Chapter 3

Materials and Methods
MATERIALS AND METHODS

(A) Collection of parasites

Live liver fluke *Fasciola gigantica* parasites were collected from the liver of freshly slaughtered domestic buffaloes (*Bubalus bubalis*), at the local zoo abattoir in Udaipur. The liver was examined for Fasciola infection and the infected part of the liver was brought to the laboratory and washed several times in tap water and transferred into 0.9% physiological saline. After removing the parasites carefully, from the liver, they were again washed several times in physiological saline to remove debris and mucus, etc. These worms were kept in 0.9% physiological saline for investigations.

(B) Preparation of three medicinal plant extracts

Fruits of *Citrullus colocynthis* (Kharatumba), seeds of *Centratherum anthelminticum* (Kalijiri) and seeds of *Trachyspermum ammi* (Ajwain). Freshly *C. colocynthis* (Kharatumba) fruits were collected from the desert area; Shriganganagar, Barmer, Jaisalmer (Rajasthan) and *C. anthelminticum* and *T. ammi* seeds were collected from local market of Udaipur.

i. All the plant material was dried in an oven at 40 °C for 3-4 days or till they become completely solid and dry.

ii. Dried plant materials were homogenized in fine powder with the help of a blender and stored in the dark at room temperature in closed containers until use.

iii. Fruits and seeds of plants were extracted by taking 20 g of each sample in 160 ml of water and organic solvent in 250 ml flask. Then continuous shaking with an orbital shaker and an occasional stirring with a glass rode manually at 4 hours interval.

iv. After 72 hours the macerates of each plant part were filtered in separate flasks using a qualitative filter paper (Whatman No 4 filter paper).

v. They were centrifuged at x 10000 g for 15 min and the supernatant was used for anthelmintic testing. The filtered supernatants were dried until constant dry weights of each extract were obtained.
Dried plant extract was reconstituted in the respective solvent. Plant extracts were reconstituted in distilled water and organic plants extract were reconstituted in 10% DMSO.

The extracts were stored in 15 ml black cap bottle, covered with aluminum foil for the prevention of plant extract directly from light. The residues were stored at 4 °C for further use.

Plant extracts were prepared at a different concentration of aqueous and alcoholic extracts of three medicinal plants; seeds of *Centratherum anthelminticum* (Kalijiri), fruits of *Citrullus colocynthis* (Kharatumba) and seeds of *Trachyspermum ammi* (Ajwain).

### C. Experimental design

Anthelmintic activity was studied by *in vitro* Petri dish method as described by Githiori et al. (2006). After thorough washing with a physiological saline solution (0.7 percent, NaCl), they were divided into five groups. At room temperature, ten flukes were taken for each group in Petri dishes. The morphological and histopathological variations of flukes were studied after the experiment.

- **The first group** of the worm was used for identification of species of *Fasciola gigantica*, with the help of whole mount preparation of *Fasciola gigantica* (Dutt, 1980).
- **The second group** of the *Fasciola gigantica* was untreated and used as control *Fasciola gigantica*.
- **The third group** of the *Fasciola gigantica* was used for evaluating the anthelmintic effect of different increasing concentrations (10, 20, 30, 40, 50 mg/ml) of aqueous or alcoholic extracts of three selected medicinal plants on *Fasciola gigantica*.
- **The fourth group** of the *Fasciola gigantica* was used to evaluate the anthelmintic effect of commonly used synthetic drug albendazole (10, 20, 30, 40, 50 mg/ml) on *Fasciola gigantica*. 
The fifth group of the *Fasciola gigantica* was given *in vitro* treatments of extracts of three selected medicinal plants at increasing concentrations (10, 20, 30, 40, 50 mg/ml) and tissues of *in vitro* treated *Fasciola gigantica* were fixed in different fixative for histological and ultrastructural studies.

(D) Evaluation of the anthelmintic activity of albendazole and aqueous and alcoholic extracts of three selected medicinal plants

10 ml of each concentration of the anthelmintic extracts and synthetic drug were applied to a group of 5 worms maintained in 10ml of selected medium and 2ml of the sterilizing solution. The experiment was performed in three replicates at the optimal temperature (37ºC) and pH 7.4. All experimented worms were examined after 3, 6, 9, 12, and 15 hours mortality was calculated for each experiment. Mortality was determined visually by activity and by mechanical stimulation using a dissecting needle after 3, 6, 9, 12, and 15 hours and worm mortality was evaluated by observation under a magnifying glass or after being removed from the experimental medium and dipped in slightly warm water and on gentle stimulation, worms confirmed by mortality.

