CHAPTER - 5

Histochemical, biochemical and kinetic studies of some tegumental and carbohydrate metabolism related enzymes
5.1. INTRODUCTION

Parasitic helminths are a phylogenetically diverse group, encompassing both the acoelomate flatworms and the pseudocelomate nematodes. Parasites from both groups inhabit a variety of habitats including the vasculature, lungs, tissue spaces, gut and bile duct of their hosts. These habitats exhibit a wide range of physicochemical characteristics, including temperature, redox potential, pH and oxygen concentration. Parasites have complex life cycles, which can comprise of multiple host species as well as free living stages (Marr et al. 2002). The absence of fully developed digestive tract in parasitic helminth compels them to search for other mechanisms whereby food and energy requirements can be obtained. The outermost covering of flatworms plays important physiological functions such as uptake of nutrients, excretion of certain metabolites, and control of motility and osmoregulation etc. (Bennett et al. 1980; Mansour 2002; Cumino et al. 2012). A large number of important enzymes are anchored in the tegument, such as glutathione S-transferase, ATP diphosphorylase, alkaline and acid phosphatases, adenosine triphosphatase, 5′-nucleotidase, β-glucuronidase, amino peptidase, acetylcholinesterase, phosphofructokinase, glucose transporters, serine hydrolases and several glycolytic enzymes (McManus and Barret 1985; Pal and Tandon 1998; Kar and Tandon 2004; Roy and Swargiary 2009; Roy et al. 2010b). Membrane enzymes such as alkaline- and acid phosphatases, 5′-nucleotidases and maltase are proteins predominantly bound to external surface membrane; on the other hand, phosphodiesterase, adenosine triphosphatase, leucine aminopeptidase and gamma-glutamyltranspeptidase were found apparently associated with the internal membrane (McManus and Barret 1985; McManus 2009). Because of its essential physiological functions in helminths, tegument has been an important target for many chemotherapeutic anthelmintics. Commercial anthelmintics like parbendazole, piperazine adipate, phenothiazine, diethazine, diethylcarbamazine, centperazine, tetramisole and levamisole alter the metabolism and disrupt mitochondrial energy formation, resulting in decrease in ATP levels (Agarwal et al. 1990; Aggarwal et al. 1992; Vinaud et al. 2009). Activities of many of these enzymes are reported to be altered by crude ethanolic extract of traditionally used medicinal plants too, like Flemingia vestita, Acacia oxyphylla, Millettia pachycarpa, Alpinia nigra, Potentilla
fulgens etc. (Tandon and Das 2007; Lalchhandama et al. 2008; Roy and Swargiary 2009; Roy et al. 2010).

The discovery of complex pathways and the details of the enzyme systems involved in the energy metabolism of helminth parasites, have encouraged biochemists to investigate the metabolism in those parasites. Biochemists who are interested in the chemotherapy of these parasites looked at these studies as a way of finding reactions that are unique to the parasites and that enzymes catalysing these reactions could be targets for new antiparasitic agents (Mansour 2002). It is now clear and well documented that striking differences do exist between the energy metabolisms of host and parasite. Such differences range from the levels of protein structure and enzyme kinetics to a higher level of metabolic pathways, nutritional requirements, and physiological functioning of corresponding tissues (Saz 1971). Metabolic adaptations normally require different enzymes that are not present in the host organisms or parasite specific modification of already present enzymes so that energy (ATP) metabolism can be carried out. Thus, identification of the differences in energy metabolism pathways between parasites and their hosts can be expected to reveal new parasite-specific drug targets (Upcroft and Upcroft 2001; Seeber 2003; Ribeiro-dos-Santos et al. 2006; Abdulla et al. 2007; Wiesner et al. 2008).

A large number of commercially available anthelmintics like PZQ, LEV, MBZ, FBZ and ALB found to interfere with the metabolic pathways involving LDH, MDH, HK, G6PDH and PEPCK of different helminth parasites (Oztop et al. 1999; Veerakumari and Munuswamy 2010). Similarly, the crude extract of various plants and their active component(s) were shown to induce flaccid paralysis and alteration in the various tegumental and glycolytic enzymes of trematodes and cestodes (Das et al. 2004; Roy and Swargiary 2009; Roy et al. 2010b; Swargiary and Roy 2011).

Nafuredin, a potentially new anthelminthic appears to specifically inhibit anaerobic helminth electron transport at the level of Complex-I (Omura et al. 2001). However, in spite of years of extensive studies many key areas and questions remain partially defined and unresolved. Clearly, energy generation in parasitic helminths differs substantially from that of the host, and remains an important target for chemotherapy. Limited knowledge of the biochemical pathways of parasitic helminth is still the major impediment to identify promising novel chemotherapeutic targets. Therefore, to continue to exploit physiological and metabolic functioning of helminth as a tool for a rational approach to chemotherapy, some of the important tegumental
as well as metabolic enzymes were investigated in the present study after treated the fluke *F. buski* with the extract of *A. nigra* and its active compound astragalin.

**5.2. MATERIALS AND METHODS**

**5.2.1. Histochemical localization of enzymes**

The following enzymes were investigated histochemically using duly processed frozen sections cut at a thickness of 10-15 μm in a Leica CM 1850 cryostat.

5.2.1.1. Acid phosphatase (AcPase)

AcPase activity was detected in cold formol-calcium fixed specimen following the modified Lead nitrate method (Pearse 1968), using sodium β-phosphoglycerate as the substrate.

5.2.1.1.1. Tissue preparation

Adult flukes (control and treated *F. buski*) were fixed in cold formol-calcium (prepared by mixing 4% formaldehyde with 1% CaCl₂ at pH 7.0) at 4°C overnight. The fixed parasites were washed extensively with distilled water and processed for frozen sectioning. Small pieces of parasite were fixed on a tissue holder and frozen for about 20 - 30 min in a Leica CM 1850 cryostat at 23-25°C, followed by cutting sections at a thickness of 10-12 μm. Cut sections were incubated in an incubation medium for 2 hours at 37 ± 1°C in an incubator [Incubation medium: 10 ml of 0.1 M sodium acetate buffer, pH 5.0, 20 ml of 2% β-glycerophosphate dissolved in the above buffer, 10 ml of 2% lead nitrate and 3 ml of 1% MgCl₂]. After staining, the sections were rinsed in distilled water and then developed in ammonical-silver nitrate solution (prepared by adding 28% ammonia water drop by drop to 5% aqueous AgNO₃ till the precipitates dissolves) for 2-3 min. Finally, the sections were rinsed in 5% sodium thiosulphate solution for 2-3 min. A brownish black deposition indicates the site of acid phosphatase activity.

5.2.1.2. Alkaline phosphatase (AlkPase)

A modified coupling azo-dye method as described by Pearse (1968) was used for the detection of AlkPase activity. The brown colour was observed with fast violet-B.

5.2.1.2.1. Tissue preparation

Adult flukes (control and treated) were fixed in 10% neutral buffered formalin (NBF) at 4°C for 10 - 16 h. Fixed parasites washed extensively with distilled water and processed for frozen sectioning. Small pieces of parasite was fixed on a tissue holder and frozen for about 20 - 30 min at 25°C. Duly frozen parasite was cut into sections.
at a thickness of 10 - 12 µm in a Leica CM 1850 cryostat at 23-25°C. Cut sections were then incubated in a freshly prepared incubation medium for 2 h at 37±1°C containing 10 mg sodium-α-naphthyl phosphate, 20 mg Fast Violet-B mixed in 20 ml 0.1 M Tris-HCl buffer, pH 9.5. After incubation, the slides were washed in running tap water for 1 - 3 min. The slides were allowed to dry and mounted in glycerine jelly and observed in Leica DM 1000 microscope. A blackish coloured deposition indicates the site of alkaline phosphatase activity.

5.2.1.3. Adenosine triphosphatase (ATPase)

ATPase activity was localised following the Calcium method as described by Pearse (1968).

5.2.1.3.1. Tissue preparation

Control and treated *F. buski* were processed directly for frozen sectioning. Small pieces of parasite was frozen and cut into sections at a thickness of 10-12 µm in a Leica CM 1850 cryostat at 23 - 25°C. Cut sections were incubated in a freshly prepared incubation medium for 10 min at 37 ± 1°C. [Incubation medium: 5 mg of Na₂3-ATP dissolved in 10 ml of 0.1 M sodium glycine buffer mixed with 0.75 M CaCl₂, pH 9.6; adjusted with 0.1 M NaOH]. After the incubation is over, the sections were rinsed well in distilled water. Immersed in 2% cobaltous chloride (CoCl₂) for 2 minutes. Rinsed well with distilled water again for 3 to 4 times with tap water. Immersed in dilute (1:10) ammonium sulphide solution for 30 seconds and rinsed well in running tap water and finally mounted in glycerine jelly. The enzyme activity was determined through observation of blackish brown deposit.

5.2.1.4. Acetylcholinesterase (AchE)

Acetylcholinesterase activity was localised following the method of Karnovsky and Roots (1964) with little modification (Pearse 1968).

5.2.1.4.1. Tissue preparation

Control and treated *F. buski* were processed directly for frozen sectioning. Small pieces of parasite were fixed on a tissue holder and frozen for about 20 - 30 min at 25°C. Duly frozen parasite was cut into sections at a thickness of 10 - 12 µm in a Leica CM 1850 cryostat at 23 - 25°C. Cut sections were then incubated in a freshly prepared incubation medium for 30 min at 37 ± 1°C containing 6.5 ml of 0.1 M sodium acetate buffer, pH 6.0, 0.5 ml of 0.1 M sodium citrate, 1 ml each of 30 mM copper sulphate, 5mM potassium ferricyanide (0.165 g/100 ml) and distilled water and 5 mg acetylthiocholine iodide was added to the mixture. After incubation, the
sections were rinsed in distilled water, mounted in glycerine jelly and viewed in Leica microscope. The enzyme activity was determined through observation of blackish brown deposit.

5.2.1.5. Lactate dehydrogenase (LDH)
LDH enzyme activity was localised following the method of Pearse (1972).

5.2.1.5.1. Tissue preparation
Control and treated *F. buski* were processed directly for frozen sectioning. Small pieces of parasite were fixed on a tissue holder and frozen for about 20 - 30 min at 25°C. Frozen pieces were cut into sections at a thickness of 10 - 12 µm in a Leica CM 1850 cryostat at 23 - 25°C and incubated in a freshly prepared incubation medium for 30 min at 37 ± 1°C [Incubation medium consists of a mixture of equal volumes of solutions (a) and (b) and 1.5 mM NAD⁺ was added to it]. After the incubation is over, sections were rinsed in distilled water, dry for some time and mounted in glycerine jelly. Stained sections were viewed in a Leica microscope. Depositions of blackish brown stain in the tissue sections indicates the activity of LDH.

*Solutions*
Solution (a): Consists of 0.2 M L-lactate, 9.8 mM nitroblue tetrazolium (NBT), 10 mM NaCN, 0.66 mM PMS, 3.3 g polyvinyl alcohol, all dissolved in 100 ml of 0.1 M Tris-HCl buffer, pH 7.2.
Solution (b): 4% polyvinyl alcohol prepared in 50 mM Tris-HCl buffer.

5.2.1.6. Malate dehydrogenase (MDH)
MDH enzyme activity was localised following the method of Pearse (1972).

5.2.1.6.1. Tissue preparation
Adult flukes (control and treated) were processed directly for frozen sectioning. Duly frozen parasites were cut into sections at a thickness of 10-12 µm in a Leica CM 1850 cryostat at 23-25°C. Cut sections were incubated in a freshly prepared incubation medium for 10 to 15 min at 37±1°C [Incubation medium contains 2 mg NAD⁺ mixed in 0.1 ml solution of (a) and 9 ml solution of (b)]. After the incubation is over, sections were rinsed in distilled water, allowed to dry for some time and mounted in glycerine jelly. Observation was carried out in Leica microscope. Depositions of blackish brown stain in the tissue sections indicates the activity of LDH.
Solution (a): 1 M L-lactate dissolved in 0.2 M Tris-HCl buffer, pH 7.4 neutralised by 40% NaOH.

Solution (b): Mixture of 2.5 ml of (0.4%) NBT, 2.5 ml of 0.2 M Tris-HCl buffer (pH 7.4), 1 ml of 5 mM MgCl₂ and 3 ml distilled water (Total volume 9 ml).

5.2.2. Biochemical studies

5.2.2.1. Acid phosphatase

AcPase activity was quantitatively measured by estimating the p-nitrophenol formation following the method of Plummer (1988) with some modifications. Kinetic study of AcPase was carried out following the method of Mazorra et al. (2002) with little modification.

\[
\text{Acid phosphatase} \quad \text{p-Nitrophenyl phosphate} + \text{H}_2\text{O} \rightarrow \text{p-Nitrophenol} + \text{Pi}
\]

In presence of the substrate p-nitrophenyl phosphate, acid phosphatase hydrolys its substrate to form a coloured compound p-nitrophenol which is measured colorimetrically at 410 nm. The substrate p-nitrophenyl phosphate as well as the substrate p-nitrophenol does not absorb at 410 nm in acidic conditions and therefore the progress in enzyme catalysed reaction can be followed after a fixed incubation time and by stopping the reaction with alkali solution like NaOH.

5.2.2.1.1. Solutions/Reagents

Stock solutions: sodium acetate buffer (0.1 M, pH 4.5), p-nitrophenyl phosphate (p-NPP) (2.5 mM), MgCl₂ (27 mM), NaOH (0.2 N) and tissue supernatant (10% w/v).

5.2.2.1.2. Tissue homogenate

A 10% (weight/volume) tissue homogenate was prepared in sodium acetate buffer (100 mM, pH 4.5) using a remi-motor driven homogenizer fitted with Teflon pestle. The homogenate was centrifuged at 5,000 rpm for 20 minutes at 4 ± 2°C. The supernatant was taken for the enzyme study.

