Materials and Methods
3. MATERIALS AND METHODS

3.1 Collection of Parasites

Adult live *Cotylophoron ctylophorum* (Fig.2) were collected from the rumen of the sheep slaughtered at a local abattoir in Chennai. The flukes were washed thoroughly in physiological saline.

3.2 *In vitro* maintenance of *Cotylophoron ctylophorum*

Hedon-Fleig solution (pH 7.0) is the best medium for *in vitro* maintenance of *C. ctylophorum* (Veerakumari, 1996; Anitha, 2007). It is prepared by dissolving 7 g of sodium chloride, 0.1 g of calcium chloride, 1.5 g of sodium bicarbonate, 0.5 g of disodium hydrogen phosphate, 0.3 g of potassium chloride, 0.3 g of magnesium sulphate and 1 g of glucose in 1000 ml of distilled water. Collected adult live flukes from local abattoir were maintained in Hedon-Fleig solution (pH 7.0).

3.3 Extract preparation of plant material

Leaves of *Adhatoda vasica* (Fig.3) and *Piper betle* (Fig.4) collected from local market and shade dried for 21 days; periodical turnover of the leaves been done later dried leaves are coarsely powdered. Leaves extraction was done following the method of Harborne (1998). Successive soaking was done for 48 h with hexane, chloroform, ethyl acetate and ethanol. Aqueous extract was also prepared with universal solvent. Intermittent agitation was
Fig. 2 *Cotylophoron cotylophorum* in the rumen of sheep

Fig. 3 *Adhatoda vasica*

Fig. 4 *Piper betle*
necessary while soaking in various solvents. Watt man filter paper no.1 was used for filtrations. Distillations have been done with rotary evaporator (EQUITRON). Extracts were kept in Lyodellyophilizer (DELVAC) to remove the solvents and dried up.

3.4 *In vitro* studies

3.4.1 Gross visual observation on the motility of the flukes and selection of effective solvent extract

Solvent and aqueous extracts of *A. vasica* and *P. betle* were prepared in different concentrations (1, 3 and 5 mg/ml) with Hedon-Fleig solution (pH 7.0). The motility of the flukes was observed at various time intervals (5, 15, 30 min, 1, 2, 4, 6, 8, 12, and 24 h) of exposure. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extracts. Based on the motility of the flukes, the observations were categorised as very active (++++), moderately active (+++), slightly active (++), sluggish (+) and dead (-). The flukes with no movement were regarded as dead. Based on the motility of the flukes the effective solvent plant extract was identified, and then five different sub-lethal concentrations were selected (0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml). The flukes were incubated for 2, 4 and 8 h in the various sub-lethal concentrations for the effective solvent plant extract for further *in vitro* studies.
3.4.2 Quantitative survey of motility response of drug treated flukes using Electronic Micromotility Meter (EMM)

The motility response of the drug-treated flukes was authentically recorded with the aid of Electronic Micro motility Meter (EMM) (Veerakumari, 2003) after 2, 4 and 8h respectively. This instrument works on the principle of spectroscopy which states that light rays passing through a quartz cuvette containing parasites in an aqueous medium are perturbed by the movement of the parasites. The perturbation to the light rays is detected by a photo detector and expressed as variations in electric signal. The extent of deviation is directly proportional to the extent of movement of the parasites. A temperature controller system maintains the temperature of the EMM. The EMM has been found to be highly sensitive, and therefore, suitable for in vitro quantitative assay of the motility of helminths. In the present study, the motility of the control and treated flukes was measured. The percentage of inhibition of motility of control and drug-treated flukes were calculated by using the formula,

\[
\% \text{ inhibition of motility} = \frac{C - T}{C} \times 100
\]

Where,

C - Deviation of voltage signal in the control fluke

T - Deviation of voltage signal in the fluke treated with plant extracts
3.4.3 Structural analysis

3.4.3.1 Light Microscopic studies

The flukes were incubated in *Adhatoda vasica* ethyl acetate extract (AvEaE) (1.0mg/ml) and *Piper betle* ethyl acetate extract (PbEaE) (1.0mg/ml) for 8h. Simultaneously, control was also maintained in Hedon-Fleig solution without the plant extract. The control and drug-treated flukes were fixed for 1h in 10% formalin. Subsequently they were washed and dehydrated in graded series of alcohol. The samples were embedded in L-shaped wax moulds. The blocks were fixed in block holder of the microtome and cut into fine sections of 5µm thickness and mounted on slides. After removing the paraffin, slides were stained in haematoxylin for 10 min and counter stained for 6 min with eosin. Histological sections were examined under high power compound microscope, LABO. Images were captured using Digital camera.

3.4.3.2 Scanning Electron Microscopic studies

The flukes were incubated in AvEaE (1.0mg/ml) and PbEaE (1.0mg/ml) for 8h and were fixed in 2.5% glutaraldehyde for 18 h, washed thrice in phosphate buffer for 15 min. The flukes were dehydrated in 30, 50, 70, 90 and 100 % alcohol each one hour. The flukes were left in 100% alcohol overnight and air-dried in vacuum desiccator for two days. The dried samples were mounted on a brass stub, coated with gold under vacuum and
observed in JEOL JSM-6360 scanning electron microscope. Photomicrographs were taken in OLYMPUS digital camera.

