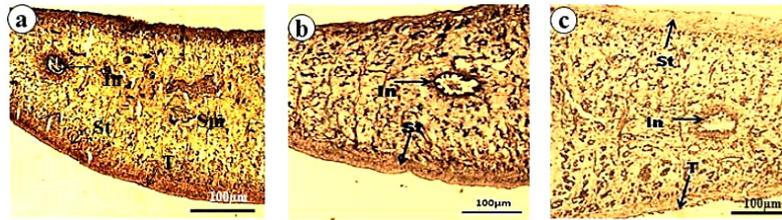


Figure 3. Acid Phosphatase Activity in *F. buski*, Photographs of Fresh Frozen Sections. (a) Control, (b) Shoot Extract Of *A. nigra* Treated Section, (c) Praziquantel Treated Section. [Photographs are adapted from Journal of Parasitic Diseases, 33(1&2), 2009, 48-53, Anthelmintic efficacy of ethanolic shoot extract of *Alpinia nigra* on tegumental enzymes of *Fasciolopsis buski*, a giant intestinal parasite, Roy B. and Swargiary A., Fig. no. 1, a, c and e, © Springer, with kind Permission Of Springer Science+Business Media]

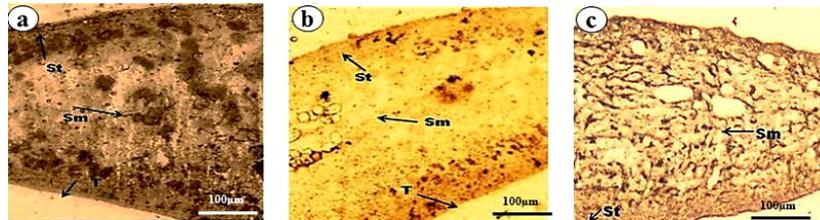


Figure 4. Alkaline Phosphatase Activity in *F. buski*, Photographs of Fresh Frozen Sections. (a) Control, (b) Shoot Extract Of *A. nigra* Treated Section, (c) Praziquantel Treated Section. [Photographs are adapted from Journal of Parasitic Diseases, 33(1&2), 2009, 48-53, Anthelmintic efficacy of ethanolic shoot extract of *Alpinia nigra* on tegumental enzymes of *Fasciolopsis buski*, a giant intestinal parasite, Roy B. and Swargiary A., Fig. no. 2, a, c and e, © Springer, with kind Permission Of Springer Science+Business Media]

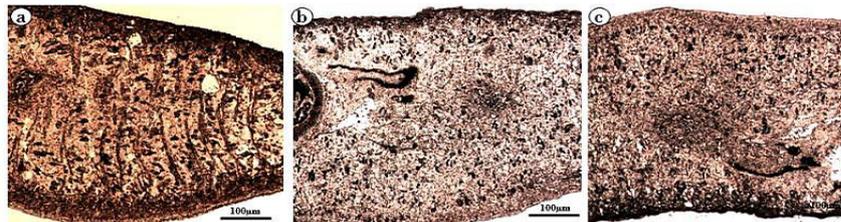


Figure 5. Adenosine Triphosphatase Activity in *F. buski*, Photographs of Fresh Frozen Sections. (a) Control, (b) Shoot Extract Of *A. nigra* Treated Section, (c) Praziquantel Treated Section

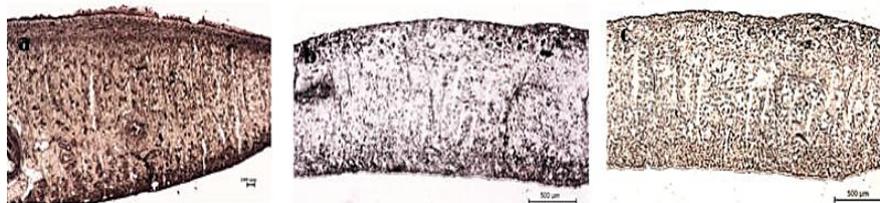


Figure 6. Malate Dehydrogenase Activity in *F. buski*, Photographs of Fresh Frozen Sections. (a) Control, (b) *A. nigra* Treated Section, (c) PZQ Treated Section Of *F. buski*