5.2.2.1.3. Assay mixture

In a total assay mixture of 2 ml, 1.45 ml of 0.1 M acetate buffer, 0.20 ml of 2.5 mM p-NPP and 0.15 ml of 27 mM MgCl₂ was mixed in a cleaned, sterilised test tube and warm up at 37 ± 1°C for 5 min. 200 µl of tissue supernatant was added to the above mixture and allowed to stand for 30 min at 37±1°C in a water bath wrapping the test tube mouth with aluminium foil. After 30 min of incubation, the reaction was stopped by adding 2 ml of 0.2 N NaOH to the assay mixture in ice-water. The change
in colour was read at 410 nm in a double beam spectrophotometer. Blank assay mixture was prepared following the same steps except NaOH was added before the addition of tissue supernatant.

The amount of acid phosphatase activity was measured by the amount of p-nitrophenol produced from a linear standard graph of p-nitrophenol. Enzyme activity was expressed as total and specific enzyme activity. Total enzyme activity was defined as the amount of µM p-nitrophenol formed per minute per gram wet tissue. Specific activity was represented as total enzyme activity/mg tissue protein.

5.2.2.1.4. Kinetic studies
Influence of pH on the enzyme activity was studied by taking acetate buffer (100 mM) with pH range 3 to 7. The effect of incubation time and temperature was analysed within a range of 1 to 60 min at 5°C to 70°C, respectively. Linearity of enzyme activity was studied within a substrate concentration of 0.5 to 10 mM p-NPP. Interference of A. nigra extract, astragalin and PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture as mentioned above.

Two concentrations were taken for each treatment,

- A. nigra - 0.5 and 1 mg (per assay mixture)
- Astragalin - 10 and 25 µM
- Praziquantel - 10 and 25 µM.

5.2.2.2. Alkaline phosphatase (AlkPase)
AlkPase activity was measured by estimating the p-nitrophenol formation following the method of Plummer (1988) with some modifications. Kinetic study of AlkPase was studied following the method of Mazorra et al. (2002).

In presence of the substrate p-nitrophenyl phosphate, alkaline phosphatase hydrolyses its substrate to form a coloured compound p-nitrophenol which is measured colorimetrically at 410 nm. The substrate p-nitrophenyl phosphate does not absorb at 410 nm and therefore the progress in enzyme catalysed reaction can be followed by measuring the increase in absorbance at 410 nm.
5.2.2.2.1. Solutions/Reagents
Stock solutions: glycine buffer (0.1 M, pH 10.5), *p*-nitrophenyl phosphatase (*p*-NPP) (50 mM), MgCl₂ (27 mM), NaOH (0.2 N) and tissue supernatant (10% w/v).

5.2.2.2.2. Tissue homogenate
A 10% (weight/volume) tissue homogenate was prepared in glycine buffer (100 mM, pH 10.5) using a remi-motor driven homogenizer fitted with Teflon pestle. The homogenate was centrifuged at 5,000 rpm for 20 minutes at 4 ± 1°C. The supernatant was taken for the enzyme study.

5.2.2.2.3. Assay mixture
In a total assay mixture of 2 ml, 1.35 ml 0.1 M glycine buffer, 0.30 ml of 50 mM *p*-NPP and 0.15 ml 27 mM MgCl₂ was mixed in a cleaned, sterilised test tube and warm up at 37 ± 1°C for 5 min. 50 µl of tissue supernatant was added to the above mixture and allowed to stand for 30 min at 37±1°C in a water bath wrapping the test tube mouth with aluminium foil. After 30 min of incubation, the reaction was stopped by adding 2 ml of 0.2 N NaOH to the assay mixture in ice-water. The change in colour was read at 410 nm in a double beam spectrophotometer.

Blank assay mixture was prepared following the same steps except NaOH was added before the addition of tissue supernatant.

5.2.2.2.4. Kinetic studies
Influence of pH on the enzyme activity was studied by taking glycine buffer with pH range 8 - 11. The effect of incubation time and temperature was analysed within a range of 1 to 60 min and 5°C to 70°C, respectively. Linearity of enzyme activity was studied within a substrate concentration of 0.5 to 10 mM *p*-NPP. Interference of *A. nigra* extract, astragalin and PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture as mentioned above.

5.2.2.3. Adenosine triphosphatase (ATPase)
Biochemical estimation of ATPase activity was done following the method of Kaplan (1957). Enzymatic activity was measured by determining the inorganic phosphate (*Pi*) liberated (Fiske and Subbarow 1925).

\[
\text{ATP} + \text{H}_2\text{O} \xrightarrow{\text{ATPase}} \text{ADP} + \text{Pi} \\
\text{Pi} + \text{Fiske and Subbaraow Reagent} \rightarrow \text{blue coloration}
\]
5.2.2.3.1. Solutions/Reagents

Tris-HCl buffer (100 mM, pH 7.5), Na$_2$-ATP (30 mM), MgCl$_2$ (30 mM), NaCl (0.5 M), KCl (0.2 M) and 15% Tri-carboxylic acid (TCA).

Fiske and Subbarow reagent: 117 ml of 100 mM Tris-HCl buffer, pH 7.5, 16.5 ml of 2.5% ammonium molybdate (prepared in 5 N H$_2$SO$_4$), 6.5 ml of 0.25% 1,2,4-aminonaphthol sulphonic acid (ANSA) (prepared by dissolving 0.25 g of ANSA in a mixture of 97.5 ml 15% sodium bisulfite and 2.5 ml of 20% sodium sulfite).

5.2.2.3.2. Tissue homogenate

A 10% (weight/volume) tissue homogenate was prepared in Tris-HCl buffer (100 mM, pH 10.5) using a remi-motor driven homogenizer fitted with Teflon pestle. The homogenate was sonicated for 30 sec. and then centrifuged at 1,000 rpm for 20 minutes at 4 ± 2°C. The supernatant was taken for the enzyme study.

5.2.2.3.3. Assay mixture

A final volume of 1 ml assay mixture consists of 250 µl 0.1 M Tris buffer, 150 µl Na$_2$-ATP, 150 µl MgCl$_2$, 200 µl NaCl, 50 µl KCl and 200 µl tissue supernatant. Assay mixture excluding the substrate (Na$_2$-ATP) was pre-incubated for 5 min at 37 ± 1°C. Reaction was started by adding the substrate to the assay mixture and incubated for 1 h at the same temperature in order to hydrolyse ATP completely. Reaction was stopped by adding 1 ml of 15% TCA holding the test tube on ice bath to cool the mixture. The mixture centrifuged at 3000 rpm for 10 min at room temperature. Precipitate discarded and the supernatant collected for the determination of inorganic phosphate following the method of Fiske and Subbarow (1925). 0.2 ml of the supernatant was taken and 2.8 ml of the Fiske and Subbarow reagent was added and the mixture allowed to stand for 5 min at 37 ± 1°C in dark. Development of blue colouration of inorganic phosphate was read at 700 nm in a spectrophotometer.

Blank assay mixture was prepared following the same assay compositions except the TCA was added to the assay mixture before the addition of tissue supernatant. The amount of ATPase activity was measured by a linear standard graph of inorganic phosphate. The ATPase activity was expressed in terms of micromoles of Pi (phosphate group) liberated/mg protein.

5.2.2.3.4. Kinetic studies

Influence of pH on the enzyme activity was studied within pH 5 to 9, (Phosphate buffer 5.0 - 7.0 and Tris-HCl buffer 7.5 - 9.0). The effect of incubation time and
temperature was analysed within a range of 2 to 60 min and 5ºC to 70ºC, respectively. Linearity of enzyme activity was studied within a substrate concentration of 0.5 to 10 mM Na₂ATP. Interference of A. nigra extract, astragalin and PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture as mentioned above.

5.2.2.4. Acetylcholinesterase (AchE)

AchE enzyme activity was measured following the method of Plummer (1988).

The substrate used in the assay system is acetylthiocholine iodide (AchI), an ester of thiocholine and acetic acid. Hydrolysis of esters produces mercaptan which reacts with an oxidising agent 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) splitting it into two products, one of which (5-thio-2-nitrobenzoate) absorbs at 405 nm. The activity of enzyme can thus be measured by following the increase in absorbance at 405 nm.

5.2.2.4.1. Solutions/Reagents

Phosphate buffer (100 mM, pH 8.0), DTNB (10 mM) and AchI (75 mM) both prepared in phosphate buffer.

5.2.2.4.2. Tissue homogenate

A 10% tissue homogenate was prepared in a phosphate buffer and Triton-X (1% w/v) using a remi-motor driven homogenizer fitted with Teflon pestle in an ice water. The homogenate was centrifuged at 15,000 rpm for 30 minutes at 4 ± 2°C. The supernatant was taken for the enzyme study.

5.2.2.4.3. Assay mixture

50 µl of tissue supernatant was added to 3 ml of phosphate buffer and incubated for 5 min at room temperature. Then 10 µl of DTNB was added followed by 20 µl of AchI. Soon after the addition of AchI, increase in absorbance was recorded in a double beam spectrophotometer at 405 nm against a blank solution prepared at the same time where the buffer was put in place of tissue supernatant. Changes in the absorbance were read for 3 minutes at 30 sec intervals at 37 ± 1°C.

5.2.2.4.4. Kinetic studies

Influence of pH on the enzyme activity was studied within pH 6 to 10, (Phosphate buffer 6.0 - 7.0 and Tris-HCl buffer 7.5 - 9.0). The effect of incubation time and temperature was analysed within a range of 2 to 60 min and 10ºC to 70ºC, respectively. Linearity of enzyme activity was studied within a substrate concentration of 0.1 to 5 mM AchI. Interference of A. nigra extract, astragalin and
PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture as mentioned above.

5.2.2.4.5. Enzyme calculation

\[
\text{AchE activity} = \frac{(\Delta E \times 1000 \times X)}{(1.36 \times 10^4 \times Y)} \, \mu\text{M/min/ml}
\]

Where, \(\Delta E\) = absorbance change per minute, 1000 = factor to obtain micro moles, \(1.36 \times 10^4\) = molar extinction co-efficient of chromophore at 405 nm, \(X\) = total volume of the assay mixture and \(Y\) = volume of enzyme (tissue supernatant) in ml.

5.2.2.5. Lactate dehydrogenase

Activities of LDH were measured following the method as described by Bergmeyer et al. (1981).

The LDH activity is measured by the rate of consumption of pyruvate and reduced NADH. The reaction velocity is determined by a decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit causes the oxidation of one micromole of NADH per minute at 25ºC and pH 7.3, under the specified conditions.

5.2.2.5.1. Solutions/Reagents

Phosphate buffer (100 mM, pH 7.4), pyruvic acid (1 mM) and NADH (1 mM).

5.2.2.5.2. Tissue homogenate

A 10% tissue homogenate (w/v) was prepared in an ice-cold 100 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 2 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 20 min at Hermle Z 233MK-2 and the resultant supernatant was used for enzyme assays. All the steps were carried out at 4ºC.

5.2.2.5.3. Assay mixture

A total of 3 ml assay mixture consists of 2.10 ml of phosphate buffer (100 mM, pH 7.4), 0.6 ml pyruvic acid (2 mM), 0.10 ml NADH (6 mM) and 100 µl tissue supernatant. Before the reaction is started, assay mixture excluding NADH was warm up (at 37 ± 1ºC) in the cuvette inside the spectrophotometer for about 2 minute. Thereafter, the reaction was started by adding 0.60 ml of NADH. Changes in absorbance were monitored at 340 nm for 3 minutes. Blank solution was prepared similar to the assay mixture except the NADH replaced by phosphate buffer.
5.2.2.5.4. Kinetic studies

Influence of pH on the enzyme activity was studied within pH 5 to 9, (phosphate buffer 5.0 – 7.0 and Tris-HCl buffer 7.5 - 9.0). The effect of temperature on enzyme activity was analysed within a range of 5 to 70°C. Linearity of enzyme activity was studied within a substrate concentration of 0.05 to 1.0 mM pyruvic acid. Interference of A. nigra extract, astragalin and PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture as mentioned above.

5.2.2.6. Malate dehydrogenase

Activities of MDH were measured following the method as described by Bergmeyer et al. (1974).

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Lactate} + \text{NAD}^+
\]

MDH enzyme activity is assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm resulting from the oxidation and disappearance of NADH. One unit oxidizes one µmole of NADH per minute at 25°C and pH 7.4 under the specified conditions.

5.2.2.6.1. Solutions/Reagents

Phosphate buffer (100 mM, pH 7.4), oxaloacetic acid (1 mM) and NADH (1 mM).

5.2.2.6.2. Tissue homogenate

A 10% tissue (F. buski) homogenate was prepared in tissue homogenising buffer (consisting of Tris-HCl buffer (100 mM, pH 7.4), 250 mM sucrose and EDTA 2 mM) using a motor pestle. The homogenate was centrifuged at 500 rpm in Hermle Z 233MK-2 for 10 min. The resultant supernatant was collected and used as crude tissue homogenate. All the steps were carried out at 4°C (Zenka and Prokopic, 1987).

5.2.2.6.3. Preparation of sub-cellular fraction

Mitochondrial and cytosolic fractions were prepared by differential centrifugation of a 10% tissue homogenate in a fractionating buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose and 2 mM EDTA following the method of Zenka and Prokopic (1987). The homogenate were centrifuged at 500 rpm for 10 min. The pellet was removed and the supernatant used as crude tissue homogenate. Half of the tissue supernatant was re-centrifuged at 10,000 rpm for 30 min. The supernatant part was collected and used as cytosolic fraction. The precipitated sediment was re-suspended in the same buffer and centrifuged at 12,000 rpm for another 30 min. The
supernatant was discarded and the pellet was suspended in the buffer and sonicated for 20s and used as mitochondrial fraction. All the steps were carried out at 4°C.