(E) Statistical Analysis

The dead worms were counted in each experimental set and the percentage of average mortality was calculated in all experiments according to the following equation: Average Mortality rate = a Total number of dead parasites/Total numbers of experiment parasites X 100.

All values were expressed as mean.
Microscopic Techniques

(A) Histology by Light Microscope (LM)

Transverse and longitudinal sections of control and treated Fasciola gigantica with three selected medicinal plant extracts of *Citrullus colocynthis* (Kharatumba) fruits, seeds of *Centratherum anthelminticum* (Kalijiri) and seeds of *Trachyspermum ammi* (Ajwain) were fixed in Bouin’s fixative (Bancroft and Stevens, 1977), dehydrate, embedded in paraffin wax, sections cut at 6µ on rotary microtome, dehydrated, stain with Haemotoxylin and Eosin, cleared in Xylene and mounted in DPX. Stained sections were examined under light microscope.

This study was carried out at the P. G. Department of Zoology, Govt. Meera Girls College, Udaipur (Raj.).

(B) Ultrastructure by Scanning Electron Microscope (SEM)

For the ultrastructural study, whole live liver fluke *Fasciola gigantica* parasites were fixed in EM fixative (The fixatives were 4 percent glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) containing 3 percent sucrose and 0.5 mm CaCl$_2$) for overnight at 4°C.

The *Fasciola gigantica* parasites were then washed three times in 0.1M cacodylate buffer (pH 7.2), postfixed for 1 hr with 1 percent osmium tetroxide (aqueous) and dehydrated in acetone series. Then fixed parasites were taken to AIIMS, New Delhi for further processing and analysis. Since SEM facilities are not available at Udaipur and in Rajasthan. The following work on SEM observation was carried out at AIIMS, New Delhi:

- Fixed *Fasciola gigantica* parasites were dried coated with gold using sputter and then they were observed with a resolution of scanning electronic microscope (SEM).
(C) **Ultrastructure by Transmission Electron Microscope (TEM)**

Transverse and longitudinal sections of live liver fluke *Fasciola gigantica* parasites were fixed in EM fixative (The fixatives were 4 percent glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) containing 3 percent sucrose and 0.5 mM CaCl₂) for overnight at 4°C.

The materials were then washed three times in 0.1M cacodylate buffer (pH 7.2), postfixed for 1 hr with 1 percent osmium tetroxide (aqueous) and dehydrated in acetone series. In some cases, materials were stored at 4°C in 0.1M cacodylate buffer (pH 7.2). Then *Fasciola gigantica* parasites were taken to AIIMS, New Delhi for further processing and analysis. Since TEM facilities are not available at Udaipur and in Rajasthan. Following research work for TEM observations were carried out at AIIMS, New Delhi:

- Block preparation
- Ultrathin sectioning and Staining
- Loading of sections on mesh copper grids and examination under TEM.
- Ultra-microphotographs were taken.

Fixed tissues of *Fasciola gigantica* were brought to Regional Electron Microscopy Facilities, AIIMS, New Delhi for SEM and TEM process for several times. As Electron Microscope facility is not available in Udaipur and Rajasthan.
Morphological Techniques

Whole Mount Preparation of *Fasciola gigantica* (Dutt, 1980)

Several species of Fasciola liver fluke in buffaloes such as *Fasciola hepatica, Fascioloides magna, Fasciola jacksoni, Fasciolopsis buski* and *Fasciola gigantica* cannot be distinguished apparently. Since different fasciola species infect the liver at a time, it is, therefore, necessary to make them whole mounts in order to identify them by light microscopy, based on their morphological characters.

Various species of Fasciola parasites have their distinct features like shape, size, the topography of the various organs and other structures distinction. The whole mounts of the Fasciola parasites were identified according to the above-mentioned characters as detailed by Dutt (1980).

The specific diagnosis of *Fasciola gigantica* is mainly based on the form, shape, and size of their body and shape, size, and topography of the various organs together with their other structural distinctions. A method for the study of the complete morphology of Fasciola parasites is given here. The various steps are a collection, relaxation, fixation, bleaching, staining, dehydration, clearing and examination.

Collection of parasites

Live fasciola species were collected from the liver of the freshly slaughtered buffaloes (*Bubalus bubalis*) at the local zoo abattoir and various shops of a meat market in Udaipur.

