Materials and Methods
Chapter-III

Materials and Methods

3.1 Materials

3.1.1 Collection of pods and leaves of *C. fistula*

Pods and leaves of *C. fistula* were collected from the month of May to June 2009 from Mathura and its adjoining areas. *C. fistula* plant was identified and authenticated by the Dr. A.K. Agarwal (Prof.), Head of department of Botany, BSA College Mathura. The pods and leaves were dried in shade and coarsely powdered and was used for the preparation of aqueous and hydromethanolic extracts.

3.1.2 Bacterial Isolates

*Escherichia coli* and *Staphylococcus aureus* cultures were obtained from the department of Microbiology and immunology, Pandit Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidylyaya Evam Go Anusandhan Sansthan, (DUVASU), Mathura and culture of *Pasteurella multocida* was obtained from Central Institute of Research in Goat (CIRG), Farah, Mathura. These cultures were used to determine the antibacterial activity of extracts of pods and leaves. Prior to use, the bacterial isolates were characterized on the basis of morphological, cultural and biochemical characteristics as described by Cruickshank, (1975). *Salmonella typhimurium* was also used as an antigen for humoral immune response.

3.1.3 Fungal isolate

*Candida albicans* culture obtained from the Department of microbiology & Immunology, Pandit Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidylyaya Evam Go Anusandhan Sansthan, (DUVASU), Mathura, was used to determine *in vitro* antifungal activity of HAEP, HAEL and HMEL of *C. fistula*. Prior to use, the fungal isolate was characterized on the basis of morphological, cultural and biochemical characteristics (Bhavan *et al.*, 2010).
3.1.4 Infectious Bovine Rhinotracheitis (Herpes virus)

To determine the antiviral activity of aqueous extract of pods and leaves against Infectious Bovine Rhinotracheitis (IBR) virus strain was obtained from Department of Epidemiology, Pandit Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, (DUVASU), Mathura was used. Before its use, IBR virus was cultivated in MDBK cell line and characterized by cytopathic effect in MDBK cell line.

3.1.5 Cell line

MDBK cell line (Madine- darby bovine kidney cells) maintained in Dulbaecos Minimum Essential Medium (DMEM) was procured from Dept. Of Epidemiology, Pt. Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, (DUVASU) Mathura.

3.1.6 Experimental Animals used

Wistar albino rats (Av. Wt. 80-100 gm) purchased from the animal house of IVRI Izzatnagar were used for haematological and biochemical parameter of blood, Humoral and cell mediated immune responses, spleen cells proliferation and cytokines (IFN-γ and IL-10) induction studies.

3.1.7 Housing

Experimental animals were housed in polypropylene cages with paddy husk bedding. All animals were given standard laboratory rodent food and drinking water and maintained as per norms of ethical committee. All animals were examined for health state prior to using them in different experiments. Each cage and individual animal was marked using 1% picric acid.

3.1.8 Media

Nutrient agar media (NAM), Muller Hinton Agar (MHA, Himedia) Sabouraud’s Dextrose agar media (SDA, Himedia) and Dulbecco’s Minimum Essential Medium (DMEM, Sigma) RPMI-1640 (Himedia) were used. Media, chemicals and reagents used in the study were of analytical grade.
3.1.9 Chemicals
BaCl$_2$, H$_2$SO$_4$, (Hi Media) fetal bovine serum (Sigma), Con-A (Genni), new born calf serum (Hi media), MTT (Sigma), Trypan blue, Dimethyl Sulphoxide (DMSO), DNCB (1-Chloro, 2,4-dinitro chloro benzene) antibiotic Antimycotic solution (100x)etc were used.

3.1.10 Biochemical Kit
Biochemical kit of Autospan for the estimation of serum total protein, albumin and globulin were employed.

3.1.11 ELISA Kits
For assaying interferon gamma (IFN $\gamma$) kit of R&D system, USA was used.

For assaying IL-10 (Invetrogen rat IL-10 CYTOSET$^\text{TM}$) was used.

3.1.12 Buffer Solution
PBS and washing buffer solution, dilution buffer, and blocking buffer as given in annexure were used for serological and other studies.

3.1.13 Solvents used for preparation of extracts
Triple distilled water for aqueous extracts and triple distilled water and methanol (7:3) for hydromethanolic extracts.

