Discussion
5. DISCUSSION

Helminths are major cause of reduced production in livestock. Control of helminth parasites is a vital part of health and production management in sheep flocks. The prevalence of amphistome parasites is very high in domestic ruminants and seen all over the world. *Cotylophoron cotylophorum* is a digenetic trematode parasite belongs to the family paramphistomidae cause the disease paramphistomosis (Qadir *et al*., 2010; Swarnakar and Kumawat, 2013). The disease causes high morbidity and mortality resulting in great economic losses through reduced productivity to poor farmers. The economy of rural people largely depends on domestic ruminants.

Chemotherapy is the efficient and effective tool to cure and control the amphistome infections; anthelmintics may interfere with parasite’s carbohydrate metabolism, inhibit respiratory enzyme, block neuromuscular action or make them susceptible to destruction by host’s macrophages. However, the gastrointestinal helminths become resistant to currently available synthetic anthelmintics due to indiscriminant use of drugs (Waller, 1997; Singh *et al*., 2002). The degree of resistance against parasitic worms has reached the point where sheep farming is unsustainable in some areas. If left unchecked, anthelmintic resistance could prove to be one of the biggest challenges to sheep production and welfare in world wide apart from
other serious problems such as residual toxicity, adverse drug reaction and relatively unaffordable to the small-scale farmers. It is imperative to decrease the reliance on these chemotherapeutic drugs for parasite control, not only because of resistance, but also because of growing concerns about the adverse consequences of these anti-parasitic drugs on the ecosystem and biodiversity. Therefore, there is a need to rethink the usage of synthetic anthelmintics, as well as to develop cheap, eco-friendly drugs, which will lead to sustainable control of parasites (Waller, 2003; Washira et al., 2009; Veeraumari, 2015).

The use of botanical anthelmintics is one of the alternative methods (Veerakumari, 2015). Plant derived drugs serve as prototype to develop more effective and less toxic medicines (Mehta and Anita, 2008; Lakshmanan et al., 2011; Dutta et al., 2012). A number of medicinal plants have been used to treat parasitic infections in man and animals (Akhtar et al., 2000; Ayyanar and Subash-Babu, 2012). Unlike synthetic anthelmintics, plant based anthelmintics with different mode of actions could be able to prevent the development of resistance. Hence an attempt has been made to reveal the anthelmintic efficacy of well-known medicinal plants Adhatoda vasica and Piper betle against C. corylophorum. The results of the present in vitro study evidently asserted AvEaE and PbEaE were biochemically effective by placing physiological challenges and cause many structural deformities in treated flukes. Furthermore, in vivo study elucidated the anthelmintic potency of PbEaE against paramphistomes.
5.1 In vitro studies

5.1.1 Motility and role of Acetylcholinesterase

The action of many anthelmintics is reflected in their ability to reduce the motility of the parasites. Assessment of drug efficacy was based on the expectation that motility would decline faster when the flukes were exposed to an effective agent (Behnke et al., 2008). Gross visual observations of motility of the parasites against aqueous and various solvent extracts revealed that the ethyl acetate extract of both the plants were effective. The flukes were exposed to five different sub lethal concentrations of AvEaE and PbEaE (0.01, 0.05, 0.1, 0.5 and 1.0mg/ml) for 2, 4 and 8h. The motility response of the control and drug-treated flukes were recorded with the aid of electronic micro motility meter for more authentication, the highest percentage of inhibition in motility and AChE was found in drug-treated parasites after 8h of exposure at 1 mg/ml concentration. Inhibition of motility of parasites exposed to various plant extracts is well documented by many researchers. Ethanolic extract of Allium sativum, Piper longum, Potentilla fulgens, Lysimachia ramose and Carex baccans caused a dose-dependent inhibition of motility and mortality of Fasciola gigantica, Gigantocotyle explanatum, Gastrothylax crumenifer, Fasciolopsis buski, Ascaris suum and Raillietina echinobothrida (Singh et al., 2007; Roy et al., 2010; Challam et al., 2010; 2012).
AChE is an important enzyme in helminths which is associated with
the neuromuscular coordination. The classical role of AChE is to terminate
transmission of neuronal impulses by rapid hydrolysis of acetylcholine.
Cholinesterase is secreted by many helminths in the alimentary tract or other
mucosal tissues. Acetylcholine has been recorded to have numerous effects
including stimulation of chemotaxis and lysosomal enzyme secretion by
neutrophils, inflammatory mediators and histamine, leukotriene release by
mast cells, and augmentation of lymphocyte-mediated cytotoxicity
(Lee, 1996). Thus, acetylcholinesterase activity would help to prevent
stimulation of cellular and humoral response to parasite infection.

Szwajgier and Borowiec (2012) found that the fruit extract of
*Carum carvi* effectively inhibited AChE. The two compounds R-carvone and
D-limonene, found in *C. carvi* were demonstrated to be a potent inhibitor
of AChE activity from larvae of several stored product insects
(Abdelgaleil *et al.*, 2009; Lopez and Pascual, 2009). The inhibition of AChE
could be the significant reason for the drastic changes in motility of the
parasites. Roy *et al.* (2012) reported that the AChE of *Fasciolopsis buski* was
inhibited when treated with *A. nigra* plant extract and praziquantel. In
*Fasciola hepatica*, inhibitors of AChE relaxed the musculature of the worms
inhibiting rhythmical movements and eventually resulted in paralysis
(Mansour, 2002). Similarly, the inhibition of AChE activity of
*C. cotylophorum* exposed to seed kernel extract of *Azadirachta indica* was
reported by Veerakumari and Priya (2006). Results of the present investigation clearly showed that the extracts of AvEaE and PbEaE had immolated the parasites by affecting their neurotransmission and dismantle the muscular coordination system.