Relaxation and Fixation

For making whole mounts, the live fasciola parasites *Fasciola gigantica* were kept in little physiological saline. Hot AFA (Alcohol 85 ml., formalin 10 ml. and acetic acid 5ml.) at (80° to 85°C) then gradually poured into the beaker, which not only fixe them but also made them completely relaxed. Such Fasciola parasites then pressed between two sides (to make them flat) and left in cold AFA till use.
**Bleaching**

Pressed fasciola parasites, as mentioned above, were removed from fixative and washed several times in distilled water, passed through ascending series of alcohol up to 70% and then transfer into chlorinated alcohol for bleaching, which is necessary to make their internal organs visible. In twelve hours the fasciola parasites were bleached completely.

**Staining**

Bleached fasciola parasites were had in 70% alcohol and stained with alcoholic borax carmine for 5 min. and differentiated in acid for a minute.

**Dehydration**

Fasciola parasites were dehydrated in ascending series of alcohol for half an hour in each series up to 90%, after which they were transferred into absolute alcohol. Two changes of 1 hour each were given in absolute alcohol.

**Clearing**

Clove oil which is the best clearing agent was used for clearing purposes. Within twelve hours the fasciola parasites became completely transparent in clove oil.

**Mounting**

Clove oil, which clears fasciola parasites were mounted in DPX, on glass slides. Whole mounts prepare in this way and then examined both in dissecting and a compound microscope to finally identify them.
**Light Microscope Histology**  
_(Bancroft and Stevens, 1977)_

**Preparation of fixatives and stains**

1. **Bouin’s Fluid**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Picric acid</td>
<td>75 ml</td>
</tr>
<tr>
<td>Formalin</td>
<td>25 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

2. **Weigert’s Iron Haematoxylin**

**Solution A**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin</td>
<td>1.0 gm</td>
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<tr>
<td>Absolute alcohol</td>
<td>100 ml</td>
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**Solution B**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric chloride</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>4.5 gm</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>298.0 ml</td>
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</table>

**Working solutions** - Equal parts of solution A and solution B

3. **Alcoholic Eosin**

<table>
<thead>
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<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>Eosin</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>1.0 ml.</td>
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</table>

4. **AFA Fixative**

<table>
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<th>Ingredient</th>
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<tr>
<td>Alcohol</td>
<td>85 ml</td>
</tr>
<tr>
<td>Formalin</td>
<td>10 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>5 ml</td>
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5. Chlorinated alcohol

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<table>
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<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Potassium chlorate</td>
<td>1 gm</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>5 ml</td>
</tr>
<tr>
<td>Alcohol</td>
<td>70%</td>
</tr>
</tbody>
</table>

Methods

Live liver fluke *Fasciola gigantica* parasites were collected from the liver of the freshly slaughtered buffaloes. Parasites were washed in tap water and transfer in 0.9% physiological saline and then they were fixed in Bouin’s fluid for 24 hrs, after which they were washed for least 24 hrs in running tap water. The *Fasciola gigantica* flukes were dehydrated for 30 minutes in each ascending series of alcohols (30%, 50%, 70%, 90% and absolute alcohol) and cleared in xylene for 15 minutes. Fasciola was transferred into a beaker containing 50% xylene and 50% wax in equal quantity at 62°C for 45 minutes. Therefore, then they were transferred into pure paraffin wax at 62°C for 12 hours, for completely remove xylene from the parasite tissues.

The worms tissues were embedded in wax at 62°C using L pieces and blocks prepared. Sections were cut at 6μ on rotator microtome; sections were cut from paraffin ribbon and taken on a albumin (solution of 50% white part of the egg and 50% glycerin) coated glass slide. Then slides containing sections thus obtained, deparaffinized and hydrated using alcohol series 30%, 50%, 70%, after 70% staining in weigert’s Iron haemotoxylin solution for 2 minutes then washed in tap water and transfer in alcoholic eosin stain for 2 minutes then washed in tap water and again transfer for quickly dehydrated up to in 70%, 90%, absolute alcohol, for 15 minutes, cleared in xylene with two successive changes of 15 minute and mounted in DPX, covered with coverslip.
Electron Microscopy

The electron microscope is scientific instruments that use a beam of highly energetic electrons to examine objects on a very fine scale. These examinations can information about the topography (surface features of an object) morphology (shape and size of the particular making up the object). Electron microscopes were developed due to the limitation of light microscopes which are limited by the physics of light to 500x or 1000x magnification and resolution of 0.2 micrometer. There was scientific desire to see the fine details of the interior structures of cells (nucleus, mitochondria, etc.). This required 10,000x plus magnification which was just not possible using light microscopes.