3.1.14 Reagents of Analar grade (Himedia, SRL) used in phytochemical analysis
Mayer’s reagent, Hager’s reagent, Dragendorff’s reagent, Ninhydrin test reagent, Ferric Chloride solution, Alkaline test reagent, Biuret test reagent, Legal’s test reagent: (1) Pyridine (2) Alkaline sodium nitroprusside. Methanol.

3.1.15 Organic solvents (SRL) used in Thin Layer Chromatography
3.1.16 Organic solvents used in High performance liquid chromatography (HPLC)

HPLC grade of methanol (Sigma) and water (SRL) were used. Standards of o-coumaric acid, p-coumaric acid, sinapic acid caffeic acid, cinnamic acid, ferulic acid, chlorogenic acid, gallic acid, kaempferol, quercetin dehydrate (Himedia) were obtained from the Dept.of Biosciences and Biotechnology, Banasthali University, Jaipur.

3.1.17 Plastic ware and glassware

Plastic ware and glass ware of Nunc, Borosil, Axygen, Tarsons, Variable and fixed micropipette of (Himedia) were employed.

3.1.18 Instruments used

Various instruments like Soxhlet apparatus (PERFIT), High performance liquid chromatography (HPLC) apparatus (waters), ELISA Reader (ECI), Electronic balance (KERN), Centrifuge (REMI), Water bath (REMI), Grinder (REMI), Vortex (GENEI), Incubator (SONAR), Inverted Microscope (Motic AE 21), Light Microscope (NIKON), pH meter (APPOLO, USA), CO2 incubator (ISHIN), Hot air oven (YORC), Laminar air flow (KLEINZEDS) and Autoclave (SONAR), etc were used.

3.2 Methods

3.2.1 Preparation of extracts of *C. fistula* using soxhlet apparatus (Goel et. al., 2008)

(a) Hot aqueous extract from dried pods (HAEP) of *C. fistula*.

(b) Hot aqueous extract from dried leaves (HAEL) of *C. fistula*.

(c) Hot Hydromethanolic extract from dried pods (HMEP) of *C. fistula*.

The extraction of *C. fistula* pods and leaves was carried out using standard procedure by Soxhlet apparatus. Dried powder of pods / leaves was placed in a porous cellulose thimble in soxhelt apparatus. The thimble was then placed in an extraction chamber, above a collection flask containing 750 ml solvent. Temperature did not exceeding the boiling point.
of the solvent. Extraction process lasted 16-18 hours and flask containing the solvent and extract was removed. The solvent in the flask was then evaporated to drying at 45°C using hot air oven and remaining extract was weighed and examined for % of dry matter yield, colour, and consistency and then kept at 4°C used for further study.

3.2.2 Extraction: Flow chart for preparation of hot aqueous/hot methanolic extract of pods and leaves of *C. fistula*

Dried pod/leaf powder in thimble.

↓

Triple distilled water 750 ml in flask.

↓

Continuous water flow in condenser.

↓

Soxhlet extraction fixed at the temperature according to boiling point of the solvent.

↓

After 72 hours withdrawal of the thimble from extraction chamber.

↓

Solvent was removed.

↓

Collection of extract in storage vial.

↓

Evaporation of remaining solvents at 45°C in hot air oven.

↓

Storage at 4°C for further use.

For the preparation of hydromethanolic extracts, mixture of methanol : water (7:3,v/v) was taken as solvent. Powdered plant material (70 gm) was extracted at 50°C taking 600 ml solvent (420 ml methanol + 180 ml triple distilled water) on collection flask. The remaining procedure is similar to the preparation of HAEP.
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The evaporated dried extracts (on dry weight basis) were calculated by following equation (Stanojevic et al., 2009).

\[
\text{The } \% \text{ of yield} = \frac{\text{Weight of extract}}{\text{Weight of powdered material}} \times 100
\]

3.3 Qualitative analysis of phytochemicals present in HAEP, HAEL and HMEP of C. fistula

Extracts were tested for the presence of active principles such as, tannins, flavonoids, alkaloids, glycoside, triterpenoides phytosteriodes and proteins by following standard and procedures as described by Debela, 2002.

3.3.1 Alkaloid

3.3.1.1 Mayer's Test

About 0.5-1 ml of extract solution was taken in a test tube and few drops of Mayer’s reagent was added. After adding mixture was shaken and allowed to stand for some time. Appearance of cream colour precipitate indicated the presence of alkaloids.