5.1.2 Structural changes

Tegument of the parasites could be a vital target for any anthelmintics. AvEaE and PbEaE caused serious morphological and anatomical deformities in C. cotylophorum. Light microscopic study revealed the distortions of the tegument in the AvEaE and PbEaE-treated flukes. The tegument acts as the vital organ of any endoparasites, which performs various functions like absorption of food materials, protection and osmoregulation; suckers which are modification of tegument, offers organ of anchorage (Swarnakar and Kumawat, 2014). Hence the modifications in the structural organization of the tegument of a parasite are essential in developing any rational drugs as stated by Panyarachun et al. (2010).

In the present study the light microscopic observations revealed that the tegumental surface of control flukes is highly corrugated with transverse folds alternating with grooves and is spineless, this is a specific character of trematodes (Swarnakar and Kumawat, 2014) whereas in drug-treated flukes wide ranges of deformities were seen in the tegumental architecture of C. cotylophorum.
Tegumental detachment due to anthelmintics may breakdown parenchymal cells, leaving vacuolated areas (Veerakumari and Paranthaman, 2004). In the present investigation AvEaE and PbEaE treated *C. ctylophorum* showed the detachment and discontinuation of tegument surface syncytium and also vacuolization and breakage in subsyncytial zone. Similarly crude extract of *Flemingia vastita* caused disorganization of cuticle and body musculature in treated *Ascaris suum* (Yadav et al., 1992) and destruction in tegument and distortion of muscles, vacuolisation in muscles of sucker in different helminth parasites (Tandon et al., 1997). The plant extract of *Lasimachia ramosa* caused mortality, shrunken body, destructive surface alteration in *Ascaris suum, Fasciola buski* (Challam et al., 2010) and the anthelmintic activity of *Trigonellafoenum-graecum* produced swelling, detachment, blebbing and discontinuation in tegument of amphistome *Gastrothylax crumenifer* (Swarnakar et al., 2014) which corroborate with our findings.

The posterior sucker (acetabulum) and oral sucker of *C. ctylophorum* were damaged by *A. vasica and P. betle*. Lesions and vacuoles were observed in the acetabulum of treated flukes. Both AvEaE and PbEaE-treated *C. ctylophorum* showed vacuolization and deformed oral sucker. Swarnakar and Kumawat (2014) compared the tegument of drug-treated parasite of *Orthocoelium scoliocoelium* with the untreated parasite by light microscopy. The microscopic observations revealed wide
scale deformity in the tegumental architecture of drug treated parasite with breakage and detachment in surface tegument and they also observed vacuolization in subsyncytial zone and parenchymatous cells, damaged acetabulum of drug treated parasites revealed the breakage and vacuolization in musculature of sucker. Degenerated acetabulum in treated flukes, retard the attachment of the parasites to the host rumen epithelium which may lead to the expulsion from the host. Rigorous and prominent lesions were observed in the gastrodermis of AvEaE and PbEaE-treated flukes result in the impairment of feeding mechanism and absorption of nutrients. AvEaE and PbEaE induced vacuolization in testes and ovary. Defects in the quality of the vitelline cells would prevent shell formation and render non-viable eggs (Hanna et al., 2006). Vacuolization in the ovary and testes affect the reproductive potential of C. cotylophorum resulting in decreased egg production and irregular embryonation.

Scanning electron microscopic (SEM) observations revealed the visible changes in the surface tegumental features of the flukes treated with AvEaE and PbEaE. An alteration in the surface topography of the test parasites could be chosen as a parameter to assess the effect of the plant-derived components (Lyndem et al., 2008). The body surface of the parasite, the interface between the parasite and its microenvironment inside the host, seems to be the foremost potential target for action of any anthelmintic. In vitro treatments of the parasites with different plant extracts
shown alteration in the structure and composition in their tegumental architecture (Roy and Tandon, 1996; Sinha Babu et al., 1997; Pal and Tandon, 1998a; Meaney et al., 2004; Nandi et al., 2004). The anthelmintic activity of albendazole, mebendazole, fenbendazole, levamisole, praziquantel (Veerakumari and Munuswamy, 1999), oxyclozanide and niclosamide (Veerakumari and Paranthaman, 2004) treated *C. cotylophorum* showed swelling of the body, disruption and detachment of tegument (Veerakumari et al., 2012). In the present study the SEM observations of the flukes treated with AvEaE and PbEaE, showed numerous blebs, furrows, rough warts and large pits on the tegumental surface after 8h exposure. Obviously the damaged tegument may impair normal physiological function of the drug- treated parasites. Similar observations were also reported in other helminth parasites treated with synthetic and plant anthelmintics (Sharma and Hanna, 1988; McConville et al., 2006; Lalchhandama et al., 2007; Ghangale et al., 2009; Dasgupta et al., 2010; Jeyathilakan et al., 2010; Panyarachun et al., 2010; Bashtar et al., 2011; Saowakon et al., 2011; Buddhachat et al., 2012; Jeyathilakan et al., 2012; Nahla et al., 2012; Shaheen and Eman, 2012; Ahmed et al., 2013; Panyarachun et al., 2013; Saowakon et al., 2013; Scantlebury et al., 2013).

Transmission electron microscopic (TEM) investigations portrayed the significant changes in the internal structure of helminths. Findings of the present study clearly proved the adverse effect of both plant extracts *i.e.*,
AvEaE and PbEaE on treated flukes. Loss of normal cellular integrity in vital tissues and also mitochondrial abnormalities and disintegration of cristae observed in drug-treated flukes, suggests the disfigurement of carbohydrate metabolism. These drugs could cause cytoplasmic autolysis, degenerative changes at the brush border area, cellular necrosis and finally the death of the parasites (Jasra et al., 1990).

5.1.3 Carbohydrate metabolism

Carbohydrates form the chief energy source in the trematodes. The inhibition of energy metabolism is the most important mode of anthelmintic action of various groups of drugs (Tielens, 1994; Martin, 1997; Priya and Veerakumari, 2011; Manoj Dhanraj and Veerakumari, 2015). Results of the present investigation disclosed the effect of AvEaE and PbEaE on the key enzymes involved in carbohydrate metabolic pathway.