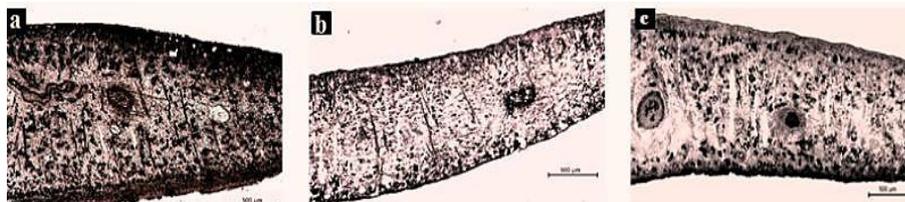


Figure 7. Lactate Dehydrogenase Activity in *F. buski*, Photographs of Fresh Frozen Sections. (a) Control, (b) *A. nigra* Treated Section, (c) PZQ Treated Section of *F. buski*

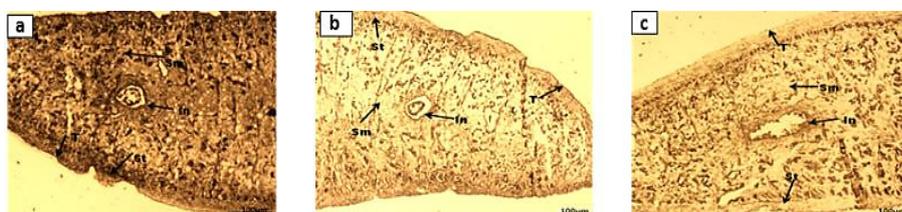


Figure 8. Acetylcholinesterase Activity in *F. buski*, Photographs of Fresh Frozen Sections. (a) Control, (b) *A. nigra* Treated Section, (c) PZQ Treated Section of *F. buski*

Table 2. *In Vitro* Biochemical Effects of Shoot- Extract of *A. nigra* and Praziquantel on *F. buski*. Values Are Given As Mean (\pm SE) From Four Replicates (N = 4), Total Activity = 1 μ mol of Product/h/g Of Wet Tissue, *Specific Activity = Total Activity/Mg Tissue Protein. [Values Of AcPase, AlkPase And ATPase Are adapted from Journal of Parasitic Diseases, 33(1&2), 2009, 48-53, Anthelmintic efficacy of ethanolic shoot extract of *Alpinia nigra* on tegumental enzymes of *Fasciolopsis buski*, a giant intestinal parasite, Roy B. and Swargiary A., Table no. 2, © Springer, with kind Permission Of Springer Science+Business Media]

Treatment (mg/ml)	Enzyme activities (Total/specific*)			
	AcPase	AlkPase	ATPase	AchE
Control	17.30 \pm 0.26/ 1.70 \pm 0.04*	37.40 \pm 0.54/ 3.93 \pm 0.09*	86.28 \pm 0.54 8.25 \pm 0.06*	22.59 \pm 0.32/ 2.63 \pm 0.12*
	9.44 \pm 0.29/ 0.97 \pm 0.04*	22.09 \pm 0.28/ 2.53 \pm 0.07*	49.17 \pm 0.53 5.06 \pm 0.09*	10.87 \pm 0.17/ 1.26 \pm 0.21*
PZQ	7.33 \pm 0.26/ 0.83 \pm 0.02*	22.04 \pm 0.27/ 2.55 \pm 0.04*	44.77 \pm 0.78 4.71 \pm 0.06*	10.01 \pm 0.24/ 1.16 \pm 0.13*

Table 3. Biochemical Effects Of *A. nigra* Crude-Extracts and PZQ on Test Parasite in *Vitro*. Values Are Given as Mean (\pm SE) From Three Replicates (N = 3), Total Activity = μ mole Of Product/min/g of Tissue Weight, *Specific Activity = Total Activity/mg Tissue Protein, P < 0.05, Statistically Significant

Treatment mg/ml PBS	Enzyme activities (Total/Specific*)			
	PEPCK	PK	MDH	LDH
Control	1.30 \pm 0.05/ 0.12 \pm 0.01*	2.13 \pm 0.12/ 0.19 \pm 0.01*	17.58 \pm 0.6/ 1.52 \pm 0.11*	7.68 \pm 0.10/ 0.71 \pm 0.02*
	0.71 \pm 0.01/ 0.07 \pm 0.01*	2.00 \pm 0.12/ 0.20 \pm 0.01*	12.90 \pm 0.03/ 1.10 \pm 0.01*	7.46 \pm 0.02/ 0.74 \pm 0.03*
PZQ	0.78 \pm 0.06/ 0.10 \pm 0.01*	1.97 \pm 0.16/ 0.20 \pm 0.02*	12.05 \pm 0.09/ 1.24 \pm 0.04*	6.84 \pm 0.07/ 0.69 \pm 0.01*

