Int J Pharm Bio Sci 2015 Oct ; 6(4): (P) 395 – 403

Research Article Pharmacognosy

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

ASTRAGALIN, THE ACTIVE COMPONENT OF *ALPINIA NIGRA* AND ITS EFFECT ON TEGUMENTAL ULTRASTRUCTURE OF FLUKE PARASITE, *FASCIOLOPSIS BUSKI*

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ABSTRACT

Alpinia nigra belonging to the family Zingiberaceae is an ethnomedicine with established anthelmintic property. In view of its medicinal property, the present study was designed to investigate the anthelmintic property of astragalin, a bioactive compound of *A. nigra* against fluke parasite, *Fasciolopsis buski*. *In vitro* exposure of flukes at varying concentrations 0.025 – 0.1 mg/ml of PBS a dose-dependent effect on motility and mortality was observed. Stereoscan observations of the astragalin exposed fluke revealed deformity of the surface architecture including shrinkage and eroded scales leading to scar formation as well as loss of the scales. Ultrastructural observations revealed distortion of glycocalyx, musculature region, basal layer and disintegration of the nucleus, nucleolus, nuclear membrane, Golgi complexes and mitochondria. A large number of vacuole formations could also be seen in flukes exposed to astragalin compared to control. The result therefore indicates the anthelmintic property of astragalin, the bioactive compound of *A. nigra* against the fluke parasite *F. buski*.

KEYWORDS: **Astragalin, *Alpinia nigra*, anthelmintic, tegumental ultrastructure, *Fasciolopsis buski***

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INTRODUCTION

Helminths are a group of organisms encompassing both acoelomate flatworms and the pseudocelomate nematodes that inhabit a variety of habitats including vasculature, lungs, tissue spaces, gut and bile duct of their hosts. Tegument (Trematode and Cestode) and cuticle (Nematode), the external covering of helminth parasites is an important organ that protects them from adverse conditions of the host and also helps in many other functions such as uptake of nutrients, excretion of metabolites, control of motility, and osmoregulation etc.[1]. It acts not only as the immediate barrier of anthelmintics but also perform as the main site of host-parasite interactions[2,3]. Any disruptions or damages to the tegumental surface are likely to cause serious consequences in the parasites because it would allow the drug and other unnecessary materials to penetrate to underlying tissues[4-6]. Therefore, it attracts great attention from the scientific community to know and study its structure and function and exploit it as chemotherapeutic target. Many parasitologists throughout the world have revealed the effectiveness of different anthelminthic drugs in altering the surface topography as well as ultrastructure of tegumental region of the helminth parasites[7-10]. Anthelmintics like clorsulon, closantel, mebendazole, artemether, thiabendazole, levamisole, pyrantel, praziquantel, ivermectin etc. were found to damage the external body surface of several helminth parasites[11-14]. However, the development of single or multiple anthelmintic drug resistance among gastrointestinal helminths has brought hurdles in effective controlling of helminthiases[15,16]. High treatment frequency, single-drug regiment or frequent use of the same anthelmintic, prophylactic mass treatments, under dosing etc. generally are considered to be some of the important factors for development of drug resistance that might allow the survival of heterozygous resistant worms[17-18]. Because of these reasons, scientists therefore are now looking for new drugs based on traditional knowledge and traditionally used medicinal plant-derived phytochemical(s) as an alternative remedies for controlling helminthiases. With a rich source of phytochemicals, plant kingdom has always been a source of healthcare system in India and world as well[19]. Several medicinal plants and plant-derived phytochemicals have been isolated and their potentiality of curing several diseases including worm infections have been identified and established by many workers throughout the world[20-25]. *Alpinia nigra* (Family Zingiberaceae) is one such medicinal plant, the aqueous extract of which is consumed by the tribe of North-East India in order to get rid of helminthiasis. The plant has also been reported to possess medicinal property against bone weakness, irregular menstruation, jaundice and gastric ulcers[26]. Astragalin and kaempferol-3-O-glucuronide are found to be the two major bioactive flavone glycosides of *A.* nigra[27,28]. Earlier studies in our laboratory have established the effectiveness of *A. nigra* crude extracts against several helminth parasites. However, to the best of knowledge, no such scientific studies have been done on the surface topographical and ultrastructural aspects in *F. buski* treated with the astragalin, the bioactive component of *A. nigra*. Therefore, in an effort to study the anthelmintic property of astragalin, the present study was designed to investigate the *in vitro* effects of astragalin against *Fasciolopsis buski*.