PK and PEPCK activity was found to be inhibited in AvEaE and PbEaE-treated flukes at their respective highest concentration after 8 h of exposure. PK is functionally linked with PEPCK. The two enzymes compete for a common substrate, PEP, channeling it to aerobic (PK) and anaerobic (PEPCK) pathways. Inhibition of PK may decrease pyruvate, phosphoenolpyruvate concentrations and reduce energy molecules production. Inhibition of both PEPCK and PK activities arrests the PEP-succinate/PEP-lactate pathways. Consequently, the energy yielding
process is impaired and deprives the parasite of its ATP production. Decreased generation of ATP proves fatal to the parasites (Jasra et al., 1990). Murray (2013) also declared in his study that cessation of ATP formation may lead to paralysis or death of the flukes. Similarly, the inhibition of PK and PEPCK activities treated with different anthelmintics has been reported in other helminths (Rahman and Bryant, 1977; Lloyd and Barrett, 1983; Srivastava et al., 1989; Roy et al., 2012; Swargiary et al., 2013).

The action of PK on PEP results in the production of pyruvate. Pyruvate so formed comes under the influence of LDH, which catalyzes the reduction of pyruvate to lactate and the oxidation of lactate to pyruvate. It is evident from the present observation that AvEaE and PbEaE inhibited the LDH catalyzing both the lactate oxidation and pyruvate reduction at highest concentration (1mg/ml) after maximum hours (8h) of exposure. It was strange to observe that the level of inhibition of LDH was predominant during the pyruvate reduction than the oxidation of lactate. Delabre-Defayolle et al. (1989) observed that the isatin treated Echinococcus multilocularis shown decreased activity of LDH and also significant declined level in glucose and glycogen stores. Filarin and diethylcarbamazine inhibited pyruvate reduction rather than lactate oxidation in Setaria digitata by altering the activity of LDH (Banu et al., 1989). Albendazole inhibits LDH activity of Fasciola hepatica (Ozcelik et al., 1992). Similar findings were also reported by Veerakumari and Munuswamy (2000) in C. cotylophorum, Veerakumari and Lakshmi
(2006) in *Haemonchus Contortus* and Swargiary et al. (2013) in *Fasciola buski* treated with synthetic and plant anthelmintics. The inhibition of LDH might arrest the carbon influx in the glycolytic pathway and the generation of the necessary energy through oxidative phosphorylation. Consequently, production of malate, which serves as main substrate for mitochondrial phosphorylation is reduced, which leads to reduced production of ATP (Srivastava *et al.*, 1989).

MDH is the rate–limiting enzyme in the phosphoenolpyruvate (PEP) metabolism. In eukaryotic cells, MDH exists in two forms. One is found in the mitochondrial matrix (mMDH), participating as a key enzyme in the citric acid cycle that catalyzes the oxidation of malate. The other is found in the cytoplasm (cMDH), assisting the malate-aspartate shuttle with exchanging reducing equivalents so that malate can pass through the mitochondrial membrane to be transformed into oxaloacetate for further cellular processes. In malate dismutation pathway, carbohydrates are degraded to PEP via classical glycolytic pathway. PEP is then carboxylated by PEPCK to oxaloacetate (OAA). OAA is converted to malate by reverse reaction of MDH (Kiyoshi Kita *et al.*, 2001). Malate formed is transported into the mitochondria, where pyruvate and fumarate are produced. The inhibition of MDH reduced the oxidation of malate to oxaloacetate and similarly the inhibition of MDH limiting the reduction of oxaloacetate to malate. Observations of the present study revealed that AvEaE and PbEaE
significantly inhibited cMDH and mMDH catalyzing both the oxidation and reduction reactions in *C. cotylophorum*, at 1mg/ml concentration after 8h exposure. Inhibition of malate results in reduced level of fumarate and succinate could be the possible reason for decreased ATP production. This results in energy deprivation among the drug-treated flukes. Findings of the present work might be correlated with results of Oztop *et al.* (1999) in *T. saginata* treated by albendazole and niclosamide. Similar inhibitory effect of *Acacia concinna* on the cMDH and mMDH activity of *C. cotylophorum* was reported by Priya and Veerakumari (2011).

FR is an enzyme that converts fumarate to succinate. It is the terminal electron acceptor in the energy metabolism of helminths. SDH is an enzyme complex, bound to the inner mitochondrial membrane. SDH has the ability to transfer electrons to the respiratory chain by catalyzing the formation of fumarate and succinate (Malkin and Camacho, 1972; Prichard, 1973; Barrett, 1981). FR and SDH activities were inhibited by AvEaE and PbEaE. SDH inhibition by anthelmintics could prevent the utilization of the chemical energy derived from electron transport for the net phosphorylation of ADP to ATP and deprive the parasite of its normal source of energy (Kumari, 2006; Lemke *et al.*, 2013; Manoj Dhanraj and Veerakumari, 2015). Disturbance in the terminal electron acceptor prevents succinate formation thereby curtail the ATP synthesis. This investigation amply demonstrates that *Adhatoda vasica* and *Piper betle* are potential inhibitor of the key enzymes
involved in carbohydrate metabolism of *C. cotylophorum*, indicating the anthelmintic efficacy of these plant extracts against *C. cotylophorum*.

### 5.1.4 Glucose and Glycogen

Helminth parasites utilize the food from the intestinal gut of host. The metabolism depends on the feeding habits and the rich nourishment available in the gut of the host. The parasites use this nourishment for their normal development and growth. Glycogen serves as the most important energy reserve in trematodes. Therefore, it is not surprising to find that carbohydrate and in particular glycogen provides a significant reserve store of energy particularly in forms, which exist in environments of low oxygen tension (Kaur and Sood, 1983). *AvEaE* and *PbEaE* exhibited a significant decrease in the glucose and glycogen content of *C. cotylophorum*. Reduction of glucose content by plant extracts could be responsible for glycogen depletion in drug-treated flukes. Observations of the present investigations are corroborated with the studies of Kushwaha *et al.* (2004) who reported the inhibition of glucose uptake in *F. gigantica* by *Azadirachta indica* and *Mallotus philippinensis*. Likewise, several chemotherapeutic and phytotherapeutic agents demonstrated the irreversible inhibition of glucose uptake and reduced the glycogen levels in parasitic helminths (Tandon *et al.*, 2003; Camurca *et al.*, 2007; Singh *et al.*, 2007; Basler, 2008 and Biradar *et al.*, 2010). The results of the present investigation portrays the declined level of glucose as well as glycogen in *AvEaE* and *PbEaE*-treated
parasites. Inhibition of glucose uptake results in energy crisis and reduced muscular activity. It also reduces the glycogen content, which suggests utilization of glycogen as a result of inhibition of glucose uptake. Disruption of glycogen metabolism is fatal to the parasites, which must maintain constancy in its energy state under fluctuations in substrate availability.