Two glycolytic enzymes, lactate dehydrogenase (LDH) and malate dehydrogenase (MDH), and one neurotransmitter enzyme, acetylcholinesterase (AchE) has also been studied through histochemical staining in order to test the efficacy of *A. nigra* against the model parasite, *F. buski*. Of the two glycolytic enzymes studied, higher staining intensity has been observed for MDH compared to LDH in control parasites which indicate the presence of higher activities of MDH enzyme in *F. buski* (Fig. 6a & 7a). On exposure to the crude extract of *A. nigra*, reduced staining intensity was observed for both the LDH and MDH enzymes (Fig. 6b & 7b). Similarly, PZQ-treated flukes also showed reduction in the staining intensity compared to the control one.

The neurotransmitter enzyme, AchE is an important enzyme which is found to be primarily associated with neuromuscular system and motor activity of the parasites[69]. Histochemically, the AchE enzyme in control parasite showed deep staining intensity of the enzyme throughout the body (Fig. 8a). Both the *A. nigra* and praziquantel treated parasites showed almost similar kind of reduction in the staining activity of the enzyme compared to their control ones (Fig. 8b, c).

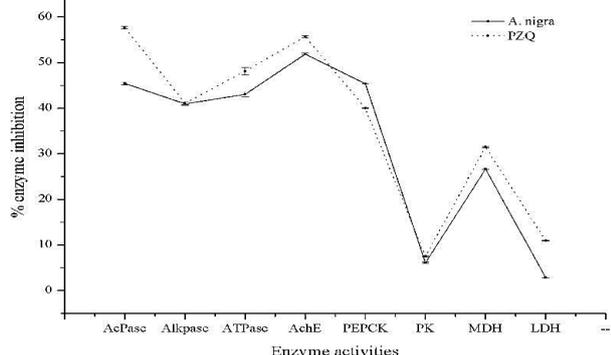
5.3. Biochemical studies

On exposure to the crude extract of *A. nigra* the activities of tegumental enzymes like AcPase, AlkPase, ATPase and AchE were found to be altered significantly in *F. buski*. All the four enzymes were found to be reduced by 40 to 50% in the parasite treated with shoot-extract of the plant (Table 2). Almost similar percentage of decrease has been recorded in the flukes treated with praziquantel compared to the control one. Biochemical analysis of large number of energy metabolism related enzymes in helminth parasites and their inhibition by different plant extract, active component of plant extract and commercial drugs suggest the extend of

their anthelmintic potential[70-73].

Glycolysis is the major energy yielding pathway in helminth parasites since the Krebs' cycle and hexose monophosphate pathways are less functional. The miracidia and cercariae are predominantly aerobic[74]. As they grow into an adult form, the energy production also shifts from aerobic to anaerobic type of metabolism. Phosphoenolpyruvate carboxykinase (PEPCK), Pyruvate kinase (PK), MDH and LDH are some of the key enzymes in the metabolic pathway of helminth parasites. Since the adult helminth parasites had to survive in a semi-aerobic or anaerobic condition of the host body, they cannot metabolise glucose completely into O₂ and H₂O. In order to survive such a harsh condition, helminth parasites change their metabolic pathway to a different one. PEPCK, PK, MDH and LDH are among the key enzymes of chemotherapeutic importance. Crude extracts of *A. nigra* were investigated to test its inhibitory activity against some of the glycolytic enzymes of fluke parasite, *F. buski*. The enzymatic activities of untreated control parasite showed high enzyme activities. MDH showed highest activity (1.52 μ M/min/mg tissue proteins) followed by LDH (0.71 μ M/min/mg tissue proteins), PK (0.19 μ M/min/mg tissue proteins) and PEPCK (0.12 μ M/min/mg tissue proteins) respectively (Table 3). Das et al.[75] studied the glycolytic enzymes of *Raillietina echinobothrida* and showed almost the similar kind of result with highest activity of MDH in control. Similarly, higher activity of LDH has been seen in the disrupted mitochondria of *Hymenolepis microstoma*[76]. The relative activities of all the enzymes studied showed more or less similar trend of reduction in the enzyme activity of *F. buski* following *in vitro* treatment to the plant extract and reference drug (PZQ). Maximum level of inhibition was observed in the activities of PEPCK, MDH and LDH (Graph 1). The activities of PEPCK and MDH were reduced by almost 31-48% and 26-36% whereas the

activities of PK and LDH were reduced to a very less extend. Both *A. nigra* crude extract-and praziquantel treated *F. buski* showed less efficacy against pyruvate kinase and LDH enzymes. Similar study was done by Navaneetha and Veerakumari[77] where the *Allium sativum* bulb-extract was found to reduce the activities of PEPCK and PK in helminth parasite, *H. contortus*. Banu *et al.*[78] screened the anthelmintic efficacy of the leaf-and flower-extract of several plants against mMDH and mME of *Setaria digitata*. All the plant-extracts were found to inhibit the activities of both the enzymes.