3.4.3.3 Transmission Electron Microscopic studies

The flukes were incubated in *Av*EaE (1.0 mg/ml) and *Pb*EaE (1.0mg/ml) for 8h, fixed immediately in 3% glutaraldehyde for 2 h, post fixed in 1% osmium tetroxide for 1 h and washed thrice in cacodylate buffer for 5 min. Tissues were dehydrated in graded series of alcohol, in filtered by propylene and embedded in siliconised rubber mould with epoxy resin. Tissue blocks were cut into fine sections using “Leica ultra-cut UCT” microtome and stained using toluidine blue. Ultrassections below 100 mm were obtained with Leica diatome. Ultrathin sections are taken on copper grid, stained in (double metallic) uranyl acetate and Reynolds solution. Sections were transmitted in electron microscope (Philips 201C by Netherlands) and photographed.

3.4.4 Biochemical analysis

3.4.4.1 Carbohydrate metabolism

Biochemical studies were carried out to observe the effect of *Av*EaE and *Pb*EaE on the enzymes of carbohydrate metabolism viz. pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), fumarate reductase (FR) and succinate dehydrogenase (SDH). The enzyme activity was expressed in terms of protein and the protein in the sample was assayed as described by Lowry *et al.*
(1951). In the present study, the activity of the enzyme is expressed as % inhibition in the enzyme activity.

Sample preparation

Adult *C. cotylophorum* were incubated in various sub-lethal concentrations of *AvEaE* and *PbEaE* extracts for 2, 4 and 8 h. Suitable control were maintained simultaneously without the plant extract in the medium. After incubation, the flukes were rinsed in distilled water. The flukes were weighed wet and a 10% (W/V) homogenate was prepared by homogenising the flukes in ice-cold 0.25 M sucrose solution containing 0.15 M Tris-HCl (pH-7.5) using a tissue homogeniser in an ice-bath. This homogenate was centrifuged at 1000 rpm for 10 min and the sediment containing the cellular particles viz. nucleus and other organelles were discarded. The supernatant was used as the enzyme source.

Preparation of ‘cytosolic’ and ‘mitochondrial’ fractions of enzyme samples (*Fry et al.*, 1983)

The cytosolic and mitochondrial fractions of *C. cotylophorum* were prepared following the method of *Fry et al.* (1983). The sample prepared as described earlier, was centrifuged at 10,000 rpm for 20 min and the supernatant thus obtained was the cytosol fraction. The sediment obtained was then washed twice by redissolving it in the homogenising medium and again centrifuged at 10,000 rpm for 10 min. The final sediment was termed as
mitochondrial fraction. The pellet was finally suspended in 1 ml of homogenising medium and used for the enzyme assays. All the centrifugation steps were carried out at 4° C using refrigerated ultracentrifuge (REMI C 24).

3.4.4.1.1 Assay of Pyruvate kinase (McManus and Smyth, 1982)

Pyruvate kinase (PK) (EC 2.7.1.4) activity in the cytosolic fraction was assayed following the method of McManus and Smyth (1982). PK catalyses the inter conversion of phosphoenolpyruvate (PEP) and pyruvate as shown below:

\[
\text{PK} \quad \text{PEP} + \text{ADP} \leftrightarrow \text{Pyruvate} + \text{ATP}
\]

The reaction mixture contained 1 ml of 300mM Tris-HCl buffer (pH 8.2) (Priya and Veerakumari, 2011), 0.5 ml of 42 mM magnesium sulphate (MgSO₄), 0.5 ml of 450 mM potassium chloride (KCl), 0.3 ml of 50 mM adenosine diphosphate (ADP), 0.3 ml of 50 mM PEP, 0.3 ml of 2 mM nicotinamide adenine dinucleotide reduced (NADH), 0.025 ml of 48 mM fructose biphosphate (FBP), 0.025 ml of 15 units of LDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n moles NADH oxidised /min /mg protein.
3.4.4.1.2 Assay of Phosphoenolpyruvate carboxykinase (McManus and Smyth, 1982)

The activity of phosphoenolpyruvate carboxykinase (PEPCK) (EC 4.1.1.32) was assayed according to the method of McManus and Smyth (1982). PEPCK catalyses the formation of oxaloacetate (OAA) from PEP.

\[
\text{PEPCK} \\
\text{PEP + CO}_2 + \text{ADP} \rightarrow \text{OAA + ATP}
\]

The assay mixture contained 1ml of 300mM imidazole buffer (pH 8.6) (Priya and Veerakumari, 2011), 0.4 ml of 300 mM MgSO4, 0.3 ml of 400 mM Kcl, 0.3 ml of 70 mM sodium bicarbonate (NaHCO₃), 0.3 ml of 20 mM ADP, 0.3 ml of 40 mM PEP, 0.3 ml of 2 mM NADH, 0.05 ml of 15 units of MDH and 0.05 ml of enzyme sample. The reaction was started by the addition of the enzyme sample and the decrease in absorbance was read at 340 nm for 3 min at an interval of 15 sec. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NADH and was expressed in n moles NADH oxidised/min/mg protein.