5.1.5 Phosphatases

Presence of acid phosphatase (AcPase), alkaline phosphatase (AlPase) reported in the body tegument, and the various organs/tissues of many flat worms (Kwak and Kim, 1996; Pal and Tandon, 1998b; Kar and Tandon, 2004; Lalchhandama et al., 2008). AcPase and AlPase are involved in various metabolic processes and believed to be involved in absorption and/or digestion in the parasite (Roy, 1982; Poljakova-Krustena et al., 1983). In the present investigation AvEaE and PbEaE diminished the activity of both AcPase and AlPases. Likewise, many commercial anthelmintics like parbendazole, piperazine adipate, phenothiazine, diethiazine, diethylcarbamazine, centperzine, tetramisole and levamisole alter the metabolism and disrupt mitochondrial energy formation by affecting the activities of phosphatases (Agarwal et al., 1990; Aggarwal et al., 1992; Vinaud et al., 2009). Similarly, aqueous extract of Butea monosperma, Embelia ribes and Roltleria tinctoria causes reduction in both AcPase and AlkPase activity in Paramphistomum cervi in vitro (Chopra et al., 1991). Roy et al. (2010; 2012) also reported the inhibition of tegumental enzymes in
**F. buski** treated with herbal extracts. Similarly, *Raillietina echinobothrida* also exhibited extensive distortion of the surface fine topography and decrease in the activities of major tegumental enzymes compared to that of control parasite on exposed to resveratrol and virosecurinine, the active compound of *Carex* Species (Giri and Roy, 2014) and *Securinega virosa* (Dasgupta *et al.*, 2013). In the present study remarkable structural damages observed in *AvEaE* and *PbEaE*-treated flukes may be the maincause for the inhibition of phosphatases which in turn might inhibit glucose uptake, since glucose undergoes phosphorylation and dephosphorylation during absorption (Jain *et al.*, 2011).

### 5.1.6 Glutathione-S-transferase

Glutathione–S–transferase is a major detoxification enzyme in parasitic helminths (Saeed *et al.*, 2013). In the present investigation dose and time dependent inhibition of GST activity was observed in *AvEaE* and *PbEaE*-treated flukes. Similar results were reported by Manoj Dhanraj and Veerakumari (2015) in *C. cotylophorum* treated with ethanolic extracts of *Areca catechu* and *Syzygium aromaticum*. Likewise, Gupta and Rathaur (2005) and Farahnak *et al.* (2006) reported that diethylcarbamazine and triclabendazole declined the level of GST in *Setaria cervi* and *F. gigantica*. Phytochemicals from *Cinnamomum verum, C. aromaticum, Allium sativum, Coriandrum sativum* and *Cymbopogon citrates* have potential to inhibit the GST in *Brugia malayi* (Shamina *et al.*, 2010). Agnieszka *et al.* (2012)
suggested that targeting the GST in anthelmintic therapy may break the defense mechanism of parasites.

5.1.7 Trace elements

Traditional Indian medicinal herbs used for strengthening the body immune system are known to have many essential and nutritional elements. Their excess or deficiency may disturb normal biochemical functions of the body (Iyengar, 1989; Kumar et al., 2011 and 2012). Gastrointestinal helminths have very specific physico-chemical requirements of their host gut environment, and nutritionally mediated changes might have a direct influence on the parasite population (Crompton and Nesheim, 1976). Trace elements like calcium and magnesium, act as the mediators that help in the functioning of the phosphatases and the free amino acid pool as they function as second messengers or as enzyme cofactors among parasites. Any significant alteration induced by a chemotherapeutic agent in the calcium influx/efflux of a cestode or trematode system disturbs the normal contraction of the parasite musculature because calcium, as second messengers, is responsible for breakdown of glycogen. Bricker et al. (1983) proved that the correlation between the vacuolization in tegument of the parasites and the concentration of calcium. The reports of the present study clearly declared that AvEaE and PbEaE-treated flukes shown the reduction in level of calcium and magnesium after 8h of exposure which could be the cause for the muscular contraction and leads to paralytic status. Likewise significant
reduction was observed in other trace elements level such as cobalt, copper, iron, potassium, manganese, molybdenum, sodium, zinc in \textit{AvEaE} and \textit{PbEaE}-treated flukes at 1mg/ml concentration after 8h exposure.