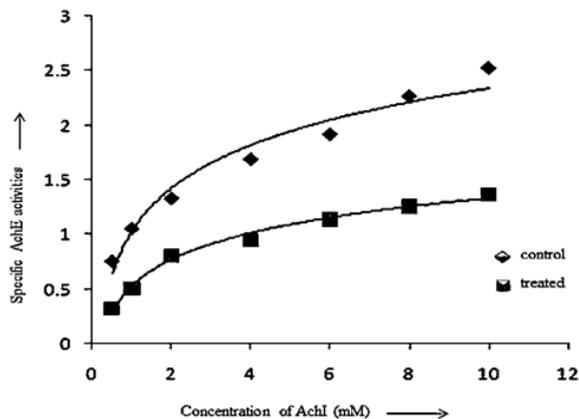


Graph 1. Showing the % Inhibition of Different Enzyme Activities on Treatment with *A. nigra* Crude-Extract and PZQ in *F. buski*

5.4. Kinetic Study

Acetylcholine (Ach) is an important cholinergic neurotransmitter in parasitic helminths which is required for proper neuromuscular activity[79]. It is synthesized from choline and acetyl coenzyme-A in presence of choline acetyltransferase. In order to continue the regular sequence of nerve impulse required for normal body mechanism, Ach is to be hydrolysed to choline and acetate with the help of acetylcholinesterase[80]. In parasites acetylcholinesterase has been found to be primarily associated with neuromuscular system and influences the motor activity. In view of the

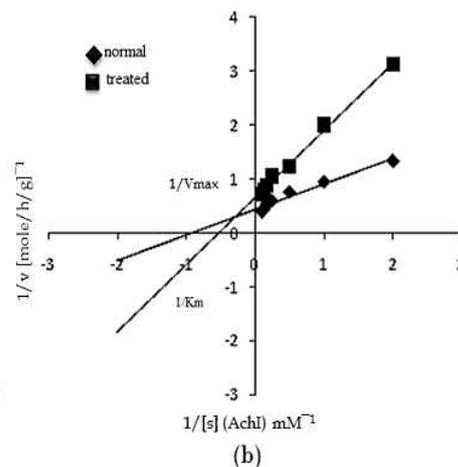
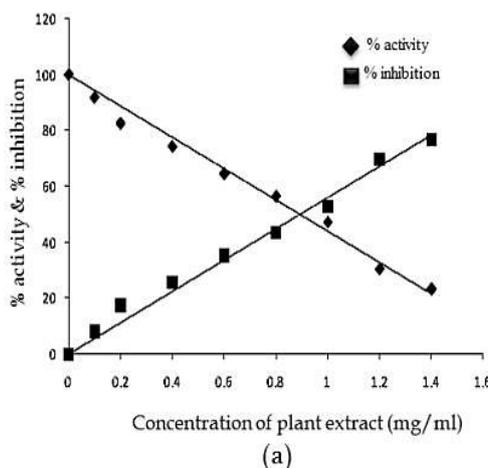
functional significance of AchE in controlling motility of parasites inside the host body, kinetic study was carried out to know the mode of inhibition through different kinetic parameters.



Graph 2. Specific Activities of Ache in Control and *A. nigra* Treated *F. buski*. [Graph was adapted from Medicinal Plants - International Journal of Phytomedicines and Related Industries, 3(2), 2011, 145-150, Kinetics of Acetylcholinesterase inhibition in *Fasciolopsis buski* and *Raillietina echinobothrida* by shoot extract of *Alpinia nigra*, an indigenous medicinal plant, Swargiary A. and Roy B., Figure no. 4, © SMP, with kind permission from SMP]

Plant extract (mg/ml)	Sp. Activities	% Activity	% Inhibition
0.0	2.69 ± 0.01	100	-
0.1	2.47 ± 0.02	91.8	8.18
0.2	2.22 ± 0.01	82.53	17.47
0.4	2.00 ± 0.02	74.35	25.65
0.6	1.74 ± 0.03	64.68	35.31
0.8	1.52 ± 0.03	56.51	43.49
1.0	1.27 ± 0.01	47.21	52.79
1.2	0.82 ± 0.02	30.48	69.52
1.4	0.63 ± 0.03	23.42	76.58