3.4.4.1.3 Assay of Lactate dehydrogenase (Yoshida and Freese, 1975)

The activity of lactate dehydrogenase (LDH) (EC 1.1.1.27) was assayed according to the method of Yoshida and Freese (1975). LDH catalyses the oxidation of lactate and reduction of pyruvate.
The conversion of pyruvate to lactate occurs in anaerobic tissues and conversion of lactate to pyruvate occurs in aerobic tissues. Therefore, LDH activity can be measured spectrophotometrically either by the reduction of nicotinamide adenine dinucleotide (NAD) in the presence of lactate or by the oxidation of NADH in the presence of pyruvate. In the present study the activities in both the directions were assayed. For oxidation of lithium lactate, 0.8 ml of 60 mM phosphate buffer (pH 7.5) (Priya and Veerakumari, 2011), 0.1 ml of 0.5 M lithium lactate, 0.05 ml enzyme sample and 0.05 ml of 20 mM NAD were placed in 1 ml cuvette. The increase of absorbance at 340 nm was recorded for 3 min at an interval of 15 sec. For the reduction of pyruvate, 0.05 ml of enzyme sample was added to 0.8 ml of 60 mM phosphate buffer (pH 6.5), 0.01 ml of 1 mM NADH, 0.01 ml of 10 mM sodium pyruvate and final volume was adjusted to 1 ml by the addition of distilled water in 1 ml cuvette. The decrease in absorbance at 340 nm was measured for 3 min at an interval of 15 sec.

The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised/min/mg protein.
3.4.4.1.4 Assay of Malate dehydrogenase (Yoshida, 1969)

Malate dehydrogenase (MDH) (EC 1.1.1.37) catalyses the oxidation of malate and reduction of oxalo acetic acid (OAA). The activity of this enzyme catalysing the malate oxidation and OAA reduction was assayed in both cytosolic and mitochondrial fractions following the procedure of Yoshida (1969).

\[
\text{MDH} \\
\text{L-Malate} + \text{NAD} \quad \text{←} \quad \text{OAA} + \text{NADH} + \text{H}^+ \\
\]

For the oxidation of malate, the reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.4 for cMDH and pH 7.2 for mMDH) (Veerakumari, 1996), 0.1 ml of 100 mM sodium malate, 0.1 ml of 10 mM NAD, 1.7 ml of distilled water and 0.1 ml of enzyme sample. For MDH catalysing the reduction of OAA, the reaction mixture contained 2.5 ml of 100 mM Tris-HCl (pH 7.4 for both cMDH and mMDH) (Veerakumari, 1996), 0.05 ml of 100 mM oxaloacetate, 0.05 ml of 10 mM NADH, 0.3 ml of distilled water and 0.1 ml of distilled water and 0.1 ml of the enzyme sample.

The activity of the enzyme catalysing oxidation and reduction reaction was measured at 340 nm for 3 min at an interval of 15 sec each. The enzyme activity was calculated from the millimolar coefficient of 6.22 for NAD and NADH and was expressed in n moles NAD reduced or NADH oxidised/min/mg protein.
3.4.4.1.5 Assay of Fumaratereductase (Sanadi and Fluharty, 1963)

Fumarate reductase (FR) (EC 1.3.1.6) catalyses the reduction of fumarate to succinate. The enzyme was assayed as detailed by Sanadi and Fluharty (1963).

\[
\text{FR} \quad \text{Fumarate} + \text{NADH} + \text{H}^+ \xrightarrow{} \text{Succinate} + \text{NAD}
\]

The reaction mixture contained 1 ml of 150 mM Tris-HCl buffer (pH 8.6) (Veerakumari, 1996), 0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.3 ml of 1 mM ethylene diamine tetra acetic acid (EDTA), 0.3 ml of 50 mM fumarate, 0.7 ml of distilled water, 0.1 ml of enzyme sample and 0.3 ml of 1.6 mM NADH in a 3 ml cuvette. After the addition of NADH, decrease in absorbance at 340 nm was measured for 3 minutes at an interval of 15 seconds. The enzyme activity was calculated by using the millimolar coefficient of 6.22 and expressed in n moles of NADH oxidised/min/mg protein.

3.4.4.1.6 Assay of Succinate dehydrogenase (Singer, 1974)

The activity of succinate dehydrogenase (SDH) (EC 1.3.99.1) was assayed according to the method of Singer (1974). Succinate is oxidised to fumarate by the flavoprotein SDH, which contains covalently bound flavin adenine dinucleotide. This reducible co-enzyme functions as hydrogen acceptor in the following reaction.
The reduced enzyme can donate electrons to various artificial
electron acceptors e.g reducible dyes. SDH assay is based on the reduction of
phenazinemethosulphate (PMS) by SDH. Reduced PMS is immediately
reoxidised by dicholoro phenol indophenol (DCPIP). Bleaching of later dye is
estimated spectrophotometrically.