5.2.2.6.4. Assay mixture

A total of 3 ml assay mixture consists of 2.55 ml of Tris-HCl buffer (100 mM, pH 7.4), 0.3 ml of oxaloacetic acid (5 mM), 0.1 ml of NADH (6 mM) and 50 µl tissue supernatant. Before the reaction was started, the assay mixture excluding NADH was warm up (37 ± 1ºC) in the cuvette inside the spectrophotometer for about 2 minute. Thereafter, the reaction was started by adding 0.60 ml of NADH. Changes in absorbance were monitored at 340 nm for 3 minutes. Blank solutions were prepared similar to the assay mixture except the NADH was replaced by buffer.

5.2.2.6.5. Kinetic studies

Influence of pH on the enzyme activity was studied within pH 5.5 to 9.0, (Phosphate buffer 5.5 - 7.0 and Tris-HCl buffer 7.5 - 9.0). The effect of temperature on enzyme activity was analysed within a range of 5ºC to 70ºC. Linearity of enzyme activity was studied within a substrate concentration of 0.05 to 1.0 mM Oxaloacetic acid. Interference of *A. nigra* extract, astragalin and PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture as mentioned above.

5.2.2.7. Pyruvate kinase (PK)

Pyruvate kinase enzyme activity was estimated following the method of McManus and Smyth (1982) with little modification.

\[
\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{Pyruvate kinase}} \text{Pyruvate} + \text{ATP} \\
\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}
\]

*The reaction velocity is determined in a lactate dehydrogenase coupled assay system by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH.*

5.2.2.7.1. Solutions/Reagents

Tris-HCl buffer (0.2 M, pH 7.4), phosphoenolpyruvate (PEP, 40 mM), MgSO₄ (42 mM), ADP (1 mM), KCl (0.4 M), LDH (6 unit) and NADH (2 mM).

5.2.2.7.2. Tissue homogenate

A 10% tissue homogenate (w/v) was prepared in an ice-cold 100 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at
10,000 rpm for 10 min at Hermle Z 233MK-2 and the resultant supernatant was used for all enzyme assays. All the steps were carried out at 4°C (Fry et al. 1983).

5.2.2.7.3. Assay mixture
A total of 3 ml assay mixture consists of 0.65 ml of Tris-HCl buffer (0.2 M, pH 7.4), 0.50 ml of PEP (40 mM), 0.30 ml of MgSO₄ (42 mM), 0.10 ml of KCl (0.4 M), 0.60 ml of ADP (1 mM), 0.30 ml of (6 unit) LDH, 0.50 ml of NADH (2 mM) and 0.05 ml tissue supernatant. Assay mixture excluding NADH was warm up (37 ± 1°C) in the cuvette inside the spectrophotometer for about 2 minute. Thereafter, the reaction was started by adding NADH. Changes in absorbance were monitored at 340 nm for 3 minutes. Blank solutions were prepared similar to the assay mixture except the NADH was replaced by buffer.

5.2.2.7.4. Kinetic studies
Influence of pH on the enzyme activity was studied within pH 5.0 to 9.0, (phosphate buffer 5.0 - 7.0 and Tris-HCl buffer 7.5 - 9.0). The effect of temperature on enzyme activity was analysed within a range of 5°C to 70°C. Linearity of enzyme activity was studied within a substrate concentration of 0.5 to 5.0 mM phosphoenolpyruvate. Interference of A. nigra extract, astragalin and PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture as mentioned above.

5.2.2.8. Phosphoenolpyruvate carboxykinase (PEPCK)
Phosphoenolpyruvate carboxykinase enzyme activity was estimated following the method of McManus and Smyth (1982) with little modification (Das et al. 2013).

\[
\text{Phosphoenolpyruvate + NaHCO}_3 + \text{ADP} \xrightarrow{\text{PEPCK}} \text{Oxaloacetate + ATP} \\
\text{Oxaloacetate + NADH} \xrightarrow{\text{MDH}} \text{Malate + NAD}
\]

The formation of oxaloacetate is monitored spectrophotometrically in a malate dehydrogenase coupled system. The reaction velocity is measured as a decrease in \( A_{340} \) resulting from the oxidation of NADH. One unit oxidizes one micromole of NADH per minute at 25°C and pH 8.5 under the specified conditions.

5.2.2.8.1. Solutions/Reagents
Tris-HCl (100 mM, pH 7.4), PEP (40 mM), MgSO₄ (0.3 M), NaHCO₃ (70 mM), KCl (0.4 M), ADP (1 mM), NADH (2 mM) and MDH 8 units.
5.2.2.8.2. Tissue homogenate

A 10% tissue homogenate (w/v) was prepared in an ice-cold 100 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. The homogenate was centrifuged at 10,000 rpm for 10 min at Hermle Z 233MK-2 and the resultant supernatant was used for enzyme assays. All the steps were carried out at 4°C (Fry et al. 1983).

5.2.2.8.3. Assay mixture

A total of 3 ml assay mixture consists of 0.95 ml of Tris-HCl buffer (0.2 M, pH 7.4, 0.60 ml of PEP (40 mM), 0.3 ml of NaHCO₃ (70 mM), 0.1 ml of MgSO₄ (0.3 M), 0.1 ml of KCl (0.4 M), 0.30 ml of ADP (1 mM), 0.10 ml of (8 unit) MDH, 0.50 ml of NADH (2 mM) and 0.05 ml tissue supernatant.

Similar to the PK, the assay mixture excluding NADH was warm up (37 ± 1°C) in the cuvette inside the spectrophotometer for about 2 minute. Thereafter, the reaction was started by adding NADH. Changes in absorbance were monitored at 340 nm for 3 minutes. Blank solutions were prepared similar to the assay mixture except the NADH was replaced by Tris-HCl buffer.

5.2.2.8.4. Kinetic studies

Influence of pH on the enzyme activity was studied within pH 5.5 to 9.0, (phosphate buffer 5.5 to 7.0 and Tris-HCl buffer 7.5 - 9.0). The effect of temperature on enzyme activity was analysed within a range of 5 to 70°C. Linearity of enzyme activity was studied within a substrate concentration of 0.5 to 5.0 mM phosphoenolpyruvate. Interference of A. nigra extract, astragalin and PZQ with the enzyme activity was studied by incubating plant extract, astragalin and PZQ with assay mixture following the above procedure.

5.2.2.8.5. Enzyme calculation

All the four glycolytic enzymes namely LDH, MDH, PK and PEPCK were calculated using the formula:

\[
\text{Enzyme activity} = \frac{(\Delta E \times 1000 \times X)}{(6.3 \times 10^3 \times Y)} \text{micromole min}^{-1} \text{ml}^{-1}
\]

Where, \(\Delta E\) = absorbance change per minute, 1000 = factor to obtain micro moles, \(6.3 \times 10^3\) = molar extinction co-efficient of NADH, \(X\) = total volume of the assay mixture and \(Y\) = volume of enzyme (tissue supernatant) in ml.

5.2.3. Preparation of standard graph

5.2.3.1. Preparation of standard graph of \(p\)-nitrophenol (Plummer 1988)
\( p \)-nitrophenol contain a nitro group at the opposite position of hydroxyl group on the benzene ring compound which give yellow colour at alkali solution.

**Reagent:** \( p \)-nitrophenol (10 mM), 0.2 N NaOH

a. Ten concentration of standard \( p \)-nitrophenol was taken in the range of 20 to 200 µM.
b. Required volume of distilled water was added to make the total up to 2 ml.
c. 2 ml of 0.2 N NaOH was added to each test tube and waited for 2-3 min.
d. Yellow colour development was read at 405 nm against a blank without \( p \)-nitrophenol in the test tube.
e. A standard curve was prepared by putting the \( p \)-nitrophenol concentrations in x-ordinate against the ODs in the y-ordinate.

5.2.3.2. Preparation of standard graph of inorganic phosphate (Fiske and Subbarow 1925)

**Reagents**

- Standard phosphate \( \text{KH}_2\text{PO}_4 \) (10 mM).
- Fiske and Subbarow reagent: 117 ml of 100 mM Tris-HCl buffer, pH 7.5, 16.5 ml of 2.5% ammonium molybdade (prepared in 5 N \( \text{H}_2\text{SO}_4 \)), 6.5 ml of 0.25% 1,2,4-aminonaphthol sulphonic acid (ANSA) (prepared by dissolving 0.25 g of ANSA in a mixture of 97.5 ml 15% sodium bisulfite and 2.5 ml of 20% sodium sulfite).

**Method**

a. From a stock solution of 10 mM \( \text{KH}_2\text{PO}_4 \), seven concentrations was prepared in the concentration range 33 to 330 µM. (volume taken 10 to 100 µM from the stock solution).
b. 2.8 ml of Fiske and Subbarow reagent was added in each test tube and made the solution up to a final volume of 3 ml with distilled water.
c. The mixture was allowed to stand for 5 minute and the blue colour developed was read at 700 nm in a double beam spectrophotometer against a blank solution without \( \text{KH}_2\text{PO}_4 \) in the assay mixture.
d. A standard curve was prepared by putting the \( \text{KH}_2\text{PO}_4 \) concentrations in x-ordinate against the ODs in the y-ordinate.

5.2.4. Estimation of protein

The protein content of the fluke parasite \( F. \text{buski} \) estimated following the method of Lowry et al. (1951) using bovine serum albumin as a standard protein.
Estimation of protein is based on the development of the blue colour by the reduction of phosphomolybdic-phosphotungstic components present in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein along with the colour development by the biuret reaction of the protein with the alkaline cupric tartrate.

Reagents/Solutions
(a) 4% sodium carbonate prepared in 0.1 N NaOH, (b) 2% potassium sodium tartrate, (c) 2% copper sulphate, Folin-Ciocalteau reagent (diluted 10 times with distilled water) and bovine serum albumin (BSA) (250 microgram/ml).

Preparation of protein reagent: 100 ml of (a): 1 ml each of (b) and (c) mixed together.

Method
1. Pipetted out 0.2 to 1.0 ml of the standard BSA in a test tube and made up to 1 ml adding distilled water.
2. 1.5 ml of protein reagent was added to the above solution and allowed to stand for 10 min.
3. Then 0.5 ml of diluted Folin-Ciocalteau reagent was added and incubated for 20 min at room temperature in dark.
4. The blue colour developed was read at 660 nm against a blank solution prepared by replacing BSA with water.

A standard curve was prepared by putting the BSA concentrations in x-ordinate against the ODs in the y-ordinate.

5.3. RESULTS
5.3.1. Histochemical localization of enzymes
The distribution and intensity of tegumental enzymes like AcPase, AlkPase, ATPase and AchE along with two glycolytic enzymes such as MDH and LDH in *F. buski* maintained as control, and treated with the ethanolic crude extracts of *A. nigra*, its ethyl acetate fraction, astragalin (bioactive compound of *A. nigra*) and reference anthelmintic praziquantel are presented in Figures 3.1-3.6. Histochemical localizations of enzymes such as AcPase, AlkPase, ATPase, AchE, MDH and LDH in the tissue sections showed variation in their staining intensities in control and treated flukes.
5.3.1.1. Acid phosphatase

The AcPase activity of brownish black depositions was observed almost throughout the whole tissue sections in control parasite (Figure 3.1a). Deep AcPase stain intensities were observed mainly in the tegumental and sub-tegumental regions on the ventral surface of control fluke compared to their dorsal surface.

Exposure of the fluke parasites to different treatments such as crude extract of A. nigra, ethyl acetate fraction and astragalin as well as reference drug, praziquantel, prominent changes were observed in the staining intensities of AcPase enzyme activity. Histochemical localisation of AcPase activity in parasites exposed to A. nigra crude extract showed high reduction in both the ventral and dorsal surfaces of the worm. Visible reduction of enzyme activity has also been noticed in the body musculature of the parasite (Figure 3.1b). Similarly ethyl acetate fraction of extract/drug treated parasites showed a more or less similar extends of reduction in the staining intensities of AcPase enzyme (Figure 3.1c-e).

5.3.1.2. Alkaline phosphatase

Similar to AcPase, AlkPase also showed a similar kind of enzyme staining. High activities of AlkPase were localized mainly in the tegument, sub-tegument and musculature regions of the control parasite. Higher stain intensities were observed in the ventral sides of the parasite compared to the dorsal surfaces (Figure 3.2a).

Exposure of the flukes to crude extract of A. nigra, ethyl acetate fraction and astragalin revealed visible changes in the stain intensities of AlkPase. Reduced stain intensities were observed almost in all the regions of the tissue such as tegument, sub-tegument and body musculature when the parasites were exposed to different treatments. Decrease in enzyme stain intensities of AlkPase has been observed though out the tissue section in crude extract of A. nigra treated fluke (Figure 3.2b) whereas, in parasites exposed to ethyl acetate fraction and astragalin, there was a slight changes in the AlkPase activity (Figure 3.2c,d). Praziquantel, on the other hand also showed higher reduction in stain intensities of AlkPase enzyme (Figure 3.2e).

5.3.1.3. Adenosine triphosphatase

High ATPase enzyme activities were noticed mainly in the tegument and sub-tegumental region of the control fluke with deep stain intensities. Body musculature region did not show much ATPase activity as per the intensity of the enzyme staining is concerned (Figure 3.3a).
Flukes, when exposed to the crude extract of *A. nigra*, its ethyl acetate fraction and astragalin, changes were observed in the staining intensities of ATPase (Figure 3.3b-e). *A. nigra* crude extract treated parasites revealed visible reduction in the ATPase stain intensity throughout the tissue section (Figure 3.3b). Ethyl acetate fraction and astragalin treated parasites also showed similar extend of reduction in their stain intensities (Figure 3.3c,d).

5.3.1.4. Acetylcholinesterase

Similar patterns of AchE enzyme staining were observed in the control tissue section. Tegumental, sub-tegumental as well as regions around the intestinal caeca showed deeper staining of the enzyme compared to the musculature region of the control fluke (Figure 3.4a).