For the study of the biological material with an electron microscope, a complex series of techniques and processes are required. These processes are similar to those used to prepare specimens for light microscopy, but some modifications are necessary because of the differences in image formation. The processes involved are a fixation, in order to preserve the structure, dehydration to remove all traces of water from the specimen and embedding to give the tissue mechanical support during ultramicrotome.
Electron Microscope Histology

(Bancroft and Stevens, 1977)

A. Preparation of fixative and buffer wash

- **Fixatives-1** 3.5% Glutaraldehyde in 0.2 M sodium cacodylate buffer.

Stock solution

1. Glutaraldehyde (25%) 7.0 ml.
2. 0.2 M sodium cacodylate buffer pH 7.2 43.0 ml.

Preparation of stock solution

0.2 M sodium cacodylate buffer pH 7.2

Solution-A

0.2 M sodium cacodylate (mol. Wt. 214.03)

0.2 x 214.03 = 42.806 gm/1000 distilled water

i.e. Dissolve 42.806 gm. Sodium cacodilate in 1000 ml. distilled water.

Solution-B

0.2N HCl

1.7 ml. con. HCl (Hydrochloric acid) in 100 ml. of distilled water.

Working Solution

Mix 50 ml. solution A and 2.7 ml. of solution B to get 0.2 M sodium cacodylate buffer (adjust pH to 7.2).

B. 0.2 M Sodium cacodylate buffer wash

Stock solution

Solution-A

0.2 M sodium cacodylate buffer pH 7.2 (42.806 gm/1000 ml. distilled Water)
**Solution-B**

0.2 N Hydrochloric acid (1.7 ml/100, dist. Water)

**Solution-C**

0.5 M calcium chloride (anhydrous) (CaCl₂) (0.5x111 = 55.5 gm/1000 ml dist. water).

**Working Solution**

1. Mixed 50 ml. of 0.2 M sodium cacodylate buffer with 2.7 ml. of 0.2 N Hydrochloric acids (HCl) and adjust pH to 7.2.

2. Make above solution up to 200 ml. by adding distilled water adjust pH to 7.2.

3. Added 1.0 ml. of 0.5 M CaCl₂ with agitation (for the preservation of membranes) and added 4 gm. Sucrose in order to increase the tonicity of the fixation.

- **Fixative-2**: 1% osmium tetraoxide (OSO₄) :
  - Osmium tetraoxide 1.0 gm
  - 0.2 M sodium cacodylate buffer 100.0 ml.

**Precaution**

This solution should be stored in dark amber coloured bottle at 4°C.

**Araldite soaking mixture (for 50 ml)**

(To be used with propylene oxide)

<table>
<thead>
<tr>
<th>Araldite (resin) (502)</th>
<th>27.0 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodeceny Succinate anhydrite (DDSA) (Hardener)</td>
<td>23.0 ml</td>
</tr>
<tr>
<td>Dibutyl phthalate (Plasticiser)</td>
<td>Two drops</td>
</tr>
</tbody>
</table>

1. *Fasciola gigantica* was fixed in 3.5% glutaraldehyde in 0.2 M sodium cacodylate buffer fixative for 1 hr. at room temp. Thereafter, they were first to cut longitudinally and transversely with a sharp blade in small pieces of less than 1.0 mm in size.
2. These small pieces of the worm were again placed in 3.5% glutaraldehyde in 0.2 M sodium cacodylate buffer for 2-6 hrs at 4°C.

3. The tissue so fixed was subsequently washed in the buffer; wash with three changes of one hour each at 4°C for removing excess fixative from the specimens.

4. The tissue was post-fixed in 1% osmium tetraoxide (OSO₄) for 2 hrs.

5. Again the tissue was washed three times with buffer wash at the interval of one hour each.

6. The small pieces of the *Fasciola gigantica* were dehydrated through ascending acetone series (30 min in each series).

7. After absolute acetone, the specimens were transferred into propylene oxide for complete dehydration. Two changes of 5 min. each in propylene oxide gave better results.

8. Before embedding in pure resin Araldite, three changes of propylene oxide plus Araldite in different proportions were given as mentioned below:

   i) **The first change** includes three parts of propylene oxide and one part of Araldite (3:1) for 30 min.

   ii) **Second change** had one part of propylene oxide and one part of Araldite (1:1) for 30 min.

   iii) **The third change** had a mixture of one part of propylene oxide and three parts of Araldite (1:3) for 12 hrs for complete evaporation of propylene oxide.