The reaction mixture included 0.5 ml of 300 mM phosphate buffer
(pH 7.5) (Veerakumari, 1996), 0.3 ml of 0.1 M succinate, 0.1 ml of enzyme,
0.3 ml of 10 mM KCN (neutralised with 0.01 N HCl), 0.1 ml of 0.75 mM
calcium chloride and 1.3 ml of water. The enzyme was incubated for 5 - 7
minutes to permit full activation. After incubation, 0.1 ml DCPIP (0.05%)
(W/V) and 0.3 ml of PMS (0.33 %) were added to initiate the reaction and
decrease in absorbance was recorded at 600nm. The enzyme activity was
calculated using millimolar extinction coefficient of 19.1 and expressed in n
moles of dye reduced/min/mg protein.

3.4.4.2 Assay of glucose (Huggett and Nixon, 1957)

The amount of glucose in the flukes was estimated following the
glucose oxidase method of Huggett and Nixon (1957). Glucose is oxidised by
glucose oxidase (GOD) to gluconic acid and hydrogen peroxide. In a
subsequent peroxidase (POD) catalysed reaction, the oxygen liberated is
accepted by the chromogen system to give a red coloured quinoneamine
compound. The intensity of the red colour so developed is measured at 505 nm and is directly proportional to glucose concentration in the sample.

Known quantity of the parasite (100 mg) was homogenized with 1 ml of 0.66 N perchloric acid. The homogenate was incubated at room temperature (31 ± 2 °C) for 1 h. The homogenate was centrifuged for 10 min at 4000 rpm. The supernatant was used as the sample. To 0.02 ml of distilled water (blank), 0.02 ml of standard and 0.02 ml of the sample, 1.5 ml of the enzyme was added. After 20 min the absorbance was measured at 505 nm in UV-visible spectrophotometer. Based on the optical density of the standard, optical density of the sample and concentration of the standard, the glucose content was estimated and expressed in mg %. The percentage decrease in the glucose content was calculated.

3.4.4.3 Assay of glycogen (Carroll et al., 1956)

Glycogen in the parasite was estimated by the method of Carroll et al. (1956). Glycogen is hydrolysed to glucose by the sulphuric acid (H₂SO₄) present in the anthrone reagent which is then dehydrated into furfurals. This compound reacts with anthrone to produce a complex coloured product, the intensity of which is proportional to the amount of glucose present in glycogen.

Known quantity of the flukes (100 mg) from the control and treated groups was homogenized with 1 ml of 5 % trichloroacetic acid (TCA) and
was centrifuged for 10 min at 2500 rpm. The supernatant was separated and to 1 ml of supernatant, 5 ml of absolute alcohol was added and kept overnight in the refrigerator. Then it was centrifuged for 15 min at 2500 rpm. The supernatant was discarded and the precipitate was dissolved in 1 ml of water. To 1 ml of the sample, 1 ml of the standard and 1 ml of distilled water, 10 ml of anthrone reagent was added. This was kept in boiling water for 10-15 min and then cooled at room temperature in dark. The colour developed was read at 620 nm using UV-visible spectrophotometer. The glycogen content was calculated and expressed in mg %. Percentage decrease in glycogen content was calculated.

3.4.4.4 Assay of Glutathione S-transferase (Habig et al., 1974)

Activity of glutathione S-transferase (GST) (EC 2.5.1.18) was assayed following the procedure of Habig et al. (1974). GST catalyses the conjugation of glutathione reduced (GSH) thiolate anion with a multitude of second substrate like 1-chloro-2, 4- dinitrobenzene (CDNB). The conjugation of CDNB with GSH was measured by disappearance of free sulphhydryl groups at 340 nm. The sample for the enzyme was prepared by homogenising 100 mg of the parasite in 1 ml of 0.2 M Tris-HCl buffer (pH 7.8). The homogenate was centrifuged at 1000 rpm for about 5 min. To 0.05 ml of the supernatant, 0.4 ml of 0.2 M Tris-HCl buffer (pH 8.0), 1.2 ml of water, 0.1 ml of 1.5 mM CDNB were added and incubated in water bath at 37 °C for 10 min. After incubation 0.1 ml of 1.5 mM GSH was added. The change in
absorbance was measured against a reagent blank at 340 nm at 30 sec interval for 5 min.

The protein content in the sample was estimated following the procedure of Lowry et al. (1951). The enzyme activity was calculated using the millimolar extinction coefficient of 9.6 of CDNB-GSH conjugate was expressed as μmoles of CDNB-GSH conjugate formed/min/mg protein.