3.3.1.2 Hager’s Test

About 0.5-1 ml of extract was taken in a test tube. Few drops of Hager’s reagent (saturated solution of picric acid) was added and shaken and allowed to stand for some time. Appearance of yellow colour precipitate indicated that alkaloids were present in the sample.

3.3.1.3 Dragendroff’s Reagent

About 0.5-1 ml of extract was taken in a test tube and few drops of Dragendroff’s reagent was added. It was then shaken and allowed to stand for some time. Appearance of orange colour precipitate exhibited the presence of alkaloids in the sample.
3.3.2 Glycosides
3.3.2.1 Legal Test

Extract was treated with small amount of pyridine in a test tube and then few drops of alkaline sodium nitroprusside solution was added. Appearance of blood red colour showed the presence of glycosides in the extract.

3.3.3 Tannins and phenolic compounds
3.3.3.1 Ferric Chloride Test

Few drops of ferric chloride were added to 0.5 – 1 ml of extract solution in the test tube. Appearance of blue green colour confirmed the presence of tannin and phenols in extract.

3.3.4 Flavonoids
3.3.4.1 Alkaline Reagent Test

About 0.5ml of the extract solution was taken and, few drops of sodium hydroxide solution (10%) were added. Formation of an intense yellow colour, which turned colourless on adding few drops of dilute acid, indicated the presence of flavonoids.

3.3.5 Protein
3.3.5.1 Ninhydrin Test

About 0.5-1 ml of extract was taken in a test tube and boiled with 0.2 of ninhydrin solution (Indane 1, 2, 3- trione hydrate). Appearance of violet colour exhibited protein in the sample.

3.3.5.2 Biuret Test

About 0.5-1 ml of extract was taken in a test tube and 2-3 drops of sodium hydroxide solution (10%) and 1-2 drops of dilute copper sulphate solution were added. After some time, appearance of dark pink colour confirmed the presence of proteins in the sample.
3.3.6 Steroids and triterpenoids

3.3.6.1 Salkowski Test

About 0.5-1 ml of extract solution was treated with chloroform in a test tube. Few drops of concentrated sulphuric acid were added, shaken well and then wait for some time. Appearance of red colour at the lower layer indicated the presence of steroids and formation of yellow lower layer indicated the presence of triterpenoides.

3.3.7 Carbohydrates

3.3.7.1 Benedict’s Test

Extract solution was (0.5-1 ml) mixed with few drops of Benedict’s reagent (alkaline solution containing cupric citrate complex) in a test tube. Upon boiling, formation of brown precipitate showed the presence of reducing sugars in the extract.

3.3.7.2 Fehling’s Test

Equal volume of Fehling’s A (copper sulphate in distilled water) and Fehling’s B (potassium tartarate and sodium hydroxide in distilled water) reagent were mixed and few drops of extract were added and boiled. A brick red precipitate of cuprous oxide showed the presence of reducing sugars.

3.4 Thin Layer Chromatography (TLC) of HAEP, HAE and HMEP of C. fistula

HAEP, HAE and HMEP of C. fistula were subjected to thin layer chromatographic analysis to know the presence of phytochemical components to supports the colour test. Method given by Edwards and Kessmann (1982) was used.

3.4.1 Preparation of samples

10mg of powdered extract of C. fistula was dissolved in 10ml of 75% methanol to prepare the concentration of 1mg/ml. This solution was centrifuged at 3000rpm for 15 minutes to obtain a clear supernatant. The pellet was discarded and the supernatant was used for TLC.
Table 3: Solvents mixtures and spraying solutions used for TLC

<table>
<thead>
<tr>
<th>Solvent mixtures</th>
<th>Ratio</th>
<th>Spraying solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform : Water-Methanol : Water</td>
<td>50:-1-10:1</td>
<td>Diazotised P nitroaniline</td>
</tr>
<tr>
<td>Acetic acid : Chloroform</td>
<td>1:9</td>
<td>(a) Vanilline H$_2$SO$_4$</td>
</tr>
<tr>
<td></td>
<td>-do-</td>
<td>(b) Folin Ciocalteu</td>
</tr>
<tr>
<td></td>
<td>-do-</td>
<td>(c) 1%Ferricchloride solution</td>
</tr>
<tr>
<td>Toluene : Ethyl acetate</td>
<td>7:2</td>
<td>UV light (366nm )</td>
</tr>
</tbody>
</table>