METHODS AND MATERIALS

In vitro Treatment

Live adult *F. buski* were collected from freshly sacrificed pig intestine in 0.9% phosphate buffered saline (PBS, pH-7.4) from a pig slaughterhouse at Mawlai, Shillong. The worms were then incubated in different concentrations of astragalin (0.025, 0.05 and 0.10 mg/ml PBS) having 0.1% dimethylsulphoxide (DMSO). Praziquantel was used as standard reference drug while simultaneously maintaining control in PBS having 0.1% DMSO at 37 ± 1ºC. The time taken for paralysis was recorded and mortality was confirmed by dipping the worm in slightly warm PBS to notice any sort of movement if any. All the experiments were carried out for three replicates and time taken for attaining paralytic state and death was recorded as described earlier[11]. The paralysed flukes were processed for electron microscopic studies.

Electron Microscopy

Both control and astragalin exposed worms were fixed in modified karnovsky's fixative for 4 hr followed by post fixation in 2% OsO_4 buffered in 0.2 M sodium cacodylate. For scanning electron microscopy, fixed specimens were dehydrated through a series of ascending grades of acetone followed by air drying after treatment with tetramethylsilane following Dey *et al.*[29] modified by Roy and Tandon[30]. Gold coated specimens were viewed using JSM-6360 scanning electron microscope. For transmission electron microscopy, the fixed specimens were dehydrated in a graded series of acetone, cleared in propylene oxide and embedded in araldite. Ultrathin sections (600 – 900Å) were collected on uncoated copper grids, stained with 5% aqueous Uranyl acetate solution followed by Lead citrate and viewed in JEM 2100 (JEOL) transmission electron microscope.

Statistical Analysis

The data are represented as mean ± SEM (standard error of mean). The comparison of the mean of the experimental data and the control was done using Student's t test taking $p < 0.05$ as the threshold of significance.

RESULTS

On exposure to different concentrations of astragalin, bioactive compound of *A. nigra* at doses 0.025, 0.05 and 0.10 mg/ml of PBS, the fluke became paralyzed at 15.79 ± 0.71, 13.56 ± 1.28 and 9.76 ± 2.05 h, while complete loss of motility and death of *F. buski* was observed within 16.70 ± 0.14, 15.44 ± 0.51 and 10.78 ± 0.77 h of incubation, respectively (Table 1). However, the reference drug PZQ paralysed the parasite earlier than astragalin taking 9.62 ± 4.18, 8.77 ± 0.81 and 6.76 ± 1.44 h leading death taking 11.26 ± 2.55, 9.57 ± 2.03 and 8.37 ± 1.27 h when incubated in an increasing concentrations 0.025, 0.05 and 0.10 mg/ml PBS, respectively. The control flukes survived for 72.03 ± 0.08 h.

Table 1

Effect of different concentrations of bioactive compound astragalin compared to reference drug praziquantel in terms of motility and mortality of helminth parasite, F. buski.

Incubation medium	Concentration of Astragalin and PZQ					
	0.025 mg/ml PBS		0.05 mg/ml PBS		0.10 mg/ml PBS	
	Paralysis	Death	Paralysis	Death	Paralysis	Death
Astragalin	15.79 ± 0.71	16.70 ± 0.14	13.56 ± 1.28	15.44 ± 0.51	9.76 ± 1.05	10.78 ± 1.47
Praziquantel	9.62 ± 1.18	11.26 ± 1.33	8.77 ± 0.81	9.57 ± 2.03	6.76 ± 1.44	8.37 ± 1.27
Control		72.03 ± 0.08				

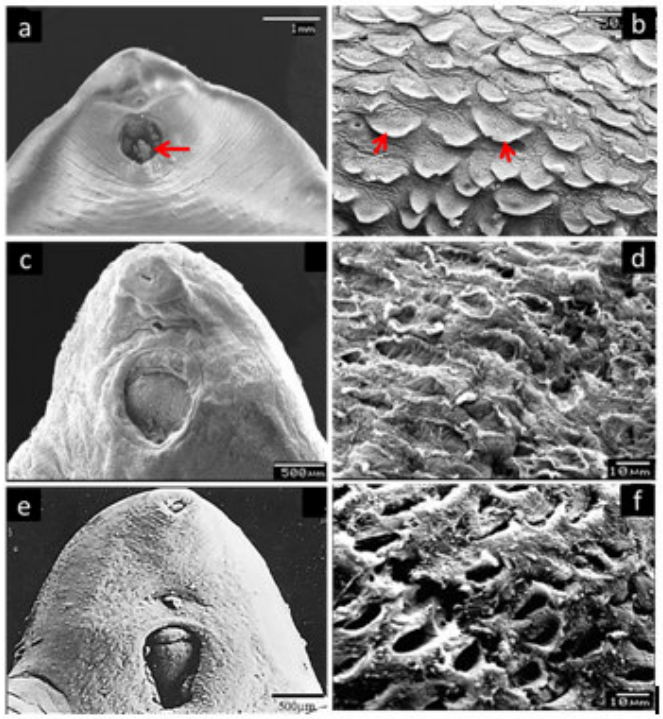
Values are expressed as mean ± standard error, (N = 3). All the values are significant at p < 0.05.