Lara \textit{et al.} (1974) reported that addition of cobalt sulphate to the diet increased the total egg output in lambs infected with \textit{Haemonchus contortus}. At the same time zinc deficiency impaired the expulsion of \textit{Trichinella spiralis} (Fenwick \textit{et al.}, 1990 a), enhanced the establishment of \textit{Strongyloides ratti} in the intestine of rats (Fenwick \textit{et al.}, 1990 b). Similarly, Boulay \textit{et al.} (1998) reported prolonged survival of \textit{Heligmosoides polygorus} in mice and El-Hag \textit{et al.} (1989) of \textit{T. spiralis}, \textit{S. ratti} in rats and \textit{H. polygyrus} in mice fed with a zinc deficient diet. In fowls diet enriched with zinc and copper salts increased \textit{Ascaridia galli} worm burdens (Gabrashanska, 1993; Gabrashanska and Timanova, 1993). Samak \textit{et al.} (1986) have indicated that adding selenium with zinc to the feed significantly increased \textit{Fasciola hepatica} egg output in ewes. Tetas and Lowenstein (1963) have demonstrated that calcium, copper, magnesium, and manganese, catalyze the hydrolysis and transphosphorylation of phosphate esters in the absence of the appropriate enzymes. Lauback (1989) reported that mice infected with \textit{A. suum} and fed low levels of dietary iron were found to harbor lower numbers of larvae in the lungs compared to mice receiving normal iron diets.
Studies on acute infection of *Nippostrongylus brasiliensis* in rats have shown that dietary iron deficiency during primary infection can increase parasite survival (Bolin *et al.*, 1977). In contrast, the addition of molybdenum to the diet of lambs exposed to infection with *Trichostrongylus vitrinus* and *Haemonchus contortus* reduced worm numbers and length of adult worms (Suttle *et al.*, 1992a and b). Smyth and McManus (1989) stated that the phosphates and inorganic ions are used during rapid synthesis of ATP for strong muscular attachment and active transport of nutrients in cestodes.

Minerals are necessary for the survival of parasites (Dvojnos and Timoshenko, 1994). The present investigations on mineral survey in AvEaE and PbEaE-treated flukes demonstrated the alteration in these elements, affecting the tegumental phosphatases and the free amino acids perhaps had a devastating effect on the transport system and cellular metabolism of the parasites eventually leading to paralysis. Obviously AvEaE and PbEaE-treated flukes thus enter a state of starvation and energy deprivation. Finally, the energy deprived flukes unable to sustain themselves *in situ* are expelled from the host system.

### 5.2 Phytochemical analysis of AvEaE and PbEaE

Phytochemical analysis of AvEaE and PbEaE revealed the presence of bioactive constituents which are known to exhibit medicinal properties. In the recent years, there is a tremendous research interest in the possible role of
phytochemical in the prevention and treatment of many diseases. The use of traditional medicine in getting rid of parasitic infections is wide spread across the world. Herbal medicine plays a vital role in health care of large sections of the population, particularly in developing countries, where they often bridge the gap between the availability and demand for modern medicines (Akerele, 1990). Anthelmintic herbal drugs are effective in killing the helminth parasites were reported by several researchers (Ghangale et al., 2009; Bashtar et al., 2011; Jeyathilakan et al., 2010; 2012; Nahla et al., 2012; Ahmed et al., 2013; Scantlebury et al., 2013).

Bioactive compounds in plants are of natural origin and serve as secondary metabolites (Kanimozhi and Bai, 2012). Secondary metabolites are an important source with a variety of structural arrangements and properties (De-Fatima et al., 2006). Biologically active components have always been of huge interest to scientists working on infectious diseases (Perumal Samy and Ignacimuthu, 2000). The preliminary phytochemical screening of *A. vasica* indicated the presence of steroid, triterpenoid, flavonoid, coumarin, sugar, quinoine, saponin, acid, tannin, phenol and alkaloids. Phytochemical screening of *P. betle* revealed the presence of steroid, triterpenoid, coumarin, sugar, quinoine, saponin, acid, tannin, phenol and alkaloids. The presence of wide range of phytochemical constituent indicates the utilization of plants in a multiple way to treat parmphistomosis. A number of studies are available for anthelmintic activity of tannins, alkaloids and flavonoids
(Athanasiadou et al., 2001; Da Silva et al., 2008; Wang et al., 2010b). The presence of these phytochemicals in AvEaE and PbEaE are responsible for the observed anthelmintic activity of plant extracts.

Alkaloids are present in both the plant extracts of AvEaE and PbEaE. Alkaloids extracted from Nauclea latifolia (Onyeyili et al., 2001) and Adhatoda vasica (Lateef et al., 2003) were effective against mixed gastrointestinal infections in sheep. Further, Nalule et al. (2013) suggested that alkaloids present in Zanthoxylum chalebium could have contributed to the paralysis and consequent death of A. suum. The present study also suggests that alkaloids could have a significant impact in deparasitization of the flukes from the rumen of sheep.

Tannins are polymeric phenolic substances having astringent property (Basri and Fan, 2005). Tannin was reported in the aqueous, ethanol and methanol extracts of Monodora myristica and Xylopia aethiopica seeds and was responsible for the significant anthelmintic activity (Niezen et al., 1995). Tannins have been shown to interfere with coupled oxidative phosphorylation thus blocking ATP synthesis in parasites (Martin, 1997). Various researchers have reported that tannins could cause mortality in parasites by binding to free proteins in the gastrointestinal tract of host animal or glycoprotein on the cuticle of the parasite (Thompson and Geary, 1995; Athanasiadou et al., 2001; Mali and Wadekar, 2008).
Saponins are natural antibiotics produced by plants as a defence mechanism to stop attacks by foreign pathogens (Okwu and Emenike, 2006). Toxicity of saponins to helminth parasites and their antifungal, antiviral, and antibacterial activity were well documented (Milgate and Roberts, 1995; Lacaille-Dubois and Wagner, 1996; Francis et al., 2002). Saponins from plant sources are also responsible for pharmacological effects like anthelmintic, antitussive and cytotoxic activities (Sparg et al., 2004). Saponins, destabilize the membranes (Gee and Johnson, 1988), affect the permeability of the cell membrane of the parasites, cause vacuolization and disintegration of tegument and elicit cell apoptosis through mitochondrial dysfunction (Cheung et al., 2005), thereby evacuating parasites completely from the host (Wang et al., 2010). Such high cytotoxic potency of saponins present in A. vasica and P. betle might be responsible for the significant anthelmintic activity and exorbitant toxicity, validating their use in treating parasite infestations.