*F. buski* exposed to the crude extract of *A. nigra* showed visible reduction in their stain intensities. Decreased intensities of AchE activity were seen throughout the regions of tissue sections (Figure 3.4b). However, no such visible reductions in the staining intensities were observed in parasite treated with ethyl acetate fraction and astragalin (Figure 3.4c,d). PZQ causes reduction in the tegumental and musculature in the treated fluke whereas sub-tegumental regions did not show any differences between the control and treated *F. buski* (Figure 3.4e).

5.3.1.5. Malate dehydrogenase

High MDH activities were observed throughout the tissue sections of control *F. buski* with high stain intensities (Figure 3.5a). Tegumental layer on the ventral surface showed deeper stain intensities compared to the dorsal surface.

Exposure of flukes to different concentration of *A. nigra* crude extract, ethyl acetate fraction, its bioactive compound astragalin as well as praziquantel, however, did not show any kind of reduction in the stain intensities of enzyme (Figure 3.5b-e). However, slight changes were observed in the astragalin treated *F. buski* (Figure 3.5d).

5.3.1.6. Lactate dehydrogenase

Deep stain intensities of LDH enzyme activities were observed in the tissue sections of control *F. buski*. Tegumental, sub-tegumental and regions around the intestinal caeca were seen to be deeply stained in control fluke (Figure 3.6a). Like other enzymes, higher enzyme activities were observed in the ventral side of the tissue section compared to the ventral surfaces.
Exposure of flukes to different concentration of *A. nigra* crude extract, ethyl acetate fraction, its bioactive compound astragalin as well as praziquantel, showed slight reduction in the stain intensities of LDH (Figure 3.6b-e).

5.3.2. Biochemical enzyme studies

The enzymatic activities of tegumental enzymes such as AcPase, AlkPase, ATPase and AchE along with four glycolytic enzymes such as MDH, LDH, PK and PEPCK in *F. buski* maintained as control in PBS were presented in Table 3.1 and 3.2. Alterations in all the enzyme activities has been observed when the parasites were exposed to the ethanolic crude extract of *A. nigra*, its solvent fraction (ethyl acetate) and astragalin as well as praziquantel which were presented in Figures 3.7 to 3.9.

5.3.2.1. Acid phosphatase

High AcPase enzyme activities (1.84 U/mg tissue protein) were seen in parasites maintained as control in PBS (Table 3.1).

On exposure of the flukes to different concentration of *A. nigra* crude extract, ethyl acetate fraction, its bioactive compound astragalin and praziquantel, a significant reduction in the AcPase enzyme activities were evident. Highest reduction in AcPase enzyme activity was observed in parasites exposed to crude extract of *A. nigra* changing the enzyme activity from 1.84±0.05 to 0.85±0.06 U/mg tissue protein with 53.50% inhibition followed by PZQ (51.72%), astragalin (43.93%) and ethyl acetate fraction (32.75%) treated flukes, respectively (Table 3.1).

5.3.2.2. Alkaline phosphatase

Biochemical study of AlkPase showed highest enzyme activity 10.62 U/mg tissue protein among all the four tegumental enzymes in control *F. buski* (Table 3.1).

Changes were observed in the AlkPase enzyme activity when the flukes were treated with different treatments. A maximum of 27.33% reduction in enzyme activity was observed in parasites exposed to the bioactive compound of *A. nigra*, astragalin (Table 3.1). A similar extent of reduction in AlkPase activity was observed in *A. nigra* crude extract treated parasites. Ethyl acetate fraction showed least enzyme inhibition (22.71%) among all the treatments. However, the reference drug PZQ showed highest inhibition in AlkPase activity with 44.96% reduction.

5.3.2.3. Adenosine triphosphatase

Similarly, ATPase showed high enzyme activity with 5.82 ± 0.07 U/mg tissue protein in control *F. buski* (Table 3.1).
Parasites exposed to different treatments showed significant reduction in their enzyme activities compared to control. Crude extract of *A. nigra* was found to be most effective against ATPase activity in *F. buski* that reduced the enzyme activity from $5.82 \pm 0.07$ to $2.93 \pm 0.1$ U/mg tissue protein with $48.89\%$ inhibition followed by ethyl acetate and astragalin treated flukes with $33.47\%$ and $30.22\%$ inhibitions, respectively (Table 3.1). PZQ treated fluke showed more or less similar extent of inhibition to that of the *A. nigra* crude extract treated parasites.

5.3.2.4. Acetylcholinesterase

Among all the four key tegumental enzymes under study, AchE activity was found to be lowest with $0.029$ U/mg tissue protein in control *F. buski* (Table 3.1).

Significant reductions were observed in the AchE enzyme activity when the parasites were treated with the crude extract of *A. nigra*, its ethyl acetate fraction and astragalin. Highest (56.8%) reduction in AchE activity was observed in *A. nigra* crude extract treated parasites followed by ethyl acetate fraction and astragalin treated flukes with 43.4 and 32.83% reductions, respectively (Table 3.1).

5.3.2.5. Lactate dehydrogenase

The untreated control *F. buski* showed high LDH activity with $3.14 \pm 0.71$ U/mg tissue protein (Table 3.2).

Flukes treated with crude extract of *A. nigra*, ethyl acetate fraction and astragalin showed changes in their enzymatic activity. *A. nigra* crude extract and astragalin treated flukes did not show any significant changes in the LDH activities between control and treated flukes. However, ethyl acetate fraction of *A. nigra* crude extracts showed statistically significant reduction in LDH activity with 22.32% reduction when the flukes were exposed to it. Anthelmintic drug, PZQ also showed significant reduction with 13.93% inhibition (Table 3.2).

5.3.2.6. Pyruvate kinase

In control *F. buski*, the PK enzyme activities were estimated to be $1.72 \pm 0.06$ U/mg tissue protein in crude tissue homogenate (Table 3.2).

Parasites exposed to different treatments showed reduction in their enzyme activities compared to control. Crude extract of *A. nigra* and PZQ treated parasites revealed significant reductions in the PK activity with 21.51 and 20.35% inhibition, respectively. Astragalin and ethyl acetate fraction treated fluke, however, did not show any significant alterations in the PK enzyme activity compared to control (Table 3.2).
5.3.2.7. Phosphoenolpyruvate carboxykinase
The specific enzyme activity of PEPCK was found to be $2.36 \pm 0.01$ U/mg tissue protein in untreated control *F. buski* (Table 3.2).

However, exposure of the flukes to different treatments showed significant alterations in the PEPCK activity. *A. nigra* bioactive compound, astragalin was found to be most effective against PEPCK in *F. buski* which reduced the enzyme activity to 27.54%. Crude extract of *A. nigra* and its ethyl acetate fraction exposed flukes also showed significant changes in the PEPCK enzyme activity with 25.84 and 18.64% reduction, respectively.

5.3.2.8. Malate dehydrogenase
Among all the four key glycolytic enzymes, MDH showed maximum enzyme activity with $11.42 \pm 0.17$ U/mg tissue protein in the crude tissue homogenate of control parasite. MDH activity was observed to be higher in cytosol compared to mitochondria with $10.81 \pm 0.05$ and $1.02 \pm 0.02$ U/mg tissue protein, respectively (Table 3.2).

Fluke parasites when treated with *A. nigra* crude extract and its ethyl acetate fraction did not show any significant variation in the enzyme activity compared to control *F. buski*. However, astragalin was found to reduce the enzyme activity in a significant manner with 13.13% inhibition (Table 3.2).

5.3.3. Kinetic studies
The acid and alkaline phosphatase, adenosine triphosphatase, acetylcholinesterase, lactate dehydrogenase, malate dehydrogenase, pyruvate kinase and phosphoenolpyruvate carboxykinase activities of the *F. buski* have been partially characterized and the *in vitro* effect of *A. nigra* crude extract, astragalin and praziquantel on all the enzymatic activity have been analyzed to see the interference of those extracts/drugs on the enzymatic activities, if any. The values of specific activities of AchE, LDH, PK and PEPCK were multiplied by 100 and MDH by 10 because of their decimal values.

5.3.3.1. Acid phosphatase
Figure 3.10 to 3.12 and table 3.3 shows the various parameters of AcPase enzyme kinetics. Highest AcPase enzyme activity was observed at pH 6.0 when the reaction mixture was incubated at temperature 40°C. Increasing the reaction temperature from 5°C up to 70°C increased the enzyme activity up to 40°C and the decreased with the increase of reaction temperature (Figure 3.10b). The effect of incubation time at a
particular concentration of substrate showed that the AcPase activity have a peak activity after 20 min of incubation at a given temperature (Figure 3.10c). The result indicates a good relationship between the AcPase activity and the substrate p-nitrophenyl phosphate (pNPP, the substrate) concentration. Increasing the substrate concentration from 0.5 to 10 mM, a linear increase in the enzyme activities (represented as units) in accordance with the normal pattern of enzymatic reactions has been observed (Figure 3.10d) with $K_m$ and $V_{max}$ values 3.59 mM and 11.02 Units (U)/mg tissue protein, respectively in untreated control $F. buski$ (Table 3.3). On exposure to different concentrations of A. nigra crude extract (0.5 and 1.0 mg), astragalin and PZQ (10 and 25 µM each in final assay volume) together with the assay mixture, alterations in the activities of AcPase have been noticed (Table 3.3). The $V_{max}$ values have been altered from 11.02 to 10.45 and 7.69 when the assay mixture was incubated with 0.5 and 1.0 mg of A. nigra crude extract. Similarly, astragalin and PZQ treated assay mixture showed changes in the $V_{max}$ values from 11.02 to 9.07 and 8.27 and 8.03 at 10 and 25 µM of drug concentrations, respectively. In a similar way, the $K_m$ values were also altered from 3.59 to 3.86 and 2.94 (A. nigra, 0.5 and 1 mg treatment), 3.24 and 3.08 (astragalin) and 3.01 and 2.90 (PZQ) at incubation dose of 10 and 25 µM, respectively. The alterations in kinetic parameters in response to the treatment of A. nigra extract, astragalin and PZQ, therefore suggest a non-competitive type of enzyme inhibition in AcPase enzyme activity.

5.3.3.2. Alkaline phosphatase

Figure 3.13 shows the influence of pH, reaction temperature, incubation time and the effects of substrate concentrations on the AlkPase enzymatic activity in vitro. The physio-chemical parameters required for the maximum enzyme activity in AlkPase include pH 9-9.5, reaction temperature 40ºC and incubation duration of 30 min (Figure 3.13). Increasing the substrate concentration showed increased enzyme activity that reached its peak at 10.24 U/mg tissue protein ($V_{max}$) at $K_m$ value 0.97 mM (Table 3.4; Figure 3.15). Assay mixture when incubated with different concentrations of A. nigra crude extract, astragalin and PZQ, changes in the kinetic parameters has been seen in AlkPase enzyme activity. The maximum enzyme activity ($V_{max}$) and the $K_m$ values were decreased in both the plant extract and astragalin treated enzyme assays showing an un-competitive type of enzyme inhibition. However, there is a difference in the alteration of kinetic parameters in
PZQ treated assay mixture which showed increase in $K_m$ value from 0.97 to 1.17 and 1.45 mM at 10 and 25 µM when the assay mixture was incubated with PZQ, thereby indicating a mixed-type of enzyme inhibition (Table 3.4).

5.3.3.3. Adenosine triphosphatase

Similarly, the influence of pH, temperature, time of incubation and substrate concentration were shown in figure 3.16. At its *in vitro* experimental conditions, the ATPase enzyme showed its maximum activity at pH 6.5 - 7.5 within a reaction temperature of 30 - 50°C. The ATPase activity increased up to 16.08 ± 0.54 U/mg tissue protein at pH value 6.5, 16.00 ± 1.58 at pH 7 and 15.12 ± 0.47 at pH 7.5 and then decreased to a lowest of 6.77 ± 0.75 U/mg tissue protein at higher pH 9.0 (Figure 3.16a). At a fixed volume of tissue supernatant and at a particular substrate concentration, the enzyme activity (specific activity) were found to be maximum when the assay mixture was incubated for 5 min, while increasing the duration of incubation time decreased the specific activity in *F. buski* (Figure 3.16c). A plot of specific activity vs. increasing substrate concentration from 0.5 to 8 mM showed a linear increase ($R^2 = 0.9826$) in the enzyme activities (represented as units) in accordance with the normal pattern of enzymatic reactions (Figure 3.17a). The maximum activity ($V_{max}$) and $K_m$ was found to be 25.61 U/mg tissue protein and 2.22 mM, respectively (Table 3.5). On incubation of assay mixture with different concentrations of plant extract, astragalin and PZQ, changes have been observed in enzyme activity of ATPase. Figure 3.18 and table 2.5 show the alterations of kinetic parameters with *A. nigra* and astragalin indicating non-competitive type of enzyme inhibition, whereas the PZQ treated tissue showed a mixed type of inhibition in ATPase activity.

5.3.3.4. Acetylcholinesterase

Investigation of suitable pH level and reaction temperature for optimum AchE activity was studied taking into account the range of pH 6 - 10 along with temperature in the range of 10 to 70°C. Figure 3.19 depicts the influence of pH and temperature on enzyme activity showing optimum activity at 7.5 - 8.0 pH range and 30°C temperature, respectively. Present studies have shown that the AchE enzyme activity decreased with the increase of pH value and temperature (Figure 3.19a,b). A good linearity ($R^2 = 0.9798$) relationship between the AchI concentrations vs. enzyme activity have been observed in the presented experimental conditions (Figure 3.20a). The kinetic parameters $K_m$ and $V_{max}$ values were 0.28 mM and 5.37 U/mg
tissue proteins, respectively in control *F. buski*. The possible mode of action of the plant extract, astragalin and PZQ tested on the parasite AchE activity revealed interference and alterations in the enzyme activity. At tested concentration 0.5 to 1.0 mg of crude *A. nigra* extract, decreased value were observed in $V_{\text{max}}$ from 5.37 to 3.95 and 3.52, whereas the $K_{m}$ value remained approximately the same both in control and *A. nigra* treated parasite. The study therefore indicates non-competitive nature of enzyme inhibition of *A. nigra* against AchE. However, the astragalin treated parasite showed slight increase in both the $V_{\text{max}}$ and $K_{m}$ values at *in vitro* experimental conditions suggesting uncompetitive type of enzyme inhibition. Similarly, PZQ showed decrease in $V_{\text{max}}$ value from 5.37 to 4.56 and 4.49 followed by increase in $K_{m}$ value from 0.28 to 0.29 and 0.4 mM and therefore indicating mixed type of enzyme inhibition (Table 3.6).