Scanning electron microscopy

Control

The untreated control *F. buski* reveals normal body contour with scale-like projections on the ventral surface. Surface topography studies revealed surface invaginations forming deep pits throughout the body surface, including the surface of scales (Fig. 1a). Structurally, the scales are broader at the base (15 - 45 µm), sharply rounded off at the margin and are posteriorly directed (Fig. 1b).

Figure 1
Scanning electron micrographs of control (a & b), astragalin (c & d) and praziquantel (e & f) treated Fasciolopsis buski



a. ***Anterior end of parasite showing normal morphology, arrow showing the acetabulum and surface invaginations (arrows).***
b. ***Tegument covered with posteriorly directed scale-like papillae (arrows).***
c. ***Anterior part of the parasite showing deformed oral and ventral sucker.***
d. ***Ventral region of body showing completely eroded scales leading to scar formation.***
e. ***Deformed anterior part of the body.***
f. ***Ventral region of the body showing totally destructed and pitted surface.***

Treated

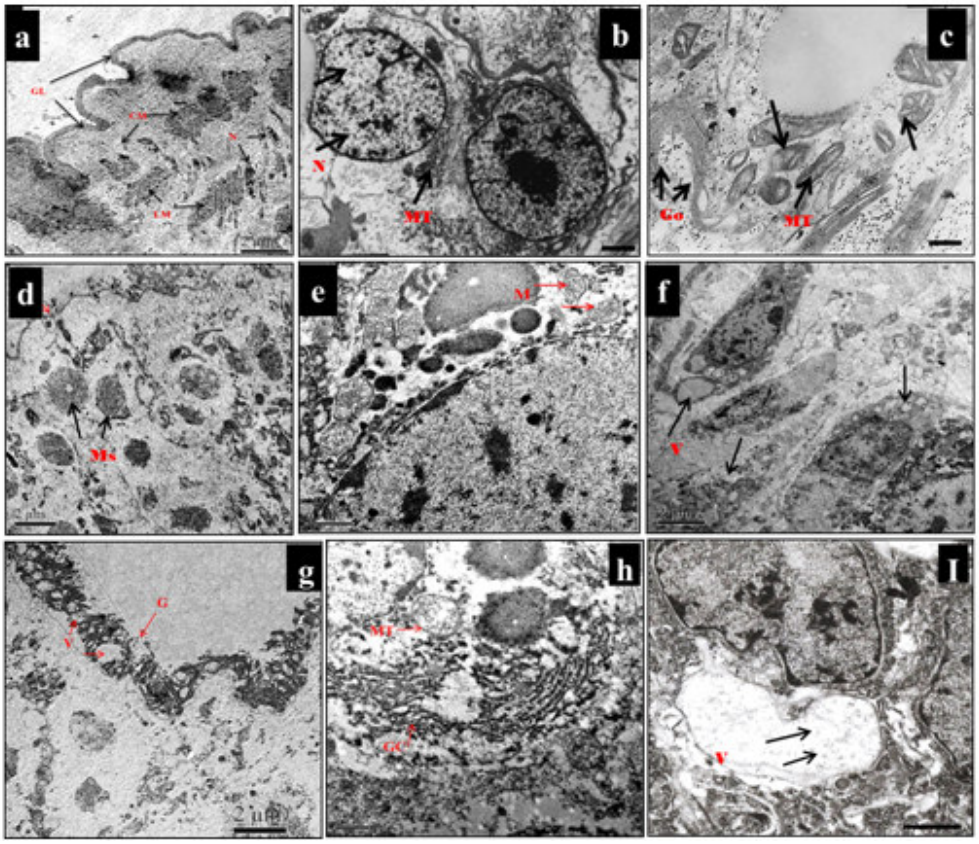
On exposed to the *A. nigra* bioactive compound astragalin, alterations in the normal surface topography have been observed (Fig. 1c, d). The treated flukes showed a deformed body with shrunken and wrinkled tegumental surface showing extensive pit formations and scarring due to sloughing off of the scale-like spines. Massive destruction and deformations were noticed in the surface topography of the parasites exposed to astragalin treatment. At the base of the scales large holes like pits were seen in treated parasites (Fig. 1d). Similarly, the commercial drug PZQ also showed anthelmintic efficacy as per the surface topographical changes in fluke parasite is concerned (Fig. 1e, f). Severe damage with complete distortion and erosion of scales has been revealed in both the treated flukes.