Coumarins are phenolic substances made up of fused benzene and pyrone rings (O’Kennedy and Thornes, 1997). They have a characteristic odor and many of them have antimicrobial properties. Fragrance of plant is carried by essential oil fractions which are secondary metabolites and highly enriched in isoprene structure based compounds. They are called terpenes but when the compound contains an additional element as oxygen they are termed as terpenoids.
Some synthetic phenolic anthelmintics like niclosamide, oxyclozanide and bithionol which are shown to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation. Alkaloids, flavonoids, saponins and tannins have been demonstrated to possess anthelmintic activities (Ekeanyawu and Etienajirhevwe, 2012). Traditionally used medicinal plants have attracted the attention of the biological scientific communities. This has involved the isolation and identification of secondary metabolites produced by plants and their use as active principles in medicinal preparations (Taylor et al., 2001). Since the gastrointestinal helminth parasite becomes resistant to currently available synthetic anthelmintic drugs, there is an increasing demand towards natural anthelmintics (Kosalge and Fursule, 2009).

On investigating the efficacy of various fractions of AvEaE and PbEaE obtained from column chromatography, A.vasica chloroform ethylacetate fraction 5 (AvCEaF-5) and P.betle chloroform ethylacetate fraction 4 (PbCEaF-4) effectively inhibited the motility of the parasites within 8h at 1.0 mg/ml concentration. Hence, the phytoconstituents present in AvCEaF-5 and PbCEaF-4 were further processed for identification by GC-MS. Column fractions from plant extracts are mixtures of different compounds and these compounds can act in combinations to produce higher antiparasitic activity than individual compounds (Suleiman et al., 2013). Dose dependent anthelmintic efficacy of many plant derivatives were tested
against different parasites and has been reported *in vitro* and *in vivo* on nematodes, cestodes and trematodes (Abdel-Ghaffar *et al.*, 2011; Klimpel *et al.*, 2011; Mehlhorn *et al.*, 2011; Navaneetha lakshmi and Veerakumari., 2012; Priya and Veerakumari, 2011; Roy *et al.*, 2012; Veerakumari *et al.*, 2012; Veerakumari *et al.*, 2013; Manoj Dhanraj and Veerakumari, 2014; Veerakumari *et al.*, 2014; Manigandan and Veerakumari, 2015; Manoj Dhanraj and Veerakumari, 2016; Veerakumari and Chitra, 2016).

Gas-chromatography mass spectrometry (GC-MS) is one of the best techniques to identify the constituents of volatile matter, long chain and branched chain hydrocarbons, alcohols, acids, esters. The more precise information in qualitative analysis can be obtained by gas chromatography coupled with mass spectroscopy. In the present study fractions obtained from column chromatography proceeded to GC-MS. The GC-MS analysis of *AvCEaF*-5 revealed 4 compounds whereas 5 phytochemicals were identified in *PbCEaF*-4. The main prevailing compounds present in *AvCEaF*-5 were Santonin, linolenic acid methyl ester, palmitoleic acid and octadecenoic acid. In *PbCEaF*-4, the prominent compounds identified were 4-isobutylhydratropic acid, oleic acid, methyl stearate, 4-O-methylnokiol and 2-methylpalmitic acid. In many respects, the mechanism of action of the herbal drugs differs from that of the synthetic drugs. Ismail *et al.* (1999) and Abdel-Hameed *et al.* (2008) confirmed that extracts and pure compounds
obtained from plants exhibiting anticestodal properties. Subhashini and Arunachalam (2011) put forth in their investigation botanical remedies provide two advantages over single compound drugs, primary active compounds in plants are synergized by secondary compounds and secondary compounds ease the side effects caused by primary active compounds. The course of searching an ethno pharmacologically active plant extract down to a single active principal ingredient may result in loss of biological activity for a number of reasons. For instance, a special compound may be unstable during the extraction or fractionation or in the purified form. The fundamental basis of ethnopharmacology does not always exist in a single active compound but rather is a result of the interaction of more than one active compound found in the extract. Similarly, in the present investigation the effective fraction AvCEaF-5 containing four compounds santonin, linolenic acid methyl ester, palmitoleic acid and octadecenoic acid, and PbCEaF-4 containing five compounds 4-isobutylhydratropic acid, oleic acid, methyl stearate, 4-O-methylonokiol and 2-methylpalmitic acid. Sometimes, the single compound potentiates the activity and it may become toxic compared to the whole plant extract. Thus, the likelihood that more than one compound present in the plant extract could contribute to a net pharmacological response of the plant extract.

Santonin was formerly used as an anthelminthic, typically administered with a purgative. Santonin was used to treat roundworm
*Ascaris lumbricoides*. It exhibits several biological activities like anthelmintic, anti-inflammatory, antipyretic, analgesic and anticancer activities (Martin *et al.*, 1988; Al-Harbi *et al.*, 1994; Kim *et al.*, 2006; Arantes *et al.*, 2009 and 2010). Linolenic acid methyl ester is the fatty acid (Rotella, 2004). Fatty acids have shown antimalarial, antimycobacterial and antifungal properties. Paula Melariri *et al.* (2012) reported that the linolenic acid methyl ester posses anti malarial activity against the parasite *Plasmodium falciparum*. Palmitoleic acid is an omega 7 fatty acid. It possesses anti-fungal activity (Kabaraa *et al.*, 1972). Octadecenoic acid contains antioxidant and antimicrobial activity (Elizabeth and Arumugam, 2014).

It is evident from the present study that santotin, linolenic acid methyl ester, palmitoleic acid and octadecenoic acid are effective against *C. cotylophorum*. Thus the phytochemicals present in AvCEaF5, individually or in combination induced paralysis of the parasite and subsequently lead in to the death of the parasites.