Table 4. Inhibition of Acetylcholinesterase Activities in *F. buski* On Exposure to Different Concentrations of *A. nigra* Plant Extracts (0.1-1.4 mg/ml PBS), Specific Activity = μmole/h/mg Tissue Protein, Values Are Presented In ± SE, N=3, P < 0.05 = Statistically Significant



Graph 3. (a) Showing The % Inhibition of Ache Following Treatment with *A. nigra* Plant Extract, (b) Lineweaver-Burk Plot Of Ache Inhibition By *A. nigra* Plant Extract (1 mg/ml) In *F. buski*. [Graphs were adapted from Medicinal Plants - International Journal of Phytomedicines and Related Industries, 3(2), 2011, 145-150, Kinetics of Acetylcholinesterase inhibition in *Fasciolopsis buski* and *Raillietina echinobothrida* by shoot extract of *Alpinia nigra*, an indigenous medicinal plant, Swargiary A. and Roy B., Figure no. 5 and 6, © SMP, with kind permission from SMP]

Biochemical studies of enzymes showed that the activity of AchE was reduced to a greater extent compared to other enzymes when treated with *A. nigra* plant extract and praziquantel (Graph 1). In *Fasciola hepatica*, inhibitors of AchE relaxed the musculature of the worms inhibiting rhythmical movements and eventually producing paralysis[81,82]. Similarly, decrease in the motility has been demonstrated in cestode *Dipylidium caninum*[83].

The crude homogenate of *F. buski* tissues showed high AchE activity of 2.69 units/mg tissue proteins. In accordance with the normal pattern of enzymatic reactions, increased reaction rates (Specific activities) has been seen with the increase of substrate concentrations (Acetylcholine Iodide) in the test parasite. A linear relationship has been observed between the reaction rates (or enzyme activities) and the substrate concentrations. The different concentrations of acetylcholine Iodide (AchI) when plotted against the enzyme activities showed hyperbolic curve (Graph. 2). Similarly, biochemical analysis of the parasites treated with *A. nigra* plant extract (1mg/ml) showed a similar kind of hyperbolic curve, but with reduced AchE activities compared to the control one (Graph 2). The kinetic parameters, V_{max} and K_m values were calculated from the Lineweaver-Burk plot (Graph 3a). In control fluke the V_{max} and K_m values were found to be 2.27 $\mu\text{M/h/mg}$ proteins and 1.09 mM whereas the *A. nigra* treated fluke has lower value of V_{max} (1.56 $\mu\text{M/h/mg}$ proteins) and higher values of K_m (1.96 mM).

The inhibition data of AchE activities with different concentrations of *A. nigra* plant extracts were presented in Table 4. The IC_{50} values of plant extracts obtained from the intersections points % activities and % inhibitions versus extract concentrations curves were found to be 0.91 mg/ml in *F. buski* (Graph 3a). The Lineweaver-Burk plots of the inhibitory activities of the plant extracts were shown in the Graph 3b. On exposure to the *A. nigra* plant extract (1mg/ml PBS), the V_{max} value was found to be decreased by 31.28%, while the K_m value was increased by 79.82%. Similar study was conducted by Sigurdsson and Gudbjarnason[84] with two medicinal herbs of Iceland, *Angelica archangelica* and *Geranium sylvaticum* against the AchE activity and were proved effective with IC_{50} values of 2.20 mg/ml and 3.56 mg/ml, respectively. Corynoline, the active component isolated from *Corydalis incisa* (Papaveraceae) showed significant AchE inhibitory effects with an IC_{50} value of 30.6 μM (Kim, 2002)[85]. Similarly, screening of six Indian medicinal plants by Mukherjee et al.[86] also showed AchE inhibitory activity with IC_{50} values ranging from 100-222.41 $\mu\text{g/ml}$. Therefore, kinetic studies on AchE of *F. buski* treated with crude extract of *A. nigra* showed a mixed and non-competitive type of enzyme inhibition.