5.3.3.5. Lactate dehydrogenase

The influence of pH, temperature and substrate concentrations on *in vitro* LDH activity is presented in the figure 3.22. Optimum enzyme activity was noticed between the pH range of 7 to 7.5 (Figure 3.22a). Lower and higher than the optimum level showed tremendous effect on the functioning of enzyme activity. Similarly, at 30 to 40°C, LDH showed maximum activity compared to 5 and 70°C which showed lowest activity $2.44 \pm 0.42$ and $0.81 \pm 0.61$ U/mg tissue protein, respectively. In accordance with the normal enzyme activity, increase in enzyme activity was observed in LDH with the increase of substrate concentration from 0.05 to 1.0 mM (Figure 3.22c). The $K_{m}$ and $V_{\text{max}}$ values obtained from the Lineweaver-Burk plot of control *F. buski* were 0.15 mM and 34.96 U/mg tissue proteins (Table 3.7). Similar to other enzymes studied alterations in the kinetic parameters have been seen when the parasites were treated with crude extract of *A. nigra*, astragalin and PZQ. Table 2.7 displays the reduced values of $V_{\text{max}}$ while the $K_{m}$ values increases in parasites treated with crude extract of *A. nigra*, indicating mixed type of enzyme inhibition. However, astragalin and PZQ showed no such enzyme inhibition against LDH indicating uncompetitive type of interference with the enzyme activity.

5.3.3.6. Malate dehydrogenase

The suitable pH level and reaction temperature for optimum MDH activity was studied taking into account the range of pH 5.5 to 9.0 along with temperature in the range of 5 to 70°C. Figure 3.24 depicts the influence of pH and reaction temperature on enzyme activity showing optimum activity at 7.5 to 8.0 pH range and 40°C
temperature, respectively. Present studies have shown that lower and higher values of pH and reaction temperature have negative effect on the MDH enzyme activity that decreased the enzyme activity (Figure 3.24a, b). A good linearity ($R^2 = 0.9986$) relationship between the oxaloacetate concentrations vs. enzyme activity have been observed in the present experimental conditions (Figure 3.25a). The kinetic parameters $K_m$ and $V_{max}$ values of control tissue showed 0.77 mM and 45.25 U/mg tissue proteins, respectively (Table 3.8). The possible mode of action of the plant extract, astragalin and PZQ tested on the parasite MDH activity revealed alterations in the enzyme activity. At tested concentration 0.5 to 1.0 mg of crude A. nigra extract and 10 and 25 µM, decreased value were observed both in $K_m$ and $V_{max}$, whereas the $K_m$ value showed little increase in PZQ treated parasite with decreased MDH ($V_{max}$) activity. The study therefore, indicates un-competitive nature of enzyme inhibition of A. nigra and astragalin against MDH. However, the PZQ showed a mixed type of MDH inhibition (Table 3.8).

5.3.3.7. Pyruvate kinase

Similarly, the influence of pH, temperature and substrate concentration on the PK enzyme activity were shown in figure 3.27. At the in vitro experimental conditions, the PK enzyme showed its maximum enzyme activity at pH 7-7.5 and within a reaction temperature of 30-40ºC. The PK activity increased up to 13.95 ± 0.54 U/mg tissue proteins at pH value 7.0 and 14.65 at pH 7.5 and then decreased with the increase of pH 9 to a lowest of 9.74 ± 0.44 U/mg tissue protein (Figure 3.27a). Similarly, the influence of temperature on the PK enzyme activity could be seen where increasing the temperature beyond 40ºC have negative effect that decreases the functioning to a lowest of 1.08 ± 0.34 U/mg tissue protein (Figure 3.27b). A plot of specific activity vs. increasing substrate concentration from 0.25 to 5 mM (Figure 3.27c) showed a linear increase ($R^2 = 0.9963$) in the enzyme activities (represented as units) in accordance with the normal pattern of enzymatic reactions (Figure 3.28a). The maximum velocity/activity ($V_{max}$) and $K_m$ was found to be 28.36 U/mg tissue protein and 2.45 mM, respectively (Table 3.9). On incubation of assay mixture with different concentrations of plant extract, astragalin and PZQ, changes have been observed in enzyme activity of ATPase. Figure 3.29 and table 3.9 show the alterations of kinetic parameters with A. nigra and astragalin indicating a mixed type of inhibition whereas, astragalin and PZQ treated tissue showed uncompetitive type of inhibition in pyruvate kinase activity.
5.3.3.8. Phosphoenolpyruvate carboxykinase

Figure 3.30 shows the influence of pH, reaction temperature, substrate concentrations and Lineweaver-Burk plot of substrate vs. PEPCK activity *in vitro*. The physicochemical parameters required for the maximum enzyme activity in PEPCK include pH 7.5 to 8.0 and reaction temperature 30ºC (Figure 3.30a,b). Similar to other enzymes, higher and lower pH as well as temperature showed opposite effect on the enzyme by decreasing their activity. Figure 2.30c showed the increase in PEPCK activity in relation to the increase in substrate concentration from 0.5 to 5 mM. The $K_m$ and $V_{max}$ values of control parasite were found to be 2.54 mM and 17.18 U/mg tissue proteins, respectively (Table 3.10). Assay mixture when incubated with different concentrations of *A. nigra* crude extract, astragalin and PZQ, changes were seen in the kinetic parameters of PEPCK enzyme activity. The $V_{max}$ values were found to be reduced on exposure to different treatments compared to control *F. buski*, while the $K_m$ value were observed to be increased in the *A. nigra* extract, astragalin and PZQ treated parasites. Alterations in the kinetic parameters on exposure to different treatments suggest a mixed type of enzyme inhibition in PEPCK under experimental conditions. However, at its tested concentrations of 0.5 and 1.0 mg, the crude extract of *A. nigra* showed non-competitive inhibition in AcPase, ATPase and AchE activities, followed by mixed type of inhibition in LDH, PK and PEPCK and un-competitive type of enzyme inhibitions in AlkPase and MDH activity. Ethyl acetate fraction of the crude extract and the bioactive compound, astragalin also causes a similar kind of alteration the kinetic parameters of all the enzymes studied.
Figure 3.1. Light microscopic photographs of transverse sections of *Fasciolopsis buski* showing histochemical localization of acid phosphatase activities, (a) control, (b) crude extract of *Alpinia nigra* treated, (c) ethyl acetate fraction treated, (d) astragalin treated and (e) praziquantel treated sections.
Figure 3.2. Light microscopic photographs of transverse sections of *Fasciolopsis buski* showing histochemical localization of alkaline phosphatase activities, (a) control, (b) crude extract of *Alpinia nigra* treated, (c) ethyl acetate fraction treated, (d) astragalin treated and (e) praziquantel treated sections.
Figure 3.3. Light microscopic photographs of transverse sections of *Fasciolopsis buski* showing histochemical localization of adenosine triphosphatase activities, (a) control, (b) crude extract of *Alpinia nigra* treated, (c) ethyl acetate fraction treated, (d) astragalin treated and (e) praziquantel treated sections.
Figure 3.4. Light microscopic photographs of transverse sections of *Fasciolopsis buski* showing histochemical localization of acetylcholinesterase activities, (a) control, (b) crude extract of *Alpinia nigra* treated, (c) ethyl acetate fraction treated, (d) astragalin treated and (e) praziquantel treated sections.
Figure 3.5. Light microscopic photographs of transverse sections of *Fasciolopsis buski* showing histochemical localization of malate dehydrogenase activities, (a) control, (b) crude extract of *Alpinia nigra* treated, (c) ethyl acetate fraction treated, (d) astragalin treated and (e) praziquantel treated sections.
Figure 3.6. Light microscopic photographs of transverse sections of *Fasciolopsis buski* showing histochemical localization of lactate dehydrogenase activities, (a) control, (b) crude extract of *Alpinia nigra* treated, (c) ethyl acetate fraction treated, (d) astragalin treated and (e) praziquantel treated sections.
Table 3.1. Biochemical changes in the tegumental enzymes of *Fasciolopsis buski* treated with *Alpinia nigra* crude extract, ethyl acetate fraction, astragalin and PZQ.

<table>
<thead>
<tr>
<th></th>
<th>AcPase</th>
<th>%IN</th>
<th>AlkPase</th>
<th>%IN</th>
<th>ATPase</th>
<th>%IN</th>
<th>AchE&lt;sub&gt;x100&lt;/sub&gt;</th>
<th>%IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.84 ± 0.05</td>
<td>-</td>
<td>10.62 ± 0.01</td>
<td>-</td>
<td>5.82 ± 0.07</td>
<td>-</td>
<td>2.96 ± 0.11</td>
<td>-</td>
</tr>
<tr>
<td><em>A. nigra</em></td>
<td>0.85 ± 0.06*</td>
<td>53.50</td>
<td>7.75 ± 0.16*</td>
<td>27.08</td>
<td>2.93 ± 0.10*</td>
<td>48.89</td>
<td>1.28 ± 0.04*</td>
<td>56.80</td>
</tr>
<tr>
<td>EA</td>
<td>1.23 ± 0.03*</td>
<td>32.75</td>
<td>8.21 ± 0.04*</td>
<td>22.71</td>
<td>3.87 ± 0.22*</td>
<td>33.47</td>
<td>1.67 ± 0.08*</td>
<td>43.40</td>
</tr>
<tr>
<td>AST</td>
<td>1.03 ± 0.05*</td>
<td>43.93</td>
<td>7.72 ± 0.04*</td>
<td>27.33</td>
<td>4.06 ± 0.54*</td>
<td>30.22</td>
<td>1.99 ± 0.19*</td>
<td>32.83</td>
</tr>
<tr>
<td>PZQ</td>
<td>0.88 ± 0.20*</td>
<td>51.72</td>
<td>5.84 ± 0.13*</td>
<td>44.96</td>
<td>3.20 ± 0.29*</td>
<td>45.06</td>
<td>1.71 ± 0.08*</td>
<td>42.29</td>
</tr>
</tbody>
</table>

Enzyme activity represented as specific activity = µmole of product/min/mg tissue protein, values are given as mean (±SE) from three replicates (N = 3); IN = inhibition, EA = ethyl acetate fraction, AST = astragalin, Students t-test *P < 0.05.
Table 3.2. Biochemical changes in the glycolytic enzymes of *Fasciolopsis buski* treated with *Alpinia nigra* crude extract, ethyl acetate fraction, astragalin and PZQ.

<table>
<thead>
<tr>
<th></th>
<th>LDH</th>
<th>%IN</th>
<th>PK</th>
<th>%IN</th>
<th>PEPCK</th>
<th>%IN</th>
<th>MDH</th>
<th>CTH</th>
<th>%IN</th>
<th>CYT</th>
<th>%IN</th>
<th>MIT</th>
<th>%IN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>3.14±0.07</td>
<td>-</td>
<td>1.72±0.06</td>
<td>2.36±0.01</td>
<td>11.42±0.17</td>
<td>-</td>
<td>10.81±0.05</td>
<td>-</td>
<td>1.02±0.02</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>2.92±0.08</td>
<td>6.84</td>
<td>1.35±0.08*</td>
<td>21.51</td>
<td>1.75±0.11*</td>
<td>25.84</td>
<td>11.10±0.17</td>
<td>2.80</td>
<td>10.53±0.12</td>
<td>2.59</td>
<td>0.69±0.03</td>
<td>32.35</td>
<td></td>
</tr>
<tr>
<td>EA</td>
<td>2.44±0.17*</td>
<td>22.32</td>
<td>1.58±0.04</td>
<td>8.14</td>
<td>1.92±0.08*</td>
<td>18.64</td>
<td>11.24±0.38</td>
<td>1.57</td>
<td>11.13±0.41</td>
<td>2.87</td>
<td>0.81±0.03</td>
<td>20.59</td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>2.85±0.05</td>
<td>8.95</td>
<td>1.80±0.05</td>
<td>4.44</td>
<td>1.71±0.11*</td>
<td>27.54</td>
<td>9.92±0.16</td>
<td>13.13*</td>
<td>9.05±0.15</td>
<td>16.28</td>
<td>1.06±0.49</td>
<td>3.77</td>
<td></td>
</tr>
<tr>
<td>PZQ</td>
<td>2.70±0.07*</td>
<td>13.92</td>
<td>1.37±0.01*</td>
<td>20.35</td>
<td>2.09±0.05*</td>
<td>11.44</td>
<td>10.97±0.31</td>
<td>3.94</td>
<td>10.42±0.13</td>
<td>3.61</td>
<td>0.95±0.09</td>
<td>6.86</td>
<td></td>
</tr>
</tbody>
</table>

Enzyme activity represented as specific activity = µM of product/min/mg tissue protein, values are given as mean (±SE) from three replicates (N = 3). PK = pyruvate kinase, PEPCK = phosphoenolpyruvate carboxykinase, AN = *A. nigra* crude extract; EA = ethyl acetate fraction; AST = astragalin; PZQ = praziquantel; CTH = crude tissue homogenate, CYT = cytoplasmic and MIT = mitochondrial fraction; %IN = per cent inhibition, students t-test *P < 0.05.
Figure 3.7. Graphical representation of enzyme activity and its percentage inhibition.