3.4.4.5 Assay of Acetylcholinesterase (Ellman et al., 1961)

The acetyl cholinesterase (AChE) (EC 3.1.1.7) activity was assayed using photometric method as described by Ellman et al. (1961). AChE in the sample hydrolyses acetylcholine, which is the substrate and forms thiocholine that will react rapidly and irreversibly with 5 thio-bis-nitrobenzoic acid.

\[
\begin{align*}
\text{AChE} & \quad \text{Acetylthiocholine} \quad \text{thiocholine} + \text{acetate} \\
& \quad \text{thiocholine} + \text{di thionitrobenzoate} \quad \text{Yellow colour}
\end{align*}
\]

The increase in colour intensity was measured spectrophotometrically at 412 nm. The enzyme samples were prepared by homogenizing 500 mg of control and drug-treated flukes in 1 ml of 0.1M phosphate buffer (pH 8.0). The homogenate was centrifuged at 1000 rpm for about 5 min. To 100 μl of the supernatant, 1.3 ml of 0.1 M phosphate buffer (pH 8.0) and 0.05 ml of 0.01M 5- thio-bis-nitro benzoic acid (DTNB) solution was added and transferred to quartz cuvette. The absorbance at 412 nm was set to zero in a UV visible double beam bio spectrophotometer. 0.02 ml of
0.075M acetylthiocholine iodide was added to the reaction mixture in the cuvette and mixed well and the absorbance was noted for 5 min at 15 seconds interval. The increase in absorbance per minute was calculated. The protein content of the sample was estimated following the procedure of Lowry et al. (1951) using BSA (100mg/ml, 200mg/ml and 300mg/ml) as a standard protein. The enzyme activity was expressed as number of moles of acetylthiocholine iodide hydrolyzed/min/mg protein.

3.4.4.6 Assay of Phosphatases (Barrett, 1972)

Alkaline phosphatase (AlPase) (EC 3.1.3.1) and Acid phosphatase (AcPase) (EC 3.1.3.2) activity of control and drug-treated flukes was determined following the procedure of Barrett (1972). The enzyme is allowed to hydrolyze an organic phosphate ester. The liberated phosphate combines with ammonium molybdate. The compound thus formed combines with elon giving a blue colour which is read at 650 nm.

3.4.4.6.1 Alkaline Phosphatase

The flukes were weighed wet (50 mg) and homogenized in 1 ml of boric acid-borax buffer and centrifuged at 2500 rpm for 10 min. The supernatant was used as the enzyme source. Two ml of 0.5 % buffered (boric acid – borax buffer pH 8.4) β-Glycerophosphate (βGP) was added to 1 ml of the enzyme sample and incubated at 37° C. After 30 min, the reaction was stopped by adding 3 ml of ice-cold 10% TCA, which resulted in the formation
of precipitate. After 15 min, the mixture was filtered in cold condition and the filtrate was used as the sample. To 2 ml of sample, 2 ml of buffer and 2 ml of standard solution, 0.8 ml of ammonium molybdate (2.5 g dissolved in 100 ml of 1 N H$_2$SO$_4$) and 0.3 ml of elon (0.5 g of elon dissolved in 10% sodium metabisulphate) were added separately which served as sample, blank and standard respectively. A suitable control was maintained by adding 10% TCA to the substrate, before the addition of the enzyme source. After 15 min, the colour intensity was measured at 660 nm in a UV-Visible spectrophotometer. The protein in the sample was estimated following the method of Lowry et al. (1951).

3.4.4.6.2 Acid Phosphatase

To determine the acid phosphatase activity, the substrate β-Glycerophosphate was dissolved in citrate buffer and the enzyme sample was prepared in citrate buffer (pH 5.0) The procedure for the enzyme assay was same as described for alkaline phosphatase. The protein in the sample was estimated following the method of Lowry et al. (1951). The activity of alkaline phosphatase and acid phosphatase was expressed in mg phosphate liberated/mg protein/min.

3.4.4.7 Estimation of Protein

The carbamyl groups of protein molecules react with copper and potassium of the reagent to give a blue coloured, potassium-biuret complex. This complex together with tyrosine and phenolic compounds present in the
protein reduce the phosphomolybdate of the folin phenol reagent to intensify the colour of the solution (Lowry et al., 1951).

**Reagents**

- **Reagent A**: 2 gm of sodium carbonate dissolved in 100 ml of 0.1 N sodium hydroxide (NaOH)
- **Reagent B**: 500 mg of cupric sulphate dissolved in 1 % sodium potassium tartrate.
- **Reagent C**: 50 ml of reagent A added to 1 ml of reagent B.
- **Standard**: 1 mg of Bovine Serum Albumin (BSA) dissolved in 1ml I N NaOH. This was serially diluted to obtain three concentrations viz., 100 mg/ml, 200 mg/ml, 300 mg/ml.

Known quantity of the tissue (100 mg wet weight) was homogenized in 10 % TCA to precipitate the protein. The sample was centrifuged at 3000 rpm for 5 min and the supernatant was decanted. The precipitate was redissolved in 1 N NaOH. To this 5 ml of reagent C was added and mixed well and allowed to stand for 10 min. To this solution 0.5 ml of Folin phenol reagent was added and mixed rapidly and allowed to stand for 30 min. 1 N NaOH and 1 % BSA were used as blank and standard solution respectively. The blue colour developed was read at 650 nm using UV-visible double beam
bio spectrophotometer (Elico), an auto analyser, which automatically quantifies the protein content in the sample.