6. Electron Microscopic Studies

Electron Microscopy has always been an important tool in biological sciences to understand the complex animal life and its functional anatomy. Tegument, being an important

organ of helminths for uptake of nutrients, excretion of certain metabolites, control of motility and osmoregulation, it attracts higher attention from scientific community to know and study its structure and function as well. Since, the tegument is one of the tissues most immediately exposed to anthelmintics; it is likely to represent a primary drug target. Any drug-induced disruption to the tegument is likely to have serious consequences for the fluke because it would allow the drug to penetrate to deeper-lying tissues and the damage would be exacerbated by the surfactant action of bile and the immune response.

Scanning electron microscopic (SEM) analysis of untreated *F. buski* revealed normal body contour (Fig. 9). The ventral surface of the parasite contains some scale-like papillae with distinct shape and size arranged in an alternate manner[87].

On exposure to crude extract of *A. nigra*, marked changes in the surface topography of *F. buski* has been noticed compared to the control one. The treated flukes showed a deformed body with shrunken and wrinkled tegumental surface that showed extensive pit formations and scarring due to sloughing off of the scale-like spines (Fig. 10). The ventral sucker present at the anterior part of the body also deformed to a great extent compared to the untreated flukes (Fig. 10a). It has also been observed that the severity of deformation increased with increasing concentration of plant extract and time of incubation. A similar pattern of alteration has been observed in the surface tegument of the fluke treated with the commercial drug, praziquantel. Destruction of absorptive surface tegument of several helminth parasites of public health importance was observed when treated with phytochemicals from different traditionally used medicinal plants[88-90]. Our recent studies on some of the plants like *Millettia pachycarpa*, *Acacia oxyphylla* and *Lysimachia ramosa* against several helminth parasites showed conspicuous deformity of the surface architecture in all the parasites exposed to the test plant extract and commercial drugs as well. A large number of commercially available anthelmintic drugs like clorsulon, closantel, praziquantel etc. are found to disrupt the external body surface of helminth parasites. McKinstry et al.[91] studied the effect of Nitroxylin (Trodax), a halogenated phenol containing anthelmintic drug on adult *Fasciola hepatica*. The parasites recovered from rat after 24h, 48h and 72h post-treatment showed extensive swelling and blebbing of the tegument on both surfaces with dorsal anterior region more severely affected than either the posterior dorsal region or entire ventral surface when observed under scanning electron microscope. Similar experiment was conducted by Keiser and Morson[92] in *F. hepatica* to see the anthelmintic property of two drugs artemether and artesunate. SEM analysis of the flukes incubated in the presence of drugs showed extensive tegumental damage, including sloughing, blebbing and eruptions, particularly in the ventral and dorsal mid-body and tail region of the body. Extensive blebbing of surface layer causing lesions and loss of spines have been observed in *F. hepatica*

after the treatment of deacetylated (amine) metabolite of diamphenethide[93]. Moreover, longer time exposure (24h) to the drug showed maximum damage of the tegument which has been stripped off to expose the basal lamina. A large number of studies have been performed to see the ultrastructural details of several helminth parasites, especially the tegumental regions because of its functional significance. Surface tegument of *F. buski* is outwardly covered by gly-

cocalyx layer followed by cytoplasmic syncytium. The syncytial zone of the tegument is separated from the muscle layer and the subtegumental cells by a membranous basal lamina. The syncytial layer is connected to the nucleated cell bodies or cytons by cytoplasmic tubes that are lined with microtubules. The cytons are the main cell bodies that contain cellular organelles like mitochondria, Golgi bodies etc. (Fig. 11).

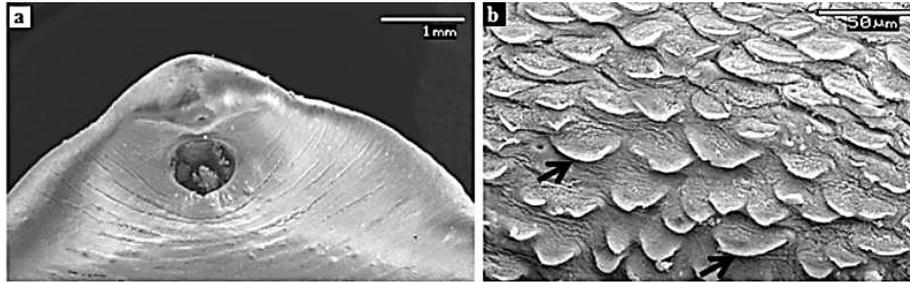


Figure 9. Surface Topography (SEM) Of *F. buski* - Control. (a) Anterior Part Of The Parasite With Normal Contour, (b) Tegument Covered With Posteriorly-Directed Scale-Like Papillae Along With Pits At Many Places.[Figure (b) was adapted from Microscopy Research and Technique, 72, 2009, 61–66, Ultrastructural Observations on *Fasciolopsis buski* and its Alterations Caused by Shoot Extract of *Alpinia nigra*, Roy *et al.*, Figure no. 1, © 2008 Wiley-Liss, Inc]