9. Finally, Araldite resin, prepared freshly as mentioned above, was poured into molds and the specimens were embedded. The molds were then placed in an oven for 48 hrs at 60°C for polymerization.

(C) **Sectioning**

Ultrathin sections were cut with help of glass knife. These ultrathin sections of resin adhered to the glass of the glass knife. They have been floated
out on the surface of distilled water. This was achieved by attaching to the knife a thin plastic through, which was filled with distilled water to form a floating out bath.

The thickness of the ultrathin section could be guessed roughly during the sectioning, from their interference colors. Table I. gives the correlation between section thickness and colors.

**Table -I**

<table>
<thead>
<tr>
<th>Thickness range</th>
<th>Interference colour</th>
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<tbody>
<tr>
<td>&lt; 60 nm</td>
<td>Grey</td>
</tr>
<tr>
<td>60 - 90 nm.</td>
<td>Silver</td>
</tr>
<tr>
<td>90 - 150 nm.</td>
<td>Gold</td>
</tr>
<tr>
<td>150 - 190 nm.</td>
<td>Purple</td>
</tr>
<tr>
<td>190 - 240 nm.</td>
<td>Blue</td>
</tr>
<tr>
<td>240 - 280 nm.</td>
<td>Green</td>
</tr>
<tr>
<td>280 - 320 nm.</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

The ultrathin sections were observed in the above-mentioned ways, which show interferences effects ranging, from bright color to fairly thick sections to dull grey for the very thin ones.

Gold, silver, and grey color sections were mounted or collected on the dull surface of the copper grids (most of the copper grids have a dull and shiny surface. They have been found in 3 mm or 2.3 mm in diameter). Then grids were allowed to air dry for 10 to 30 minutes before further treatment.

**D. Staining**

The aims of staining electron microscopy are to increase the ability of the sections to deflect the electrons and thereby improve the contrast. This is achieved by increasing the electron density of the specimen, by depositing metals of the high atomic number on to the tissues structures in the sections. Uranyl acetate and Lead citrate strains were used for the contrast.
Uranyl acetate stain

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</table>
| Uranyl acetate| 0.2 gm.
| Distilled water| 40.0 ml.

Solution shacked for 10 minutes and pH adjust to 3.4

Lead citrate stain

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<table>
<thead>
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</thead>
<tbody>
<tr>
<td>Lead nitrate Pb (NO₃)</td>
<td>1.33 gm.</td>
</tr>
<tr>
<td>Sodium citrate Na₂(C₆H₈O₇)₂H₂O</td>
<td>1.77 gm.</td>
</tr>
</tbody>
</table>

Method for Staining

1. Place a drop of Uranyl acetate stains and a drop of distilled water side by side on a clear wax surface of the Petri dish.

2. Each grid immersed in the drop of Uranyl acetate stains for 8 to 10 minutes, after which it was rinsed in a drop of distilled water placed.

3. Dry the grid by placing it on a piece of filter paper.

4. Transfer this grid now into Lead citrate stain for 5 min., rinse vigorously in distilled water for at least 2 minutes to remove the stain and put on the Whatman filter paper in a covered.

5. Stained ultrathin sections were examined under a Hitachi H300 or an AEIEM 801 or Philips electron microscope and taken an observation.
Fig.1 A. Cyst (CY) formation in bile duct (BD) of infected liver (IL) by *F. gigantica* (F. g.).

Fig.1 B. *Fasciola gigantica* (F. g.) are present in the infected liver (IL) of buffalo.
Explanation of Photographic Plate 2

Fig.2 A. *F. gigantica* (F. g.) present in bile duct (BD) of infected liver (IL) of buffalo.

Fig.2 B. Liver fluke *F. gigantica* (F. g.) parasites in saline solution.

Fig. 2 C. Full mature *F. gigantica* (F. g.).
Explanation of Photographic Plate 3

Fig.3 A. Seeds of *Centratherum anthelminticum* (C. a.).

Fig.3 B. Seeds of *Trachyspermum ammi* (T. a.).

Fig.3 C. Fruit of *Citrullus colocynthis* (C. c.) with plant in desert area.

Fig.3 D. Transverse section of *C. colocynthis* (C. c.) fruit.

Fig.3 E. Plants material dissolving in alcohol and filter by Whatman filter paper.

Fig.3 F. Plant extracts prepared with different concentrations.