3.4.2 Procedure

TLC aluminum silica gel 60 (sorbent type) F254 (a fluorescent indicator with a 254nm excitation wavelength), 20 X 20cm plates (Merck, Germany) were used. A line was drawn on the TLC plate at a distance of 1cm from the base. Marks were made on the line for sample application. The samples (8µl of each extract) were spotted on the line with the help of capillary tube and then allowed to dry for 20minutes. The plates were dipped in solvent system for about two hours as shown in table 3. The TLC plates were dried in oven at 37$^\circ$C for overnight. Then TLC plates were sprayed with different spraying agents according to the solvent used (table 3). After the complete development of colour, the plates were visualized in UV 366 nm light system (DESAGA, Germany). The spots were identified, labeled and their distances from the base line were measured in centimeters. The Rf value were calculated using the formula

$$Rf = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$
3.5 High Performance of Liquid Chromatography (HPLC) of HAEP, HAEL and HMEP

3.5.1 Preparation of samples

10 mg of powdered plant extracts of *C. fistula* was dissolved in 10 ml of methanol to get final concentration of 1 mg/ml. Before use, solution was filtered using 0.45µm syringe filter (millipore).

1 mg of each standard was dissolved individually in 1 ml of methanol. These solutions were also filtered by 0.45µm syringe filter.

3.5.2 Procedure

Above prepared samples of extracts and standards were used for HPLC. HPLC binary system (Waters) equipped with PDA detector connected to system processor. The system used Empower software with standard certification for analysis of results. A maximum pressure of 2500 psi and minimum of 1500 psi was maintained. The HPLC of solvents was run at 200 nm to 600 nm wavelength using reverse phase C-18 column. During the run, a flow rate 1 ml/min was maintained using binary mode of gradient system. Various combinations of the solvents; 20:80, 80:20, 60:20, 50:50 of methanol and water were used respectively for achieving best resolution of peaks. Finally, for the experiment 50:50 ratio of the solvent (methanol and water) was used for duration of 30 minutes.

In order to know the identity of the compounds, standards of various compounds i.e flavonoids (kaempferol, quercetin dehydrate) and phenolic acids (o-coumaric acid, p-coumaric acid, sinapic acid, caffeic acid, chlorogenic acid, cinnamic acid, ferulic acid and gallic acid) were used. The peaks were identified by comparing the retention time (RT) of the standard compounds with the RT of different peaks of the samples of the extracts.
3.6 Determination of safe and non toxic dose of HAEP and HAEL of *C.fistula*

To determine the safe and non toxic dose of aqueous extract of *C. fistula*, seven groups of albino rats, comprising five rats in each group. Group II, III and IV were consisted of HAEP treated rats, while albino rats of group V, VI and VII were fed orally with HAEL of 125 mg / 250 mg / 500 mg/Kg body weight respectively for 21 day. Group I served as control and was given triple distilled water. All the groups of albino rats were monitored for the development of clinical signs, changes in health conditions, weight gain/loss and hematological and biochemical changes.

3.6.1 Apparent health condition and toxic signs

Rats of test groups (HEAP/HAEL fed rats and unfed rats) were kept separately and observed every day for any toxic sign / Symptoms / abnormal changes throughout the experiment. The dose causing no adverse effect was taken as safe and nontoxic dose (NTD) for further studies.

3.6.2 Body weight gain/loss

Rats fed with hot aqueous extract of pods/leaves and control rats weighed at weekly interval of experiment. The mean body weight was recorded and compared with the control group.

3.6.3 Effect of HAEP and HEAL on hematological estimation

Blood samples were collected intraocularly in sterile vials containing EDTA on 21st day from all the test groups of albino rats fed with different doses of HAEP/HAEL of *C. fistula* including control group and immediately processed for Hb, PCV, RBCs, TLC and DLC as described by Schalm *et al.* (1975).

3.6.4 Effect of HAEP and HEAL on serum Protein estimation

Serum samples were also collected on 21st day from albino rats fed with different doses of HAEP/HAEL of *C. fistula* and control group albino rats. The samples were analyzed for total protein, albumin and globulin and compared in relation.
3.7 Determination of effect of HAEP and HAEL of *C. fistula* on immune response in albino rat model

3.7.1 Determination of humoral immune response against *S. typhimurium* “O” antigen

Humoral immune response was studied in 7 groups of albino rats with 5 rats in each group by sensitizing them with *Salmonella typhimurium* ‘O’ antigen which were subsequently fed with 125 mg/ 250 mg/ 500 mg/Kg body weight of HAEP/HAEL of *C. fistula* for 21 days.