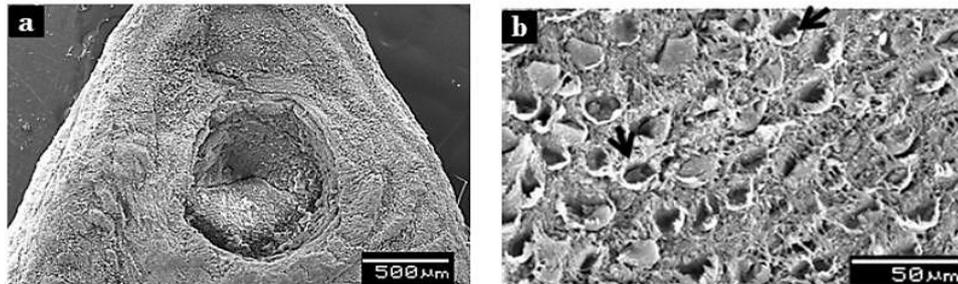


Figure 10. Surface Topography (SEM) Of *F. buski*: *Alpinia nigra* Treated. (a) Anterior Part Of The Parasite Showing Distorted And Wrinkled Ventral Sucker And Its Surrounding Parts, (b) A Portion Of The Ventral Surface Of Body Under Higher Magnification Showing Sloughed Scales (Arrows) And Scars Formation

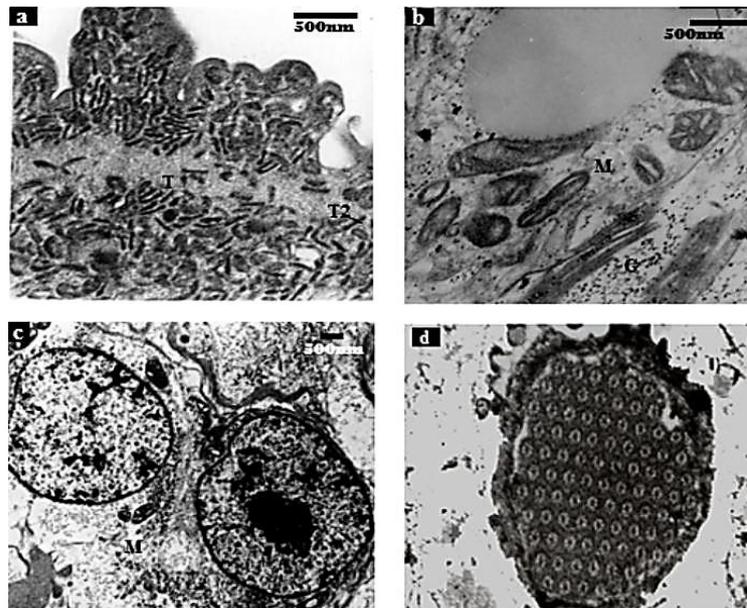


Figure 11. Transmission Electron Micrographs Of *F. buski* - Control. (a) Invegnated Tegument Showing Glycocalyx Layer, Distal Cytoplasm And Electron-Dense With T1 And T2 Tegumental Discs, (b) Photographs Showing Normal Mitochondria And Other Cell Organelles Inside The Cell, (c) Two Tegumental Cytons Lying Beside Each Other, (d) Flame Cells.[Figures (a), (b) and (c) were adapted from Microscopy Research and Technique, 72, 2009, 61–66, Ultrastructural Observations on *Fasciolopsis buski* and its Alterations Caused by Shoot Extract of *Alpinia nigra*, Roy *et al.*, Figure no. 4, 5 and 6, © 2008 Wiley-Liss, Inc]