### 3.4.4.8 Trace elements analyses

The efficacy of AvEaE and PbEaE extracts on trace elements of *C. cotylophorum* was studied by following the method of Dasgupta *et al.* (2013). Known quantity of the flukes from the control and treated groups (exposure to highest concentration 1mg/ml for 8 h) was thoroughly washed with deionized double distilled water. Whole flukes were dried in an incubator at 50 °C. The dried flukes were finely pulverized to powder. The powdered tissue (1 mg) was digested in 5 ml of concentrated nitric acid (HNO₃) in an airtight corked conical flask for overnight at 50 °C. The fully digested solution was transferred to a beaker and kept on a hot plate at 70 °C for ~2 h to allow complete evaporation of the acid. After cooling down, 10 ml of deionized water was then added, vigorously mixed with magnetic stirrer, and filtered through Whatman filter paper (110 mm Φ). The volume was finally made to 100 ml by adding more deionized water to the filtrate. The final solution was directly used for quantitative measurement of the trace elements using PERKIN ELMER OPTIMA 5300 DV ICP-OES at specific wavelengths for specific minerals.
3.5 Phytochemical analysis

3.5.1 Phytochemical screening of *A. Vasica* ethyl acetate and *P. betle* ethyl acetate extracts

Qualitative tests were performed to assess the nature of phytochemicals present in *A.vasica* and *P.betle* ethyl acetate extracts.

a) Liebermann-Burchard Test

Extract is dissolved in minimum of chloroform. Acetic acid was added and heated. Few drops of acetic anhydride and concentrated $\text{H}_2\text{SO}_4$ were added. Green colour shows the presence of Steroid.

b) Noller’s Test

Extract is treated with tin and thionyl chloride and was heated in a water bath. Purple colour shows the presence of Triterpenoid.

c) Shinoda Test

Extract is dissolved in alcohol. Magnesium bits and concentrated hydrochloric acid was added. It was heated in a water bath. Majenta colour shows the presence of Flavonoid.

d) Test for Furan

Extract is dissolved in alcohol. p-dimethylaminobenzaldehyde and concentrated hydrochloric acid was added and was heated in a water bath. Pink colour shows the presence of Furanoid compound.
e) Test for Sugar

Extract is treated with anthrone and concentrated H₂SO₄. It was heated in a water bath. Green colour shows the presence of Sugar.

f) Test for Coumarin

Extract is shaken with 10% NaOH. Yellow colour shows the presence of Coumarin. The substance regenerates when concentrated H₂SO₄ is added.

g) Test for Quinone

Extract is treated with concentrated H₂SO₄. Red colour shows the presence of Quinone.

h) Test for Saponin

Extract is shaken with water. Frothing shows the presence of Saponin.

i) Test for Tannin

Extract is shaken with water and lead acetate solution was added. White precipitate shows the presence of Tannin.

j) Test for Acid

Extract is treated with sodium bicarbonate solution. Effervescence shows the presence of acid.
k) Test for Phenol

Extract is dissolved in alcohol. Ferric chloride is added. Bluish colour shows the presence of Phenol.

l) Test for Alkaloid

Extract is taken in acetic acid and few drops of freshly prepared Dragendorff’s reagent is added. A brick red or orange precipitate shows the presence of alkaloids.

3.5.2 Isolation of fractions of plant extracts

Column chromatography

About 40 g of ethyl acetate extracts of A. vasica and P. betle was dissolved separately in ethanol and mixed thoroughly with silica gel. It was dried and made into powder form. Column chromatographic separation was carried out using glass columns packed with 200 g of silica gel. AvEaE and PbEaE along with silica gel was made into a slurry using chloroform and packed in two columns separately. It was covered on the top with a layer of cotton. The column was eluted with solvents of increasing polarity in the order of chloroform, ethyl acetate, acetone and ethanol. Elutes of AvEaE and PbEaE were collected in conical flasks at regular intervals. A total of 201 elutes were collected for AvEaE and 199 elutes were collected for PbEaE.
Thin layer chromatography

5-10 µl of elutes were spotted on silica gel pre-coated thin layer chromatography (TLC) plates of 0.2 mm thickness and developed in various solvent systems. Ratio of TLC chamber solvents used for developing TLC plates containing AvEaE elutes were chloroform: methanol (9:1), ethylacetate: methanol (9.5:0.5), butanol: acetic acid (6.3:2.7) and butanol: acetic acid: water (4:1:1). Ratio of TLC chamber solvents used for developing TLC plates containing PbEaE elutes were toluene: ethylacetate (9:2.5, 9:4, 6:4, 1:1), chloroform: methanol (9:1, 9:2.5, 9:4, 1:1) and butanol: acetic acid: water (4:1:0.5). Similar fractions were identified using TLC. Fractions with similar TLC bands were pooled together. A total of 11 fractions of AvEaE and 8 fractions of PbEaE were collected.

3.5.3 Effect of various fractions of AvEaE and PbEaE on the motility of C. cotylophorum

Efficacy of 11 fractions of AvEaE and 8 fractions of PbEaE was tested against C. cotylophorum by observing its motility visually at regular time intervals (5, 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h) and also by using Electronic micromotility meter.