3.7.1.1 Preparation of *Salmonella typhimurium* ‘O’ antigen for raising antisera

*S. typhimurium* “O” antigen was prepared as described by Bhatia *et al.* (2003). Smooth colonies of *S. typhimurium* grown on Tryptose agar medium were selected and inoculated in nutrient broth. Inoculated broth was incubated for eight hours at 37°C and then centrifuged at 3000 rpm for 20 minutes and supernatant was discarded and washed with the normal saline and then boiled at 100°C for two hours thirty minutes. This heated culture was then used as ‘O’ antigen for determination of humoral immune response in albino rats.

3.7.1.2 Immunization of rats

Experimental rats were divided in seven groups as described above. In I group *Salmonella typhimurium* ‘O’ antigen was inoculated subcutaneously without plant extract. Group II, III and IV were consisted of HAEP treated rats, while albino rats of group V, VI and VII were fed orally with HAEL respectively for 21 days along with ‘O’ antigen as shown in immunization schedule (table- 4). One week after last dose of ‘O’ antigen, blood serum was collected for determining antibody titer in rats against *Salmonella* ‘O’ antigen by serum/agglutination test.
Table 4: Immunization schedule for raising antiserum against *S. typhimurium* 'O'antigen in albino rats

<table>
<thead>
<tr>
<th>Day</th>
<th>Group I O antigen (Route)</th>
<th>O antigen fed with different concentrations of HEAP of <em>C. fistula</em></th>
<th>O antigen fed with different concentrations of HAEL of <em>C. fistula</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group II 125 mg/kg body weight</td>
<td>Group III 250 mg/kg body weight</td>
</tr>
<tr>
<td>1st Day</td>
<td>1 ml S/C</td>
<td>1 ml S/C</td>
<td>1 ml S/C</td>
</tr>
<tr>
<td>7th Day</td>
<td>1 ml S/C</td>
<td>1 ml S/C</td>
<td>1 ml S/C</td>
</tr>
<tr>
<td>14th Day</td>
<td>1 ml S/C</td>
<td>1 ml S/C</td>
<td>1 ml S/C</td>
</tr>
</tbody>
</table>
3.7.1.3 Determination of humoral response by Tube agglutination test

Tube agglutination test was used for determination of serum antibody level, as per conventional method. Clean dry agglutination tubes were racked in rows of 11 tubes, 1 ml of normal saline was put in the first tube and 0.5 ml in the remaining tubes with the help of micropipette. 0.5 ml of antiserum was added to 1st tube and mixed thoroughly and then 0.5 ml was transferred to second tube. The contents of the second tube were mixed and 0.5 ml transferred to the third tube and so on except the last 11th tube. From the 10th tube 0.5 ml of contents was discarded to make the equal volume in all agglutination tube. 0.5 ml of Salmonella typhimurium ‘O’ antigen was added in each tube and mixed thoroughly. The last 11th agglutination tube was kept as antigen control. The tubes were then incubated at 37°C for 24 hours. Agglutination titer was calculated as the highest serum dilution showing visible clumping of cells. In negative test, agglutination had not occurred and the antigen remained in suspension. For reading the end titer, the last antigen control tube always kept along with other tubes for comparison.

3.7.2 Determination of Cell mediated immune (CMI) response in albino rats using DNBC as an allergen

This study was designed to assess the effect of HAEP/HAEL of C. fistula on DNBC (1-Chloro, 2, dinitro chloro benzene) sensitized albino rats by the method as described by Tiwari and Goel (1985). In this experiment DNBC was used as an allergen. Seven groups having five rats in each group were made. Group II, III and IV were consisted of HAEP treated rats, while albino rats of group V, VI and VII were fed orally with HAEL for 21 day. Group I served as control and was given triple distilled water.

3.7.2.1 Procedure

Hairs were removed from thigh of rats of all groups and cleaned properly. 100 µl of 0.2% of DNBC (in acetone) was applied on hairless part of thigh skin of albino rats of all groups on day 7, 14, 17 respectively. Challenge dose was applied on 22nd day at the same site. Skin thickness
was measured with the help of Vernier Caliper at 0, 24, 48 and 72 hours post challenge.