4-Isobutylhydratropic acid possesses antipyretic, analgesic, anti-inflammatory and antipyretic effect. It is used to treat a wide range of illnesses such as headaches, backache, menstrual cramps, dental pain, neuralgia, rheumatic pain, muscular pain, migraine, arthritis and athletic injuries. 4-isobutylhydratropic acid is also used to reduce fever and to relieve minor aches and pain caused due to common cold or flu. (Christian Nordqvist,
Oleic acid is the mono-unsaturated fatty acid in plants. It occurs naturally in greater quantities than any other fatty acid. It lowers heart attack risk, artherosclerosis and aids in cancer prevention (Rotella 2004). Oleic acid is also used to induce lung damage in certain types of animals, for the purpose of testing new drugs and other means to treat lung diseases (Julien, 1986). Oleic acid also possesses antioxidant properties (Chia-Cheng Wei, 2016). Methyl stearate is a volatile oil used to treat against Salmonella typhi and E. coli (Motamedi et al., 2009). 4-O-Methylnokiol is a neolignan, a type of phenolic compounds. It possesses anticancer, antifibrosis, antithrombotic, and anti-inflammatory activities. Anthelmintic effects were also reported in the phenolic content of ethanolic and aqueous extracts of stem and roots of Oenothera rosea (Dahiya et al., 2012). 2-Methylpalmitic acid is a fatty acid. It posses larvicidal and antibacteraial activites (Fumiyuki kiuchi, et al., 1987; Mishra and Sree, 2007). It is apparent from the present study that 4-isobutylhydratropic, oleic acid, methyl stearate, 4-O-methylnokiol and 2-methylpalmitic acid are effective against C. cotylophorum. The present investigation amply demonstrates that the compounds obtained from AvCEaF-5 and PbCEaF-4 contributes to the flukicidal effect and could be used as a pharmacagnostical tool in the identification of a novel drug against paramphistomes.
5.3 *In vivo* studies

*In vivo* studies are intermittently engaged over *in vitro* studies considering an investigation within animals is more desirable as it contributes more insight in the countenance of the studies. In the present study, *Piper betle* ethyl acetate extract (*PbEaE*) was tested for its anthelmintic efficacy in naturally paramphistome infected sheep. At present various *in vivo* techniques are applied to gauge the toxicity of medicinal plants, endorse its anthelmintic properties against gastrointestinal parasites (Pawan, 2009) and to initiate its dosage for sheep, goats and cattle (Simon *et al.*, 2012). *In vivo* studies on the efficacy of *P. betle* has inculcate more assurance on its use as anthelmintic against *C. cotylophorum*.

Maximum reduction in EPG was recorded in infected sheep treated with 50 mg/ml of *PbEaE*. An egg count of 500 eggs per gram (EPG) is generally premeditated high enough to require treatment concerning limit pasture contamination and sub-clinical disease (Priya *et al.*, 2013). The efficacy of *PbEaE* against *C. cotylophorum* was evaluated on the basis of reduction in number of paramphistome eggs in the faeces of the host animal. Treatment of the infected sheep with two different concentrations (30 mg/ml and 50 mg/ml) of *PbEaE* brought down the EPG significantly. The egg counts reduced on day 7 post-treatment and continued to decline further till day 21 post-treatment. The maximum reduction was recorded as 89.21 % on day 21 after treatment with 50 mg/ml of *PbEaE* desiring as persuasive anthelmintic
drug. Analogously, reduction in faecal egg count of sheep infected with paramphistomes after treatment with aqueous extract of pods of *Acacia concinna* was also reported by Priya *et al.* (2013). The anthelmintic efficacy of both synthetic and plant based anthelmintics were reported by many parasitologists. Scientists have demonstrated the reduction of EPG in *Carum copticum, Terminalia arjuna, Adhatoda vasica* and *A. indica* treated sheep, naturally parasitized with mixed species of gastrointestinal parasites (Lateef *et al.*, 2006; Iqbal *et al.*, 2010). Furthermore, complete inhibition of egg hatching was reported in *Spigelia anthelmia* treated *H. contortus* (Assis *et al.*, 2003). Medicinal plants have the ability to reduce EPG and inhibit egg hatching (Hubert and Kerboeuf, 1992; Lalhmingchhuanmawii *et al.*, 2014) which in turn reduces pasture contamination during grazing by livestock. This anthelmintic effect could be due to the presence of active phytochemicals in the plant extracts (Rates, 2001) which is of practical importance in overall helminth control.

The faecal egg count reduction test is appropriate for the evaluation of all types of anthelmintics (Verma *et al.*, 2006). FECR% showed a dose dependent activity suggesting that *PbEaE* has a pharmacological basis. Onyeyili *et al.* (2001) reported a dose response activity in *Nauclea latifolia* on strongyloid nematodes of small ruminants. FECR is the recommended method for *in vivo* evaluation of plant anthelmintic activity. The reduction in faecal egg count in sheep infected with *H. contortus* following oral administration of
alcoholic extract of *A. indica* was reported by Swarnkar *et al.* (2008). Pandit *et al.* (2009) also reported faecal egg count reduction of gastrointestinal nematodosis in sheep treated with ivermectin, levamisole and albendazole.

Haematological parameters such as haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leucocyte count (TLC) and differential count (DC) of neutrophil (N), lymphocyte (L) and eosinophil (E) are important diagnostic tools to assess healthiness of host animals. Biochemical profiles such as serum glucose, total serum protein (TSP), albumin (A), globulin (G), aspartate aminotransferase (AST) and alanine amino transferase (ALT) are liable to be affected because of infection and therefore may reveal quantitative changes prior to and after the treatment (Navaneetha *et al.*, 2011; Priya *et al.*, 2013).

In the present investigation, the haematological indices such as Hb, PCV, TEC and E levels significantly decreased in infected sheep prior treatment. Similar reductions on the Hb, PCV and TEC levels in paramphistome infected lambs, cattle and sheep have been reported by several investigators (Misra *et al.*, 1996; Diaz *et al.*, 2006; Verma *et al.*, 2006; Priya *et al.*, 2013). Reductions in Hb, TEC, PCV and L in sheep infected with *H. contortus* are well documented (Sharma *et al.*, 2000; Al-Qarawi *et al.*, 2001; Navaneetha *et al.*, 2011). Jas *et al.* (2008) also reported a significant reduction in Hb, PCV and TEC levels in Bengal goat infected with nematodes. The fall in Hb, PCV and TEC probably implies that the animals
were suffering from anaemia, because of the haemorrhage caused by the immature parasites. The anaemic condition of the infected animals could also be a reflection of delayed host’s erythropoietic response to compensate for the loss of blood.