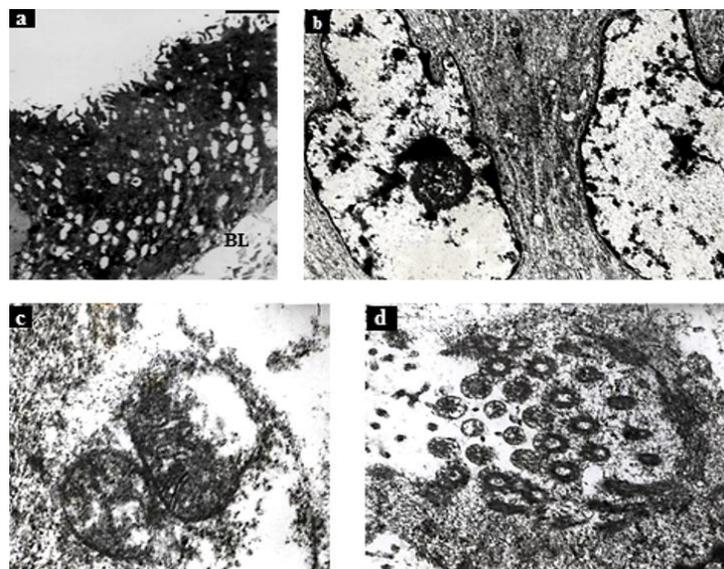


Figure 12. Transmission Electron Micrographs of *F. buski* Treated With *A. nigra*. (a) Shredded Tegument With Intensely Vacuolated Distal Cytoplasm, (b) Abnormal Shape Of Nucleus With Condensation Of Chromosome Material, (c) Distorted Mitochondria With Disrupted Mitochondrial Membrane And Cristae, (d) Disrupted Flame Cells.[Figure (a) was adapted from Microscopy Research and Technique, 72, 2009, 61–66, Ultrastructural Observations on *Fasciolopsis buski* and its Alterations Caused by Shoot Extract of *Alpinia nigra*, Roy et al., Figure no. 12, © 2008 Wiley-Liss, Inc]

Parasites treated with crude extract of *A. nigra* revealed disintegrated and vacuolated tegument at many places. Release of internal tissue materials to the exterior could also be observed through the disrupted tegument. In the tegumental syncytium, no discs were evident as it has been seen in control *F. buski*. The vacuoles in the distal cytoplasmic region were observed to move downward towards the basal lamina (Fig. 12a). The basal laminal layer has also swelled up due to treatment. In the sub-tegumental region, there was a decline in the number of tegumental cell bodies. The damage in the cytons could be seen as having highly deformed and swollen-up nuclei, and the cytoplasmic tubules connecting them with the syncytium were not prominent (Fig. 12b). The whole cytoplasmic mass was encroached upon with big vacuoles and the cytons had no defined boundaries. Although some treated cells contained normal nuclei, most had lost their double-layered membrane and were edematous, no nucleolus and had big patches of heterochromatin. Very few mitochondria were found, which did not have prominent membranes and showed electron-lucent matrix due to loss of cristae (Fig. 12c). An increased number of autophagic vacuoles were observed in the sub-tegumental cells as well as in the surrounding parenchyma. A similar kind of surface alteration also been observed among soft bodied cestode *Raillietina ehinobothrida* when exposed to the root peel-extract of *Millettia pachycarpa*, stem bark of *Acacia oxyphylla*, roots of *Potentilla fulgens*. Artemisin, one of the active principles of Chinese traditional medicine *Artemisia annua* when treated against *S. mansoni* resulted in ultrastructural alteration. The alteration could be noticed by the formation of large number of vacuoles throughout the body, focal lysis of the tegument and underlying muscles, disappearance of granular endoplasmic reticulum, lysis of mitochondria, cytoplasm etc. Beugnet et al.[94] studied the *in vitro* ultrastructural changes induced by thiabendazole, le-

vamisole, pyrantel and ivermectin in the free living larval stages of two trichostrongyles, *Heligmosomoides polygyrus* and *Haemonchus contortus*. The observed damage for each anthelmintic is related to the alteration in cellular organization, changes in mitochondria, contraction of muscle fibres, fusion and accumulation of secretory granules. Similar type of cellular alteration has also been seen in deacetylated metabolite of diamphenethide against *Fasciola hepatica*[95].

10. Conclusions

Tegumental layers of the flat worms are known to be involved in many vital functions like nutrient uptake, osmoregulation, digestion and protection from the immune attack from host. Considerable structural alteration and changes in enzyme activities, interfering with the glycolytic pathway and neuromuscular activities are suggestive of an efficient vermifugal activity of *A. nigra* shoot extract against the trematode parasite. Thus, there is a considerable evidences that the crude extract of the plant have the potential to act as anthelmintic. Further, *in vitro* and *in vivo* investigation involving active components of the plant, namely astragalins and kaempferol-3-o-glucuronide is under way to fully assess the anthelmintic potential of the plant and their cytotoxic activity, if any, in the host.

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