Difference in the skin thickness measured at 0, 24, 48 and 72 hours was calculated by subtracting the thickness measured before challenge from that of post challenge. The inoculated site was also examined for erythema, induration and vesicle formation.

3.8 Determination of effect of HAEP & HAEI of *C. fistula* on albino rat splenocytes proliferation/inhibition

*In vitro* effect of HAEP & HAEI of *C. fistula* on splenocytes proliferation of rat spleen cells was studied with their four concentrations i.e. 62.5, 125, 250 and 500µg/ml.

*Ex-vivo* effect of HAEP on splenocytes proliferation of spleen cells was also studied. Four groups each having five rats were made and group I unfed (control) rats and group II, III and VI were fed with 125, 250 and 500 mg/ml body weight HAEP respectively. After 21 days of feeding, spleen cells were harvested and prepared as describe below.

3.8.1 Splenocytes Preparation

Rats were sacrificed and spleens were harvested aseptically using separate sterilized petridishes for each spleen containing RPMI-1640 medium. Spleen was macerated with the help of sterilized toothed forceps and macerated material was filtered through nytex membrane to remove the large particles. Cells were then resuspended in RPMI-1640 medium containing 10% fetal calf serum and centrifuged splenocytes at 2500 rpm for 10 minutes. Centrifuged cells were then treated with 0.15M NH₄Cl (lysis buffer) for erythrocytes lysis and then washed with RPMI-1640 medium. Cells viability was determined by the trypan blue dye (0.1% solution) cells were counted by haemocytometer and cells concentration was adjusted to 2 x 10⁶ cells / ml. This concentration was used for splenocytes proliferation assay and cytokine analysis using, 96 wells tissue culture plates.
Protocol for splenocytes preparation

Sacrifice of albino rats

Collection and maceration of spleen

Filtration through nytex membrane

Suspension of cells in RPMI -1640 medium with 10% fetal calf serum

Centrifugation at 2500 rpm for 10 minutes

Lysis of erythrocytes by lysis buffer (0.15 M NH₄Cl)

Three times washing with PBS (pH-7.4) and RPMI 1640 medium

Viability examination with 0.1% trypan blue

Counting of cells by haemocytometer

Adjustment to 2 x 10⁶ cell/ml

Used for splenocytes proliferation / inhibition, IFN-4, IL-10 cytokine analysis

3.8.2 Splenocytes Proliferation

For in vitro effect or ex vivo effect of HAEP/HAEL of C. fistula 200 µl of 2x10⁶ cells/well were used and cultured in triplicate in RPMI-1640 medium supplemented with 10% fetal bovine serum and 5µg/ml of Con A was added in this mixture as a mitogen to stimulate proliferation of splenocytes.

3.8.2.1 In vitro effect of HAEP and HAEEL on Splenocytes Proliferation

For in vitro effect, splenocytes were prepared from control rat as described above and 50µl of each concentration from (62.5µg/ml, 125µg/ml,
250/5µg/ml, 500µg/ml) HAEP/HAEL was added to respective wells of plate according to experimental design given in (table- 5). The culture plate was then incubated for 72 hours at 37°C in 5% of CO\(_2\) incubator. After incubation, 20µl of MTT solution (5mg/ml, for formazone crystal formation) was then added in each well. The plate was reincubated for 4 hours at 37°C in CO\(_2\) incubator containing 5% CO\(_2\) under 80% relative humidity. Following incubation, supernatant was removed and 100 µl of DMSO was added in each well to dissolve the formazone crystals. OD was taken at dual wave length 560-670 by ELISA reader. Mean value was calculated as compared to control.

Table 5:  Experiment design for \textit{in vitro} splenocytes proliferation/inhibition and cytokines IFN\(\gamma\) and IL-10 analysis