Enzyme activity represented as specific activity = µmole of product/min/mg tissue protein, values are given as mean (±SE) from three replicates (N = 3); CN = control, AN = A. nigra, PZQ = praziquantel, IN = inhibition, EA = ethyl acetate fraction, AST = astragalin.
Figure 3.8. Graphical representation showing LDH, PK and PEPCK enzyme activity and its percentage inhibition on exposure to crude extract of *Alpinia nigra* (AN), ethyl acetate fraction (EA), astragalin (AST) and praziquantel (PZQ). Enzyme activity represented as specific activity = µmole of product/min/mg tissue protein, values are given as mean ±SE from three replicates (N = 3).
Figure 3.9. Graphical representation of malate dehydrogenase activity and its percentage inhibition on exposure to crude extract of *Alpinia nigra* (AN), ethyl acetate fraction (EA), astragalin (AST) and praziquantel (PZQ). Enzyme activity represented as specific activity = µmole of product/min/mg tissue protein, values are given as mean ±SE from three replicates (N =3).
Figure 3.10. Effects of (a) pH, (b) temperature, (c) incubation time and (d) substrate concentration on the acid phosphatase activity of control Fasciolopsis buski.
Figure 3.11. Graphical representation of (a) Lineweaver-Burk plot showing substrate vs. acid phosphatase activity in control tissue and (b) effect of crude extract of *Alpinia nigra* (AN = 0.5 & 1.0 mg), astragalin (AST = 10 & 25 µM) and praziquantel (PZQ = 10 & 25 µM) on acid phosphatase activity.
Figure 3.12. Graphical representation of Lineweaver-Burk plots showing (a) the effect of *Alpinia nigra*, (b) astragalin and PZQ on kinetic parameters ($K_m$ & $V_{max}$) of acid phosphatase activity.

Table 3.3. Alteration in kinetic parameters ($K_m$ and $V_{max}$) of acid phosphatase in control and treated *Fasciolopsis buski*.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th><em>A. nigra</em></th>
<th>Astragalin</th>
<th>Praziquantel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 mg</td>
<td>1 mg</td>
<td>10 µM</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>$V_{max}$</td>
<td>11.02</td>
<td>10.45</td>
<td>7.69</td>
</tr>
<tr>
<td></td>
<td>$K_m$</td>
<td>3.59</td>
<td>3.86</td>
<td>2.94</td>
</tr>
</tbody>
</table>

Inferences: Non-competitive

$V_{max}$ – maximum enzyme activity or µM product formation/minute/mg tissue protein, $K_m$ – substrate concentration.
Figure 3.13. Influence of (a) pH, (b) temperature, (c) incubation time and (d) substrate concentration on the alkaline phosphatase activity in control *Fasciolopsis buski*. 
Figure 3.14. Graphical representation showing (a) Lineweaver-Burk plot of substrate vs. alkaline phosphatase activity in control Fasciolopsis buski and (b) effect of crude extract of Alpinia nigra (AN = 0.5 & 1.0 mg), astragalin (AST = 10 & 25 µM) and praziquantel (PZQ = 10 & 25 µM) on the enzyme activity.
Figure 3.15. Graphical representation of Lineweaver-Burk plots showing (a) the effect of *Alpinia nigra*, (b) astragalin and PZQ on kinetic parameters (K_m & V_max) of alkaline phosphatase activity.

Table 3.4. Alteration in kinetic parameters (K_m and V_max) of alkaline phosphatase in control and treated *Fasciolopsis buski*.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th><em>A. nigra</em></th>
<th>Astragalin</th>
<th>Praziquantel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 mg</td>
<td>1 mg</td>
<td>10 µM</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>V_max</td>
<td>10.24</td>
<td>8.44</td>
<td>7.45</td>
</tr>
<tr>
<td></td>
<td>K_m</td>
<td>0.97</td>
<td>0.88</td>
<td>0.86</td>
</tr>
<tr>
<td>Inferences</td>
<td>Un-competitive inhibition</td>
<td>Mixed inhibition</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

V_max – maximum enzyme activity or µM product formation/minute/mg tissue protein, K_m – substrate concentration.
Figure 3.16. Effects of (a) pH, (b) temperature, (c) incubation time and (d) substrate concentration on the enzyme adenosine triphosphatase activities in *Fasciolopsis buski*.
Figure 3.17. Graphical representation showing (a) Lineweaver-Burk plot of substrate vs. adenosine triphosphatase activity in control *Fasciolopsis buski* and (b) effect of crude extract of *Alpinia nigra* (AN = 0.5 & 1.0 mg), astragalin (AST = 10 & 25 µM) and praziquantel (PZQ = 10 & 25 µM) on the enzyme activity.
Figure 3.18. Graphical representation of Lineweaver-Burk plots showing (a) the effect of *Alpinia nigra*, (b) astragalin and PZQ on kinetic parameters ($K_m$ & $V_{\text{max}}$) of adenosine triphosphatase activity.

Table 3.5. Alteration in kinetic parameters ($K_m$ and $V_{\text{max}}$) of adenosine triphosphatase enzyme on control and treated *Fasciolopsis buski*.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th><em>A. nigra</em></th>
<th>Astragalin</th>
<th>Praziquantel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 mg</td>
<td>1 mg</td>
<td>10 µM</td>
</tr>
<tr>
<td>Adenosine triphosphatase</td>
<td>$V_{\text{max}}$</td>
<td>25.61</td>
<td>21.14</td>
<td>20.31</td>
</tr>
<tr>
<td></td>
<td>$K_m$</td>
<td>2.22</td>
<td>2.10</td>
<td>2.32</td>
</tr>
<tr>
<td>Inferences</td>
<td></td>
<td>Non-competitive inhibition</td>
<td>Mixed inhibition</td>
<td></td>
</tr>
</tbody>
</table>

$V_{\text{max}}$ – maximum enzyme activity or µM product formation/minute/mg tissue protein, $K_m$ – substrate concentration.
Figure 3.19. Influence of (a) pH, (b) temperature and (c) substrate concentration on the acetylcholinesterase activities of control Fasciolopsis buski.
Figure 3.20. Graphical representation showing (a) Lineweaver-Burk plot of substrate vs. acetylcholinesterase activity in control *Fasciolopsis buski* and (b) effect of *Alpinia nigra* extract, astragalin and praziquantel on acetylcholinesterase activity in *F. buski*.
**Figure 3.21.** Graphical representation of Lineweaver-Burk plots showing (a) the effect of *Alpinia nigra*, (b) astragalin and PZQ on kinetic parameters (K<sub>m</sub> & V<sub>max</sub>) of acetylcholinesterase activity.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th>A. nigra (0.5 mg)</th>
<th>A. nigra (1 mg)</th>
<th>Astragalin (10 µM)</th>
<th>Astragalin (25 µM)</th>
<th>Praziquantel (10 µM)</th>
<th>Praziquantel (25 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>5.37</td>
<td>3.95</td>
<td>3.52</td>
<td>5.7</td>
<td>5.74</td>
<td>4.56</td>
</tr>
<tr>
<td></td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>0.28</td>
<td>0.27</td>
<td>0.28</td>
<td>0.34</td>
<td>0.53</td>
<td>0.29</td>
</tr>
<tr>
<td>Inferences</td>
<td>Non-competitive</td>
<td>Uncompetitive</td>
<td>Mixed type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

V<sub>max</sub> – maximum enzyme activity or µM product formation/minute/mg tissue protein, K<sub>m</sub> – substrate concentration.
Figure 3.22. Influence of (a) pH, (b) temperature, (c) substrate concentration and (d) Lineweaver-Burk plot of substrate vs. lactate dehydrogenase activities in control *Fasciolopsis buski*. 

\[ R^2 = 0.9873 \]
Figure 3.23. Graphical representation showing (a) effect of *Alpinia nigra* extract, astragalin and praziquantel on enzyme activity and (b & c) Lineweaver-Burk plots showing the effect of *A. nigra* extract, astragalin and praziquantel and alterations in kinetic parameters ($K_m$ & $V_{max}$) of lactate dehydrogenase in *Fasciolopsis buski*.
Table 3.7. Alteration in kinetic parameters ($K_m$ and $V_{max}$) of lactate dehydrogenase in control and treated *Fasciolopsis buski*.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th>A. nigra</th>
<th>Astragalin</th>
<th>Praziquantel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 mg</td>
<td>1 mg</td>
<td>10 µM</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>$V_{max}$</td>
<td>34.96</td>
<td>29.96</td>
<td>23.78</td>
</tr>
<tr>
<td></td>
<td>$K_m$</td>
<td>0.15</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>Inferences</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$V_{max}$ – maximum enzyme activity or oxidation of µM of NADH per minute per mg tissue protein, $K_m$ – substrate concentration.
Figure 3.24. Influence of (a) pH, (b) temperature and (c) substrate concentration on malate dehydrogenase activities in *Fasciolopsis buski*.
Figure 3.25. Graphical representation showing (a) Lineweaver-Burk plot of control *Fascioloopsis buski* and (b) effect of *Alpinia nigra* extract, astragalin and praziquantel on malate dehydrogenase activity in *F. buski*. 
Figure 3.26. Graphical representation of (a) Lineweaver-Burk plots showing the effect of *Alpinia nigra* extract, (b) astragalin and praziquantel on the kinetic parameters ($K_m$ & $V_{max}$) of malate dehydrogenase activities.
Table 3.8. Alteration in kinetic parameters \((K_m\text{ and } V_{max})\) of malate dehydrogenase enzyme on control and treated *Fasciolopsis buski*.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th>A. nigra</th>
<th>Astragalin</th>
<th>Praziquantel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 mg</td>
<td>1 mg</td>
<td>10 μM</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>(V_{max})</td>
<td>45.45</td>
<td>32.25</td>
<td>23.98</td>
</tr>
<tr>
<td></td>
<td>(K_m)</td>
<td>0.77</td>
<td>0.74</td>
<td>0.68</td>
</tr>
<tr>
<td>Inferences</td>
<td></td>
<td>Un-competitive type inhibition</td>
<td>Mixed type inhibition</td>
<td></td>
</tr>
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</table>

\(V_{max}\) – maximum enzyme activity or oxidation of 1 \(\mu\)M of NADH per minute per mg tissue protein, \(K_m\) – substrate concentration.
Figure 3.27. Influence of (a) pH, (b) temperature and (c) substrate concentration in the pyruvate kinase activities of control *Fasciolopsis buski*.
Figure 3.28. Graphical representation of (a) Lineweaver-Burk plot showing substrate vs. pyruvate kinase activity in control *Fasciolopsis buski*, (b) effect of *Alpinia nigra* extract, astragalin and praziquantel on the enzyme activity in *F. buski*. 
Figure 3.29. Graphical representation of Lineweaver-Burk plots showing effect of (a) plant extract and (b) astragalin and praziquantel on the kinetic parameters \((K_m \& V_{max})\) of pyruvate kinase activity.

Table 3.9. Alteration in kinetic parameters \((K_m \& V_{max})\) of pyruvate kinase enzyme on control and treated *Fasciolopsis buski*.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th>A. nigra</th>
<th>Astragalin</th>
<th>Praziquantel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5mg</td>
<td>1mg</td>
<td>10µM</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>(V_{max})</td>
<td>28.36</td>
<td>25.62</td>
<td>24.53</td>
</tr>
<tr>
<td></td>
<td>(K_m)</td>
<td>2.45</td>
<td>3.05</td>
<td>3.28</td>
</tr>
<tr>
<td>Inferences</td>
<td></td>
<td>Mixed type inhibition</td>
<td>Uncompetitive inhibition</td>
<td></td>
</tr>
</tbody>
</table>

\(V_{max}\) – maximum enzyme activity or oxidation of 1 µM of NADH per minute per mg tissue protein, \(K_m\) – substrate concentration.
Figure 3.30. Influence of (a) pH, (b) temperature, (c) substrate concentration and (d) Lineweaver-Burk plot of substrate vs. phosphoenolpyruvate carboxykinase activities in control *Fasciolopsis buski*.
Figure 3.31. Graphical representation showing (a) effect of *Alpinia nigra* extract, astragalin and praziquantel on phosphoenolpyruvate carboxykinase activity of *Fasciolopsis buski* and (b & c) Lineweaver-Burk showing the alterations in kinetic parameters ($K_m$ & $V_{max}$) of phosphoenolpyruvate carboxykinase in treated *Fasciolopsis buski*. 
Table 3.10. Alteration in kinetic parameters ($K_m$ and $V_{max}$) of phosphoenolpyruvate carboxykinase enzyme on control and treated *Fasciolopsis buski*.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Control</th>
<th>A. nigra</th>
<th>Astragalin</th>
<th>Praziquantel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mg</td>
<td>1 mg</td>
<td>10 µM</td>
<td>25 µM</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>$V_{max}$</td>
<td>17.18</td>
<td>16.33</td>
<td>11.31</td>
</tr>
<tr>
<td></td>
<td>$K_m$</td>
<td>2.54</td>
<td>3.42</td>
<td>2.73</td>
</tr>
</tbody>
</table>

Inferences: Mixed type of inhibition

$V_{max}$ – maximum enzyme activity or oxidation of µM of NADH per minute per mg tissue protein, $K_m$ – substrate concentration.
5.4. DISCUSSION

All living organisms including parasitic helminths have hundreds of metabolic pathways that are vital for normal functioning of their body cells. Each pathway comprised of a number of enzymes that catalyze different steps in the process. In each pathway there are one or more enzymes that are considered to be rate limiting or pacemaker enzymes. These enzymes are usually endowed with allosteric control mechanism and are subject to feedback inhibition or activation by one or more of the substrates in the pathway (Mansour 2002). Helminth parasites have a complex life cycle changing from free living larval stages (miracidia and cercaria that depends predominantly on aerobic respiration) to adult forms (that adapt to the hypoxia, or even anoxia type of microenvironment in host gastrointestinal tract) that draws their energy via anaerobic or semi-aerobically evolves mechanisms (Von Brand 1950, 1979; Tielens et al. 1992; van Hellemont et al. 2003).