Transmission electron microscopy

Control

The tegument has a deeply invaginated apex and is outwardly covered by 50 - 70 nm thick glycocalyx layer followed by distal cytoplasm and sub-tegumental layer (Fig. 2a). Distal anuclear syncytium is followed by a 300 - 500 nm thick fibrous basal lamina. Below the basal layer, musculature region consisting of circular and longitudinal muscle could be seen. Tegumental cells lying beneath the sub-tegumental muscle maintain good connections with each other and with the surrounding parenchyma as well. These cells as well as the muscle cells have abundant cell organelles including mitochondria which have prominent cristae and dense matrix (Fig. 2a-c). Ultrastructural studies in control fluke revealed normal, rounded and double-layered nuclear membrane nuclei in the cytons (with no swelling of the perinuclear space), granular nucleolus, nucleoplasm, and chromatin material (Fig. 2b). Golgi complexes containing dense flocculent material composed of stacked concave cisternae and a few vesicles could also be observed (Fig. 2c).

Figure 2
Transmission electron micrographs of control (a-c), astragalin (d-f) and praziquantel (g-i) treated Fasciolopsis buski



a. *Typical tegumental structure showing outer glycocalyx layer (GL), circular (CM) and longitudinal muscle (LM) and cyton showing large number of nuclei (N).*
b. *Enlarged view of cyton showing nuclei (N), and mitochondria.*
c. *Tegumental cyton with normal Golgi complex (Go) and mitochondria (MT) with distinct cristae.*
d. *Shredded tegument with normal musculature (Ms).*
e. *Enlarged view of the tegumental cyton showing damaged mitochondria (MT).*
f. *Cyton showing vacuolation (V).*
g. *Shredded glycocalyx layer (G) with vacuolated (V) distal cytoplasm.*
h. *Tegumental cytons showing damaged mitochondria (MT) and Golgi complex (GC).*
i. *Distorted nuclei with large vacuole (V) formation.*

Treated

F. buski exposed to different treatments viz., astragalin and reference drug PZQ showed changes in the tegumental ultrastructure of the parasites. Astragalin treated fluke revealed shredded tegumental layer with large number of vacuole formation, swelling of basal lamina layer and damage of mitochondrial structures (Fig. 2d-f). In the tegumental syncytium distorted nuclei with fragmented chromatin materials as well as increased number of vacuoles were observed in the sub-tegumental cells (Fig. 2e, f). Similarly, PZQ also showed ultrastructural changes with shredded glycocalyx layer of severe vacuolization as well as damage in mitochondria and nuclear membrane (Fig. 2g-i). Damages in the Golgi complex have also been seen the *F. buski* treated with PZQ (Fig. 2h).

DISCUSSION

In trematodes, the general body surface acts as a vital structure for attachment to the host, nutrient uptake, immunoprotection, osmoregulation, and sensation[1]. Present study revealed major injuries in the tegumental surface of the test parasite along with severe disruption of the underlying structures. Similarly, destruction of absorptive surface have been seen in many helminth parasites on exposed to different drugs - mebendazole, praziquantel, oxyclozanide and extract of several botanicals and their phytochemicals[31-32]. Studies have also showed conspicuous deformity in the surface