From the present study, it is apparent that Hb, PCV and TEC, increased significantly, and the level of TLC, N, L and E decreased in PbEaE-treated Groups. Shahadat et al. (2008) stated that TEC increased because of partial/full destruction of parasites by the anthelmintic action. The effect of PbEaE showed a significant (P<0.01) effect on PCV and Hb level. The PCV and Hb content were highest on 21st day, after treatment with 30 and 50 mg/ml of PbEaE extract. Both Hb and PCV values gradually increased in PbEaE–treated sheep. This could be due to the decrease in the number of paramphistomes in PbEaE–treated groups than untreated groups.

The mean eosinophil (E) counts (%) were significantly (P<0.01) higher in infected–untreated sheep. Similar results for eosinophil percentage were reported in sheep infected with T. colubriformis by Dawkins et al. (1989) and Kyriazakis et al. (1996). The increased TLC value might be attributed to neutrophilia and eosinophilia as a result of the host’s immune response to the infection (Ghulam et al., 1995; Siham et al., 1997; Pal et al., 2001). Heavy paramphistome infestations were also associated with increased infiltration of eosinophils (Rolfe et al., 1994). Also, Misra et al. (1996) reported leucocytosis and increased eosinophil counts in lambs infected with
paramphistomes. Normal haematological values were recorded by Kumar et al. (2007) when the parasites were removed from the host by suitable treatment, indicating improvement in health parameters. He also suggested that the pathogenic effects caused by gastrointestinal parasites were the reasons for reduction in haematological values. In addition, an increase in blood eosinophils associated with the immune responsiveness of the animals to gastrointestinal nematodes, for anthelmintics was reported by Pathak and Tiwari (2013). Restoration of haematological indices of sheep, post treatment with P*b*EaE suggests that the plant extract possesses anthelmintic activity against paramphistomes.

In the present study, significant alterations in the levels of total serum protein (TSP), albumin, globulin, glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were observed in P*b*EaE–treated sheep. TSP and albumin were significantly (P<0.01) increased in P*b*EaE–treated sheep as compared to healthy sheep. Globulin level was also found to be statistically higher (P<0.01) in 30 and 50 mg/ml in P*b*EaE–treated sheep when compared to untreated infected sheep. Present results are acquiescence with the findings of many workers (Bariyar, 2004; Lakra et al., 2007; Pathak and Tiwari, 2013) implying a major role for TSP and albumin in the synthesis of specific proteins for tissue repair and for immunological reaction to infection (MacRae, 1993). The fall in the protein level observed in the
untreated infected sheep might be due to haemodilution, a compensatory mechanism for intestinal haemorrhage caused by migrating flukes and later on due to loss of large quantities of serum protein in the gut through exudation. Hypoproteinemia in helminthosis is primarily the result of hypoalbuminaemia. Hypoproteinemia, coupled with loss of appetite, seems to be the most important pathophysiological consequence of paramphistomosis (Priya et al., 2013). Ahmad et al. (1990) stated that the decreased level of TSP and albumin in infected untreated group could have occurred due to abomasal haemorrhage caused by H. contortus. Decreased serum protein is attributable to, loss of total serum proteins in the gut, impairment in synthesis of albumin (Sharma et al., 2001). Further, the damage caused by parasites might have affected the intestinal absorption, assimilation metabolism resulting in reduction in their values.

The reductions in blood biochemical parameters during infection and return at about normal ranges after treatment have also been reported by several workers (Rajguru et al., 2002; Bariyar, 2004; Lakra et al., 2007) in parasitized goats. Present results of serum glucose (mg/dL) level, in all experimental sheep, were within the normal range suggested for sheep. This finding is in agreement with the reports of Boyd (1984) and Cenci et al. (2007) who observed no difference in blood glucose level of sheep infected gastrointestinal nematodes, treated with A. mearnsii.
The level of alanine transaminase and aspartate transaminase were found to be decreased in PbEaE–treated sheep. The high values of AST and ALT in cattle and buffaloes infected with paramphistomes were reported by Bharti and Prasad (2001). Rani and Hafeez (2007) also reported increased ALT levels in Bubalus bubalis infected with biliary amphistomes. A similar finding on the elevated levels of AST and ALT in sheep infected with paramphistomes was also reported by Priya et al. (2013). AST and ALT are liver specific enzymes catalysing the interconversion of aminoacids and α-oxoacids by the transfer of amino groups. The changes in serum biochemical constituents during Fasciola / Paramphistomum infection reflect disturbances in liver function caused due to tissue damage and fluid loss caused by the parasites in situ. Recovery from the damages of this vital organ following treatment of the infected animals may result in the resumption of liver functions and restoring the body fluid balance (Bharti and Prasad, 2001). Significant alterations in the levels of AST/ALT may indicate changes in the kidney and hepatic functions.

Medicinal activity of the plants is based on their phytoconstituents and they can be used as a pharmacognostical tool in the identification of a novel drug against paramphistomes. As suggested by Shirwaikar et al. (2011) that secondary metabolites could be exploited for a potent anthelmintic against helminth parasites. The possible mechanism of phytoconstituents as
anthelmintics have been documented by several researchers (John et al., 2009; Borba et al., 2010; Patel et al., 2010; Roy et al., 2010). The present in vitro and in vivo studies on Adhatoda vasica and Piper betle elucidate the anthelmintic efficacy against Cotylophoron corylophorum. Secondary metabolites present in AvCEaF-5 and PbCEaF-4 singly are in combination reduced the motility and ultimately resulted in the death of the parasites. With respect to increasing interest in the therapeutic use of natural products with increased potency at low cost and to solve the problem of anthelmintic resistance in livestock industry, this study strongly recommend that the both the plant extracts Adhatoda vasica ethyl acetate and Piper betle ethyl acetate which showed a remarkable anthelmintic potential against paramphisomtes, could be used in formulation of novel organic anthelmintic drug to combat paramphistomosis in livestock.