Further, absence of proper digestive tract in parasitic helminths compels them to look for alternatives through which food material and other requirements could be absorbed. The helminth body surface or tegument is known to be a dynamic cellular structure that plays vital role in the physiology of trematodes and cestodes, being involved in nutrient absorption, defense against enzymatic and immunological attack by the host, in excretion and ionic exchange. In the present study, activities of several tegumental enzymes such as AcPase, AlkPase, ATPase and AchE were detected in different sites such as tegument, sub-tegument, body musculature and intestinal regions of the fluke, F. buski. Biochemical studies have quantified large amount of AlkPase activity (10.62 U/mg tissue proteins) in the fluke among all the four tegumental enzymes studied. Membrane enzymes such as alkaline and acid phosphatases, 5’-nucleotidases and maltase are proteins predominantly bound to external surface membrane; on the other hand, phosphodiesterase, adenosine triphosphatase, leucine aminopeptidase and gamma-glutamyltranspeptidase were found apparently associated with the internal membrane. Cytochemical studies at the ultrastructural level have indicated that phosphatases are localized in or on the tegumentary brush border of cestodes (Rothman 1966; Lumsden et al. 1968). Fine electron dense precipitates of ATPase activity was seen within the tegumental regions of S. mansoni, apparently in the apical and invaginated regions of the double surface membrane and on the cytoplasmic ide of the basement membrane and its intra-tegumentary digitations. In a similar kind of study, Shaw (1987) has revealed
variations of staining intensities of ATPase with higher activity localized apparently on the dorsal side of male tegument; whereas the distribution was uniform in the tegument of female worms. Acid phosphatase activity was localized in the caeca of *Haematoloechus medioplexus* and *Paragonimus kellicotti*, and was associated with granular and vesicular membrane-bound inclusions, the Golgi complex, the granular endoplasmic reticulum, and the luminal protoplasmic projections (Dike 1969).

Phosphatases are important tegumentary proteins whose association with the membrane transport has been suggested by their ubiquitous presence in the tissues of secretory or absorptive function. The view that physiological transport activity is mediated directly by enzymes located on or within cytomembranes has received much support in recent years (Bernardi 1999). The widespread and impressive amounts of certain tegumental enzymes demonstrated in several cestodes suggest that they might play a highly significant role in digestion and/or absorption in the distinctive tissues of helminth parasites (Smyth and McManus 1989). The absorptive property of tegumental enzymes have also been highlighted by the fact that the inhibition of AlkPase activity by 23% in *Echinococcus multilocularis* metacestodes following treatment with isatin, a known phosphatase inhibitor have caused complete inhibition of glucose uptake (Duriez et al. 1989). High activities of both acid and alkaline phosphatase have been confirmed in the tegument, sub-tegument or subcutaneous cells and parenchyma of the various helminth parasites (Arme 1966; Niemczuk 1993). Arme (1966) and Niemczuk (1993) established a clear dependence of the activity of these enzymes on the degree of maturity of tapeworm segments with greater activity observed in the mature segments. It is interesting to note that the cytochemical localization of surface-associated non-specific alkaline and acid phosphatase activities shows regional localization, with AlkPase activity being on the tegument but not in the cecum portion of the esophagus, whereas the AcPase activity is located in the posterior esophagus and the cecum (Ernst 1976). The AcPase in the mammalian small intestine is believed to participate in the degradation of organic phosphatase to free phosphate. Although there is no lucid evidence, the enzyme has been known to act in the substances transportation (Kaplan 1972). Dusanic (1959) observed AlkPase activity in *S. mansoni* and reported that AlkPase supports the absorption and secretion of the parasites. Among the majority of nematodes, the activity of AcPase in the tegument is high and is co-related with the absorption of glucose through the body wall (Maki and Yanagisawa 1980). According to
Skotarczak (1987), both AcPase and AlkPase enzymes play an important role in the metabolic processes of helminth embryos, and their activity depends on the metabolic intensity. These enzymes are also believed to be responsible for the transport of substances across the walls of excretory canals and also in ion regulation. The histochemical localization of AlkPase activity in *Leucochloridiodorpha constantiae* cultivated from the metacercari to the ovigerous adult on the chick chorioallantois observed AlkPase activity in the suckers and was more intense in the acetabulum than the oral sucker of whole worms (Fried 1985). The occurrence of vital tegumental enzymes viz. AcPase, AlkPase and ATPase have recently been demonstrated both histochemically and biochemically in a number of helminth parasites viz., *H. diminuta*, *E. multilocularis*, *Spirometra erinacei* etc. (Pappas 1991; Kwak and Kim 1996; Stettler et al. 2001). It has also been hypothesized that the decrease in the tissue ATPase activity disrupts the permeability of the glycocalyx-plasmalemma system causing damage to the plasmalemma parts which in turn causes the AcPase to diffuse out of the cell to digest the damaged membrane parts leading to a decrease in the tegumental AcPase levels. Damage of the parasite membrane also causes an increase inflow of salts from the external media which accumulates in the basal infolds (Fairweather and Boray 1999).

All the tegumental enzymes studied in the present investigation showed reduced stain intensities when exposed to crude extract of *A. nigra*, astragalin and the reference drug praziquantel. In a similar kind of study, Pal and Tandon (1998) studied some important tegumental enzymes in the cestode parasite *R. echinobothrida* both histochemically and biochemically and observed reduction in enzyme activity. Kinetics study of AcPase has shown the influences of various physical parameters such as pH, temperature, incubation time and effects of various treatments on the enzyme activity (Figure 3.14). Every enzyme or protein work on a particular range of conditions, lower or higher than that range have negative effect on the functioning of enzymes. Similar to our observations, Parshad and Guraya (1978) have also studied the influence of pH and various chemicals on AcPase and AlkPase activity in several helminth parasites and found the optimum pH for AcPase activity was 5.4, 4.5, 4.7 and 5.0 in *Ascardia galli*, *Centorhynchus corvi*, *Raillietina cesticillus* and *Cotylophoron cotylophorum*, respectively; while for AlkPase the pH was 9.1, 9.5, 8.7 and 8.4 in *A. galli*, *C. corvi*, *R. cesticillus* and *C. cotylophorum*, respectively. In *A. galli* and *C. cotylophorum* the AcPase showed more activity than
AlkPase, whereas the latter was more active in *R. cesticillus* and *C. corvi*. Histochemically, a pronounced decline in the staining intensities was observed in all the enzymes. Decreased or inhibition of AcPase, AlkPase, ATPase and 5’-Nu enzyme activities have also been noticed when the parasites were treated with root peel extract of *Flemingia vestita*, its active principle genistein and PZQ. Extracts from certain medicinal plants, like *Butea monosperma*, *Embelia ribes* and *Roltlesia tinctoria* drastically decreased activities of both AcPase and AlkPase in the trematode, *Paramphistomum cervi* (Chopra et al. 1991). The root tuber peel extract of *F. vestita* and its active principle genistein similarly caused significant reduction of the enzymes in *R. echinobothrida* (Pal and Tandon 1998), and in the giant intestinal fluke, *Fasciolopsis buski* (Kar and Tandon 2004), comparable to those of the standard pharmaceuticals like praziquantel and oxyclozanide. *F. vestita* and its bioactive isoflavone, genistein was also shown to cause 39-49% decline in the concentration of calcium in *R. echinobothrida* (Das et al. 2006). Similarly, the alcoholic crude stem bark of *Acacia oxyphylla* and root bark extract of *Millettia pachycarpa* have been demonstrated to exhibit profound anthelmintic effects on the avian gastrointestinal cestode, *R. echinobothrida* through significant reduction in the activities of AcPase and AlkPase (Lalchhandama et al. 2007, 2008). Histological studies have revealed the potency of anthelmintic agents that induce considerable changes in the internal structural features of helminths, resulting loss of normal cellular conformity in vital tissues (Roy et al. 2008; Roy and Swargiary 2009; Roy et al. 2010). Recent studies have shown the potential anthelmintic properties of alcoholic crude extracts of *A. oxyphylla* and *S. virosa* that reduced the AcPase, AlkPase and ATPase in *R. echinobothrida* both histochemically and biochemically (Dasgupta and Roy 2010). Similar types of studies carried out by Challam (2012) has revealed the anthelmintic properties of several traditionally used medicinal plants such as *L. ramosa*, *C. baccans*, *Artemisia indica* and *Spilanthes oleraceae*, all of which got effective inhibitory activities against all the three enzymes.

The presence of neurotransmitters in the tegument, sub-tegument and body musculature in several helminth parasites has been extensively investigated. The present investigation through histochemical and biochemical studies have established the presence of AchE activity concentrated mainly in the tegument, sub-tegument and intestinal regions of control fluke, *F. buski*. Quantitatively, the AchE activity was estimated to be 0.029 U/mg tissue proteins. However, on exposure to crude
extract of *A. nigra* and astragalin, a reduction in the enzyme activity/staining intensities were observed indicating anti-AchE activity of the crude as well as its active compound astragalin of the plant. Similarly, cholinesterase activity has been demonstrated histochemically in the nervous system, tegumental and sub-tegumental musculature of oncomiracidium of *Pricea multae* (Venkatanarsaiah 1981). In the present study, a highest (56.8%) inhibition of AchE activity was observed in the parasites treated with *A. nigra* ethanolic crude extract, whereas the astragalin treatment showed least activity among all the treatments. In a similar kind of study, Pal and Tandon (1998) could localize the AchE activity in the tegument, sub-tegument, muscle layers and reproductive systems of *Raillietina echinobothrida* with intense activity mainly in the tegumental region of the body. Biochemical studies by the same authors have reported inhibition of AchE activity when the cestode parasite *R. echinobothrida* was treated with crude extracts of *F. vestita*, genistein and PZQ. Several studies have reported the reduction of AchE activity following *in vitro* exposure to many anthelmintics of plant origin (Kaur and Sood 1982; Win et al 1994). In contrast, Fallon et al. (1994) showed a progressively enhancing cholinesterase activity along with alkaline phosphatase activity in praziquantel-treated *S. mansoni*. It has also been believed that anthelmintics including organophosphates and PZQ have a neuromuscular mode of action (McKay et al. 1989; Cox 1994) that interfere with transmission at nerve-nerve synapse or at neuromuscular junction by inhibiting the enzymes responsible for inactivating the neurotransmitter at the synapse (Raether 1988; Bryant and Behm1989). The cholinesterases of different flatworms like *F. hepatica*, *S. mansoni*, *H. diminuta* and *H. nana* is predominantly an acetylcholinesterase with lesser or no non-specific esterase activity depending on the species showing most pronounced histochemical staining in central ganglia and associated commissures/nerve cords of the pharynx and suckers. Stain is also observed in fine ramifications throughout the parenchyma/sub-tegumental muscle regions presumably associated with neuromuscular innervation, and in muscularized reproductive structures. Since the helminth AchE differs in sensitivity to inhibition from the mammalian counterpart, the enzyme appears to be a potential target for chemotherapy (Barrett 1981). In *Fasciola hepatica* acetylcholine, its congeners and AchE inhibitors relaxed the musculature of the worms inhibiting rhythmical movements and eventually producing paralysis (Chance and Mansour 1949, 1953). Similar decrease in the
motility has been demonstrated in cestode Dipylidium caninum (Terada et al. 1982). Experiments involving plant-based anthelmintics or extracts of different medicinal plants have been tested in vitro and in vivo against AchE activity and have been found to be effective against different helminths. For example, the use of male fern (Dryopteris felix mas) against cestodes and trematodes and that of arecoline (Areca catechu) against tapeworm of dogs and poultry has been reported (Reinemeyer and Courtney 2001). Dose and time dependent inhibition of motility and AchE activity were reported in Azadirachtin-treated worm Cotylophoron corylophorum (Veerakumari and Priya 2006). Filho et al. (2006) studied 309 plant extracts and 260 compounds isolated from plants were investigated for their inhibitory effect against AchE. The hexane fruit extract of Schizandra chinensis (Schisandraceae) was found to show significant inhibition of AchE activity (Hung et al. 2007). Recent studies on the methanolic flower extract of Rangoon creeper (Quisqualis indica Linn.) showed an inhibitory activity of electric eel acetylcholinesterase in a dose-dependent manner (Wetwitayaklung 2007). The alteration in AchE activity also observed in the present investigation after treatment with shoot extract or astragalin suggests that A. nigra and its active compound may be of anthelmintic interest.