3.5.4 Analysis of A.vasica and P.betle effective fractions by Thin Layer Chromatography (TLC)

Effective fractions were also analyzed by thin layer chromatography (TLC). Five milligrams of the extract was dissolved in ethanol and used for
analysis. The sample (5µl) was applied as bands using micro syringe on precoated silica gel plates. The plates were developed in twin trough chambers after sample application. Fractions were analysed by TLC using ATS4 on pre-coated silica gel 60 F254 of 0.2 mm thickness. The plates were also examined under UV light 254 nm and 366 nm, sprayed with vanillin sulphuric acid reagent, heated at 105 °C till the colour of the spots appear and the photo documentation of TLC plates was carried out. Butanol: acetic acid: water is used as solvent system. The plates were dried and scanned under UV (254nm), UV (366nm), sprayed with Vanillin sulphuric acid reagent and then scanned. Camag TLC scanner was used for scanning and the photo documentation of TLC plates was carried out.

3.5.5 Analysis of A. vasica and P. betle effective fractions by Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS technique was used in this study to identify the phytocomponents. GC-MS analysis of the fractions was performed using GC-MS-QP 2010 (SHIMADZU) and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column (Length : 30.0 m, Diameter : 0.25 mm, Film thickness : 0.25 µm composed of 100% Dimethyl poly siloxane). For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2µl was employed (split ratio: 20), Injector
temperature 200°C; Ion-source temperature 200°C. The oven temperature was programmed from 70°C (isothermal for 2 min), with an increase of 300°C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 – 1000 m/z. Total GC running time was 35 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a GC-MS solution ver. 2.53.

3.5.6 Identification of phytocomponents

Interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST08) and WILEY8. The spectrum of the unknown components was compared with the spectrum of known components stored in the library. The name, molecular weight and structure of the components of the test materials were ascertained.

3.6 In vivo studies

In vivo studies were carried out to investigate the anthelmintic effect of the effective extract of *P. betle* against paramphistomes. Twenty five naturally infected sheep were divided into four groups - group I, group II, group III and group IV. Group I animals dewormed prior to experimental period with albendazole (at the manufacturer-recommended dose) served as uninfected control. Group II served as infected untreated control. Group III
and IV animals were treated orally with effective extract of *P. betle* at two
different concentrations, 30 and 50 mg/ml for each group.

### 3.6.1 Faecal examination

Faecal samples for examination, collected per rectum were stored in
disposable polyethylene bags and brought to the laboratory in a thermacool
box. Clinical diagnosis of paramphistomosis was carried out by counting the
number of paramphistome eggs in the faecal sample. Quantitative estimation
of eggs per gram (EPG) was done following the McMaster’s method
(Soulsby, 1982). One gram of the faecal sample was mixed thoroughly in 12
ml of saturated salt solution. The solutions were thoroughly mixed by
continuous aspirations. Using a pipette the two counting chambers of the
McMaster’s slide were completely filled with the supernatant of the above
mentioned mixture. The slide was kept undisturbed for 20 min, which allows
the eggs and cyst to float to the top of the solution in the chamber. Over each
chamber is an etched square divided into 6 equal columns. The etched lines
were focused and the amphistome eggs were counted under the low power of
the microscope. The area marked off by the square over each chamber
represents a specific volume of solution (0.15 ml) in the chamber. The
number of paramphistome eggs in both the chambers was counted.
Paramphistome eggs per gram of faces were calculated. Percent reduction in
faecal egg count (FECR %) was calculated.
3.6.2 Haematological studies

Blood sample for the evaluation of the haematological parameters was drawn from the jugular vein with the help of a sterilized dry syringe and transferred to disposable blood collecting tubes (BD Vacutainer Sodium Heparin 68 USP units). These samples were transported to the laboratory in thermacool box. The haematological parameters viz., haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leucocyte count (TLC) and differential count (DC) were evaluated following standard methods (Jain, 1986).

3.6.2.1 Haemoglobin (Hb)

The haemoglobin in the blood sample was estimated following the cyanomethaemoglobin (CMG) method (Hailine, 1958). Potassium ferricyanide converts hemoglobin iron from ferrous to ferric state to form methemoglobin. Methemoglobin combines with potassium cyanide to produce a stable red cyanomethaemoglobin complex. The intensity of the colour is proportional to the haemoglobin concentration. To 0.02 ml of distilled water (blank), 0.02 ml of standard and 0.02 ml of the sample, 5 ml of Drabkin’s reagent (50 mg of potassium cyanide and 200 mg of potassium ferricyanide dissolved in 500 ml of distilled water and the volume made up to 1000 ml) was added. After 10 min the absorbance was measured spectrophotometrically at 540 nm. Based on the optical density (OD) of the
standard, OD of the sample and concentration of the standard, the hemoglobin content was estimated and expressed in g/dl.

3.6.2.2 Packed cell volume (PCV)

Packed cell volume was determined by Wintrobe method. One milliliter of blood was taken in a Wintrobe tube and filled to the graduation mark. Care was taken during filling to avoid the formation of air bubbles. The Wintrobe tube is spun for 10 min at 3000 rpm in a centrifuge. The volume of packed cells is read directly from the graduation mark (descending order) on the Wintrobe tube. PCV was measured in millimeter and expressed as percentage of the total blood volume.