architecture of parasites on exposed to the *Lysimachia ramosa* plant extract[33]. Alcoholic extracts of *Acacia oxyphylla* and *Securiniga virosa* were found to cause alternation in the tegumental morphology of cestode parasite, *R. echinobothrida*[34]. In a similar way, surface blebbing and severe lesions were observed in *F. gigantica* when the parasite was incubated with extracts of *Siwa propolis*[35]. Surface changes to the adult fluke *Fasciola hepatica*, when treated with genistein, comprised swelling and blebbing, especially in the posterior region and there was particular disruption to the spines, accompanied by loss of spine[36]. *Toxocara vitulorum* when incubated with 10 µg/ml albendazole showed swelling of their anterior ends, retraction of the lips which exhibited some focal areas of severe swelling. The cuticular surface of the body including the lips showed prominent wrinkles, and erosion extended to some areas[37].Ultrastructurally, the tegumental architecture of *F. buski* conformed to generalized structure of digenean trematodes[38]. Formation of numerous vacuoles, distortion of nucleus, nuclear membrane, damage of mitochondria etc. on exposure to different treatment is evident from our study. Similar to our study, *in vitro* ultrastructural changes were found to be induced by thiabendazole, levamisole, pyrantel and ivermectin in the free living larval stages of two trichostrongyles, *Heligmosomoides polygyrus* and *Haemonchus contortus*[14]. The disruption of surface tegument in *Opisthorchis viverrini* on exposure in amoscanate have been correlated with the osmotic imbalance in parasite that resulted impaired ion transfer[39]. In accordance to our present study which revealed a large number of vacuole formation in the tegument and sub-tegumental regions of *F. buski* on treatment astragalin and reference drug PZQ; a similar kind of vacuolization as well as severe distortion with disorganization of tegumental musculature was seen in cestode parasite, *R. echinobothrida* when exposed to extracts of *F. vestita*, genistein and PZQ[33]. Extensive structural alteration of tegument indicates that the plant extract and its active component alter membrane permeability of the parasite leading to paralysis and subsequent death. The plant-derived chemicals also caused extensive vacuolization in the parasite's tegument, which led to the release of tissue material to the exterior. The vacuolization of the tegument leads to disruption of the apical tegumental layer which eventually caused the parasite to die. The surface blebbing of the parasite as seen in the present study may have led to the vacuolisations in tegument[33]. It has been known that the vacuolization and loss of tegumental matrix are because of the formation of surface blebs[40]. This vacuolization with early signs of separation of the syncytium from the basal lamina probably corresponds to the scar formation on the surface of the fluke along with the deformity of the spines[41]. Fine structural changes within the tegumental syncytium and tegumental cells were revealed in *F. hepatica* when the parasites were exposed to triclabendazole and clorsulon[42,43]. Recent studies have revealed wide scale destruction of the tegument with intense vacuolization of the syncytium and swellings of the basal lamina accompanied by deformities in the cell organelles in *R. echinobothrida* exposed to *S. virosa* and *Amomum maximum* extract, respectively[36,25]. A pronounced damage was observed in the mitochondrial membranes and cristae in the present study, which might be due to a general stress brought by the effect of the plant-derived components[13]. Onset of flaccid paralysis, disintegration of the surface tegument and necrosis of the worm tissue, occurring under the influence of anthelmintics have been correlated with inhibition of neuromuscular activity, disturbance in ion flux across the membrane and altered membrane transport, and osmoregulation together with accelerated myelin degeneration, and autophagy by host immune system[44-47]. Drugs like albendazole and its related compounds enter the parasite body through simple diffusion and cause disruption in the tegument and muscle layers in helminth parasites leading to vacuolization or pit formation due to disturbances in the Ca^{2+}-ion flux across the membrane[48].

CONCLUSION

Tegument, ultimate boundary between the flatworm parasite and its host is of crucial importance to helminth parasites that is known

to be involved in many vital functions. The severe topographical and ultrastructural changes brought about in the surface body or tegument of the fluke parasite on exposure to *A. nigra* bioactive compound, astragalin as well as reference drug praziquantel may account for the loss of body movement, which ultimately led to death through a preceding paralytic state. The tegumental interface of *F. buski* seems to be a target organ for the active components of *A. nigra* to exert its vermicidal effect. The activity of any anthelmintic drug or plant extract depends not only on its binding to the specific receptor (pharmacodynamics) but also on its ability to reach high and sustained concentrations at the location of the parasite to enable the delivery of effective drug concentrations at the receptor in the parasite cells and in sufficient time to induce the anthelmintic effect. The various ultrastructural changes observed in the present study may be due to the interference of astragalin with the functioning of numerous receptors or protein or enzymes and thereby leading to death of the treated parasites within a particular time period. However, in view of the potential anthelmintic efficacy of the plant-derived components, the anthelmintic active principle(s) of *A. nigra* needs to be isolated and identified to understand their mode of action.

ACKNOWLEDGEMENT

SEM and TEM facility of SAIF, NEHU is gratefully acknowledged.

CONFLICT OF INTEREST

Authors declare conflict of interest none.

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