Glycolysis is the major energy-yielding pathway in the parasites since the Krebs’ cycle and hexose monophosphate pathways are less functional. In most helminth parasites, glucose and other simple carbohydrates are metabolized following type 2 glucose fermentation, which is characterized by a CO₂-fixation step (by phosphoenolpyruvate carboxykinase) and malate dismutation (Bryant and Flockhart 1986; Omar et al. 1996; Lakhdar-Ghazal et al. 2002). Several glycolytic enzymes, such as hexokinase (HK), phosphofructokinase (PFK), aldolase, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase (LDH), and malic enzyme (ME) are reported to be present in various helminth species, and the operation of the Embden-Meyerhof pathway has been confirmed (Arme and Pappas 1983; Smyth and McManus 1989). Present histochemical study displayed high stain intensities for both LDH and MDH activities in the untreated control F. buski compared to the treated flukes. However, no such visible changes in the stain intensities were observed for MDH activity on treatment with the crude extracts of A. nigra, astragalin and PZQ. LDH activity showed slight changes in their depositions of stain. Similarly, quantitative enzyme assays of four important and key glycolytic
enzymes such as MDH, LDH, PK and PEPCK showed high enzyme activities in control tissue homogenate of *F. buski*. However, a highest (56.8%) inhibition of AchE was observed in *A. nigra* crude extract treated parasite followed by AcPase (53.50%), ATPase (48.89%), AlkPase (27.08%), PEPCK (25.84%), PK (21.51%), LDH (6.84%) and MDH (2.8%). Reference anthelmintic drug, praziquantel showed more or less similar extend of enzyme inhibition as that of crude extract of *A. nigra* treated parasite. Similarly, commercial drugs like sumarmin, centperazine, diethylcarbamazine and LEV were showed marked inhibition of several glycolytic enzymes (Hussain et al. 1990; Xiao et al. 1998). Pampori and Srivastava (1987) recorded a significant inhibition of different glycolytic enzymes activities like PEPCK, HK, G6PDH and MDH in *Cotugnia digonophora* when treated with different anthelmintics like MBZ, niclosamide and PZQ. Ahmad and Nizami (1987) showed that the commercial anthelmintic mebendazole has no effect on HK in *Avitellina lahorea* but it influenced the activities of some glycolytic enzymes such as phosphorylase, phosphoglcomutase and G6Pase, and also caused glycogen depletion and the inhibition of glucose uptake in vitro. In *Hymenolepis microstoma*, the specific activity of HK, PFK and PK was not stimulated by 5-hydroxytryptamine, but CO₂ fixation by PEPCK was found to be inhibited (Rahman and Mettrick 1982). PEPCK’s main role in helminth parasites is in the degradation of glucose rather than its synthesis through the gluconeogenic pathway (Smyth and McManus 1989). However, in *S. mansoni*, experiments with inhibitors of PEPCK gave no indication that this enzyme is involved in the degradation of glucose, and it was confirmed that lactate is formed from phosphoenolpyruvate via the actions of PK and LDH (Tielens et al. 1991). In the present study, PEPCK activity was found to be higher than PK in *F. buski*, averaging about 2.36 units/g tissue protein of control parasites. It is also observed that the activity of PEPCK was reduced by 11-27% in the parasites treated with the various test materials.

Pyruvate kinase, another potential regulatory enzyme of glycolysis, converts phosphoenolpyruvate to pyruvate. In the present study, the PK activity decreased by about 4 to 21% in *F. buski* treated with *A. nigra* extracts, astragalin and PZQ. Thus, the branch point of PEPCK/PK perhaps forms the basis of the anthelmintic attack by the plants and plant derived components. Enzymatic activities of PK were also inhibited by artemether both in male and female *Schistosoma japonicum* (Xiao et al. 1998). Commercial anthelmintics like Sumarmin and centperazine caused a marked
inhibition in most of the enzymes of phosphoenolpyruvate-succinate pathway in *Setaria cervi*, although diethylcarbamazine and levamisole were found to be more or less ineffective at a lower concentration against all enzymes of the glycolytic pathway (Hussain et al. 1990). In the present investigation the ethyl acetate fraction treated parasite showed highest reduction (22.32%) in LDH activity with least enzyme inhibition (8.95%) was observed among astragalin treated flukes. In contrast to our result, the activity of LDH increased by 67% with genistein treatment in *R. echinobothrida*, and by 55% and 58% by treatments with crude root peel extract of *Flemingia vestita* and PZQ, respectively (Das et al. 2004). Many anthelmintics are believed to work via an alteration in the activity of parasite LDH. For example, benzimidazoles act primarily via activation of LDH, catalysing the conversion of pyruvate to lactate (Veerakumari and Munuswamy 2000). Artmether, a derivative of Artemesia and well known for its antimalarial properties, was shown to exert a potent inhibitory action on the LDH and G6PDH activities in *S. japonicum* (Xiao et al. 1998). Filarin (a drug of herbal origin and used in Siddha medicine) and diethylcarbamazine inhibited pyruvate reduction rather than lactate oxidation in *S. digitata* by altering the activity of LDH (Banu et al. 1989). With treatment by isatin, the LDH activity decreased in the metacestodes of *Echinococcus multilocularis*, in which glucose and glycogen stores also declined significantly (Delabre-Defayolle et al. 1989). In the present study the activity of MDH (11.42 U/mg tissue protein) was found to be higher compared to LDH (3.14 U/mg tissue protein). However, on exposure to different treatments, changes in the enzyme activities were noticed although not statistically significant at P < 0.05. Drugs like oxyclozanide, hexachlorophene, nitroxynil, rafoxamide and diamphenethide were reported to inhibit the MDH activity in different trematodes like *F. hepatica*, *F. buski*, *Paramphistomum explanatum* (Probert et al. 1981). Similarly, following *in vitro* treatment with levamisole, albendazole, cambendazole and tiabendazole, inhibition of different enzymes like AcPase, AlkPase and MDH activity was reported in fowl nematodes *Ascaridia galli* and *heterakis gallinae* (Sharma et al. 1986, 1989). Though the diethylcarbamazine citrate did not appreciably changed the activity of either mitochondrial MDH or mitochondrial ME, the anthelmintic agent filarin effectively inhibit mitochondrial MDH in *S. digitata*. The leaf extracts of *Ocimum sanctum*, *Lawsonia inermis* and *Calotropis gigantean*, and leaf and flower extracts of *Azadirachta indica* were, however, found to inhibit both mitochondrial MDH and
mitochondrial ME (Banu et al. 1992). The MDH activity was also suppressed by mebendazole, albendazole and PZQ in hydatid cysts of *E. granulosus* (Xiao et al. 1993).

There is a good correlation between the ratio of PK and PEPCK activities and the relative amounts of lactate vs. succinate accumulated in the cell (Bueding and Saz 1968). It has been postulated that the parasites that rely and synthesize energy rich ATP via normal glycolytic process have a PK/PEPCK ratio of 2-10 (Barrett 1981; Bera and Manna 2007). In our present study, the PK/PEPCK ratio was found to be 0.12 which is an indication of anaerobic type of respiration in adult. Higher value of PK/PEPCK ratio indicates that the adult fluke are more lactate producer than malic acid. In another study Rafi and Raj (1991) studied all enzymes of phosphoenolpyruvate-succinate and their inhibition with an antifilarial drug diethylcarbamazine in *S. digitata*. The result showed higher ratio of PK/PEPCK and the drug were found to cause general inhibition of all enzymes of the pathway. In the present study, the PEPCK activity was found to be inhibited more compared to PK activity under various treatment agents, suggesting that glucose breakdown followed the PEPCK-malate pathway. Thus, from the results obtained in the present *in vitro* experiments, it may be postulated that the phytochemicals of *A. nigra*, which seem to target the tegumental interface of the parasite as the primary target, also influence carbohydrate metabolism towards PEPCK-malate formation in *F. buski*.

**5.5. CONCLUSION**

Currently, there is no effective vaccine available to control helminth parasites due to complex life cycle and immunological interactions occurring during helminth infections. As such, chemotherapeutic drugs are the only direct intervention currently available. There is also no denying about the fact that the use of commercial anthelminitics follows hand in hand with side effects of the drugs or development of drug resistance and this is the driving force for developing newer drugs against helminth infections. The present study has revealed the presence of high activity of some of the key tegumental enzymes such as acid and alkaline phosphatase, adenosine triphosphatase, acetylcholinesterase that are established to be functioning in absorption and transportation of glucose via., tegument of fluke, *F. buski*. Presence of glycolytic enzymes such as pyruvate kinase, phosphoenolpyruvate carboxykinase, lactate dehydrogenase and malate dehydrogenase are also indicative of the semi-
aerobic or anaerobic type of respiration in adult *F. buski*. The observed alterations in the enzyme activities on exposure to different treatments have exposed the anthelmintic efficacy of *A. nigra* and its phytochemicals. However, more detailed study regarding the isolation of other active compound(s), their mode of action, binding and inhibition of enzymes/proteins are needed in order to reveal its actual anthelmintic potency of the plant. There are remarkable differences in the physiology of various parasitic helminths, and therefore target prioritization could be given according to the structural features as well as uniqueness of enzymes, receptors, ion channels, or biochemical pathways and the sensitivity of the these parasites towards small molecules that are potential drugs against these targets. Moreover, the studies of enzyme properties, its specificity, regulation, kinetics and other physical parameters cannot be studies with certainty in crude tissue homogenate. The prospects for specific and effective anthelmintic development therefore will require enzymes/proteins in pure form that can be obtained by cloning the enzyme and obtaining the amino acid sequence to control helminthiases. However, the present preliminary investigation established the fact that the plant derived ethanol extract, its ethyl acetate fraction and active compound of the plant astragalin acts transtegumentally, alter surface architecture and activities of vital tegumental enzymes leading to paralysis and death of the fluke, *F. buski*. 
SUMMARY

Indigenous system of herbal therapy is becoming an increasingly attractive approach to control parasitic infections, particularly in developing countries like India. Among parasites, helminths (Cestodes, Trematodes and Nematodes) and helminthic infections remain to be one of the major health problems affecting billions of people all over the world. They are the most common infectious agents of human beings of tropical countries contributing wide spread occurrence of undernourishment, anemia, eosinophilia and pneumonia. They are also responsible for considerable economic losses to the livestock industry of marginal farmers, particularly in developing countries. The poor hygienic conditions, limited availability of health care services, unsafe drinking water, poverties as well as favorable conditions of the tropics for the growth and development of helminths contributes enormously to the spread and propagation of helminthic infections. The use of commercial anthelmintic drugs of various types such as mebendazole, albendazole, praziquantel, levamisole etc. have been used over the last few decades as the most common method of controlling helminthiases all over the world. Although, a large number of effective and potential anthelmintic drugs have been developed, controlling of helminthiases still is one of the most challenging tasks because of the limited availability of commercial drugs to the rural areas and the resistance capacity developed by most of the helminths against those synthetic drugs.

Contribution of plants to fight against various diseases dates back several centuries, and has been documented by the ancient civilizations. 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 different helminths. Several such studies based on traditional medicinal knowledge were done throughout the globe as well as in Indian sub-continent to test the putative anthelmintic properties of different plants. *Alpinia nigra* belonging to the family Zingiberaceae is one such traditionally used medicinal plant, the shoot part along with a part of rhizome of which is used by the indigenous tribal people of Tripura, India, as vegetable; whereas the aqueous juice of shoot of the plant is consumed to get rid of intestinal helminth infection. Although earlier studies carried out on *A. nigra* have confirmed the anthelmintic potential of crude
extract of the plant against giant intestinal fluke *Fasciolopsis buski*, details of histomorphological and biochemical alterations in *F. buski* caused by the crude and its different fractions of the crude extract of the plant are not known. Therefore, in the present study detailed investigations was carried out involving crude extract of the plant and its different fractions responsible for anthelmintic properties and their possible mode of action on *F. buski*, a parasite of zoonotic importance in Northeast India.

It is observed that the shoot extracts of *A. nigra* possessed potential anthelmintic efficacy against the trematode parasite, *F. buski*. The ethanolic crude extract showed stronger anthelmintic property by paralyzing the parasites within 2.14 ± 0.48 h of incubation at a test concentration of 20 mg/ml PBS. Out of all the four solvent fractions of *A. nigra* crude extract used for treatment, ethyl acetate fraction showed better anthelmintic activity that paralyzed the parasites within 3.12 ± 0.42 h of incubation at similar concentration. Astragalin, the active compound of *A. nigra* also showed good anthelmintic property at its tested concentrations. The time taken for paralysis was 9.76 ± 1.05 h at its highest concentration of 0.10 mg/ml PBS, while at the same concentration the paralysis time for the reference drug praziquantel was 6.76 ± 1.44 h. The crude extract and its different fractions of *A. nigra* were also found to cause significant changes in the quantity of trace elements. Ultrastructurally, *F. buski* exposed to different treatments viz., *A. nigra* crude extract, and its ethyl acetate fraction, astragalin and reference drug PZQ showed changes in the tegumental ultrastructure of the parasites. *A. nigra* crude extract treated fluke revealed shredded tegumental layer with large number of vacuole formation, swelling of basal lamina layer and release of internal tissue materials to the exterior. Similar extent of damages and distortions were observed in parasites exposed to ethyl acetate fraction and astragalin. Histochemical localizations of enzymes such as AcPase, AlkPase, ATPase, AchE, MDH and LDH in the tissue sections showed variations in their staining intensities between the control and treated flukes. Reductions in the staining intensities of almost all the enzymes were observed under study. Biochemical quantification of enzymes activities also revealed changes in their enzyme activities when the parasites were exposed to different treatments. The crude extract of *A. nigra* causes highest reduction in the activities of AchE, AcPase and ATPase whereas the ethyl acetate fraction of *A. nigra* crude extract and bioactive
compound astragalin showed more or less similar extent of enzyme inhibition. The mechanism of enzyme activity of all the enzymes such as AcPase, AlkPase, ATPase, AchE, PK, PEPCK, MDH and LDH under study was found to be influenced by various physico-chemical properties such as pH, temperature, incubation time and substrate concentrations. Enzyme assay mixtures when incubated with different doses of treatments, alterations were observed in the kinetic parameters of all the enzymes. At its tested concentrations of 0.5 and 1.0 mg, the crude extract of *A. nigra* showed non-competitive inhibition in AcPase, ATPase and AchE activities, followed by mixed type of inhibition in LDH, PK and PEPCK and un-competitive type of enzyme inhibitions in AlkPase and MDH activity. Ethyl acetate fraction of the crude extract and the bioactive compound, astragalin also causes a similar kind of alteration the kinetic parameters of all the enzymes studied.

Our preliminary *in vitro* investigations on bioactivity of crude extract of *Alpinia nigra* and its different fractions along with bioactive compound astragalin have revealed the potent anthelmintic property of the plant. However, in order to pinpoint the specific principal ingredient of the plant as an anthelmintic, it is suggestive that the ethyl acetate fraction would be a good choice to start with. Astragalin, an active component of the plant showed potential anthelmintic efficacy and therefore, there is a possibility of astragalin being the active principle of the plant responsible for anthelmintic activity. However, it is prerequisite to isolate and identify other active anthelmintic component(s) of ethyl acetate fraction of the plant, if any, to confirm the present finding and to study its exact mode of action.