3.6.2.3 Total erythrocyte count (TEC)

The total erythrocyte count was performed using the haemocytometer (Neubauer chamber). Twenty microlitres of blood was diluted with 0.38 ml of diluting fluid (0.85 % NaCl- physiological saline). The diluted blood was then filled in the haemocytometer. Caution was taken to prevent overfilling and the formation of air bubbles. The cells in the four large corner squares of the ruled area were counted under the high power of the microscope. The number of red cells per cubic mm in diluted blood was calculated.

3.6.2.4 Total leucocyte count (TLC)

The total leucocyte count was performed using the haemocytometer (Neubauer chamber). Twenty microlitres of blood was diluted with 0.38 ml of
diluting fluid (1.5 ml glacial acetic acid and 1 ml of aqueous solution of gentian violet in 97.5 ml of distilled water). Diluted blood was then filled in the haemocytometer. Caution was taken to prevent overfilling and the formation of air bubbles. The cells in the four large corner squares of the ruled area were counted under the low power of the microscope. The number of white cells per cubic mm in diluted blood was calculated.

3.6.2.5 Differential count (DC)

For differential count a thin blood smear was prepared on glass slide. The slide was air dried and stained with Leishman’s stain. After drying for 5 min the smear was examined for white cells viz., neutrophils (N), lymphocytes (L) and eosinophils (E) and each cell type was counted and recorded.

3.6.3 Biochemical profiles

Blood sample for the evaluation of biochemical parameters was drawn from the jugular vein and transferred to disposable blood collecting tubes (BD Vacutainer Serum) and allowed to coagulate at 37 °C for 30 min. These samples were transported to the laboratory in a thermacool box. The serum was separated by centrifugation at 3000 rpm for 10 min. This serum was used for various biochemical assay including glucose, total serum protein (TSP), albumin (A), globulin (G), A/G ratio, aspartate aminotransferase
(AST) and alanine amino transferase (ALT). A, G, AST, and ALT were assayed following the standard methods (Burtis and Ashwood, 1998).

3.6.3.1 Glucose

The glucose in the serum sample was estimated by glucose oxidase method of Huggett and Nixon (1957) as described in the in vitro study.

3.6.3.2 Total serum protein (TSP)

The total protein in the serum sample was estimated by Lowry et al. (1951) as described in the in vitro study.

3.6.3.3 Albumin and Globulin

Determination of albumin in serum or plasma is usually based on the binding behaviour of the protein with anionic dye Bromocresol green (BCG). Albumin binds with the BCG in a buffered medium to produce a green colored albumin-BCG complex, which is measured spectrophotometrically at 628 nm. The intensity of the color formed is directly proportional to the concentration of albumin present in the sample. To 0.01 ml of distilled water (blank), 0.01 ml of standard and 0.01 ml of the sample, 1 ml of albumin reagent was added and mixed well. The mixture was incubated at room temperature for 5 min and the absorbance was measured at 628 nm in UV-visible spectrophotometer. Based on the OD of the standard, OD of the sample and concentration of the standard, the albumin content was estimated
and expressed in g/dl. The globulin content in the serum was calculated from the following formula, Globulin in g/dl of the test serum = Total protein (g/dl) – Albumin (g/dl). Further, the A/G ratio was calculated.

3.6.3.4 Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST, EC 2.6.1.1) catalyses the transamination of L-Aspartate to 2-oxoglutarate forming L-glutamate and OAA. The OAA formed is reduced to malate by MDH with simultaneous oxidation of reduced NADH to NAD. The change in absorbance with time due to the conversion of NADH to NAD is directly proportional to AST activity. The reaction mixture included 2.3 ml of 228 mM aspartate, 0.1 ml of 4.2 mM NADH, 0.1 ml of 3.4 mM pyridoxamine-5’-phosphate, 0.05 ml of MDH (36,000 U), 0.05 ml of LDH (72,000 U), 0.2 ml of serum sample. The reaction was measured by the decrease in absorbance at 340 nm for 3 min at an interval of 15 sec. The AST activity was calculated from the millimolar coefficient of 6.22 for NADH and the activity was expressed as U/L.

3.6.3.5 Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT, EC 2.6.1.2) catalyses the transamination of L-Alanine to 2-oxoglutarate forming L-glutamate and pyruvate. The pyruvate formed is reduced to lactate by LDH with simultaneous oxidation of reduced NADH to NAD. The change in absorbance with time due to the conversion of NADH to NAD is directly proportional to
ALT activity. The reaction mixture included 2.3 ml of 610 mM alanine, 0.1 ml of 4.2 mM NADH, 0.1 ml of 3.4 mM pyridoxamine-5'-phosphate, 0.1 ml of LDH (72,000 U), 0.2 ml of serum sample. The reaction was measured by the decrease in absorbance at 340 nm for 3 min at an interval of 15 sec. The ALT activity was calculated from the millimolar coefficient of 6.22 for NADH and the activity was expressed as U/L.

3.7 Statistical analysis

All the data obtained in the present study were statistically analysed using the statistical software SPSS version 16.0. One-way ANOVA using Bonferroni test was applied to find out the significant difference between the different concentrations of plant extracts and hours of incubation. The same statistical tests were applied for comparing the data obtained in the in vivo study.