<table>
<thead>
<tr>
<th>Con A + spleen cells</th>
<th>Con A + spleen cells</th>
<th>Con A + spleen cells</th>
<th>Con A + spleen cells</th>
<th>Con A + spleen cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A + spleen cells +62.5µg/ml HAEP</td>
<td>Con A + spleen cells +62.5µg/ml HAEP</td>
<td>Con A + spleen cells +62.5µg/ml HAEP</td>
<td>Con A + spleen cells +62.5µg/ml HAEL</td>
<td>Con A + spleen cells +62.5 µg/ml HAEL</td>
</tr>
<tr>
<td>Con A + spleen cells +125 µg/ml HAEP</td>
<td>Con A + spleen cells +125µg/ml HAEP</td>
<td>Con A + spleen cells +125µg/ml HAEP</td>
<td>Con A + spleen cells +125µg/ml HAEL</td>
<td>Con A + spleen cells +125µg/ml HAEL</td>
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<td>Con A + spleen cells +250 µg/ml HAEP</td>
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<td>Con A + spleen cells +250µg/ml HAEP</td>
<td>Con A + spleen cells +25µg/ml HAEL</td>
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<tr>
<td>Con A + spleen cells +500µg/ml HAEP</td>
<td>Con A + spleen cells +500µg/ml HAEP</td>
<td>Con A + spleen cells +500µg/ml HAEP</td>
<td>Con A + spleen cells +500µg/ml HAEL</td>
<td>Con A + spleen cells +500 µg/ml HAEL</td>
</tr>
</tbody>
</table>

3.8.2.2 \textit{Ex vivo} effect of HAEP on Splenocytes proliferation

To study \textit{ex vivo} effect of HAEP on splenocytes proliferation, spleens were harvested from control rats and rats fed orally with 125mg / 250 mg / 500 mg / kg body weight for 21 days. Splenocytes were prepared from each rat as described above. The experiment was designed as given in table-3 and then incubated for 72 hours at 37°C in 5% CO\(_2\) incubator as described above. After incubation supernatant was removed and 100 µl of DMSO was
added in each well to dissolve formazone crystals. OD was taken at dual wave length 560-670 nm by ELISA reader. Mean value was calculated and compared to control.

**Table 6: Experiment design for ex vivo splenocytes proliferation/inhibition**

<table>
<thead>
<tr>
<th>Con A+ spleen cells of unfed rat</th>
<th>Con A+ spleen cells of unfed rat</th>
<th>Con A+ spleen cells of unfed rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A+ spleen cells of 125 mg/kg body weight HAEP fed rat</td>
<td>Con A+ spleen cells of 125 mg/kg body weight HAEP fed rat</td>
<td>Con A+ spleen cells of 125 mg/kg body weight HAEP fed rat</td>
</tr>
<tr>
<td>Con A+ spleen cells of 250 mg/kg body weight HAEP fed rat</td>
<td>Con A+ spleen cells of 250 mg/kg body weight HAEP fed rat</td>
<td>Con A+ spleen cells of 250 mg/kg body weight HAEP fed rat</td>
</tr>
<tr>
<td>Con A+ spleen cells of 500 mg/kg body weight HAEP fed rat</td>
<td>Con A+ spleen cells of 500 mg/kg body weight HAEP fed rat</td>
<td>Con A+ spleen cells of 500 mg/kg body weight HAEP fed rat</td>
</tr>
</tbody>
</table>

3.9 *In vitro* effect of HAEP/HAEL of *C.fistula* on induction of cytokines

Splenocytes were prepared from normal rats similarly and cultured in presence of Con-A 5µg/ml and subjected to different concentrations (125µg/250µg/500µg /ml) of HAEP/HAEL of *C. fistula*.

Procedure followed for cytokine assay was same as that for splenocytes proliferation assay of spleen cell culture was done for 48 hours and then supernatant was collected for determination of IFN-γ /IL-10 cytokine in it. The quantitation of IFN-γ cytokine in cell culture was done according to kit protocol.
Protocol of cytokine induction

Preparation of splenocytes

Incubation of splenocytes for 48 hours at 37°C in 5% CO\textsubscript{2} atmosphere under humid condition

Centrifugation at 2500rpm for 10 minutes

Collection of supernatant for quantitation of IFN-\gamma/IL-10 cytokine using ELISA

3.9.1 Quantitation of cytokines (IFN-\gamma) using sandwich ELISA

Experimental plan was made as given in table-4. Cytokine analysis was done by ELISA method. Different concentrations of IL-10 std. 1-3500 pg/ml, std. 2-1750 pg/ml, std. 3-875 pg/ml, std. 4-437.5 pg/ml, std. 5-218.5 pg/ml and 109.75 pg/ml and 54.875 pg/ml were made. The collected supernatant from Con A activated spleen cells cultures were thawed and used for ELISA as per format given below. Optical density was measured at dual wavelength (450-570 nm). Each experiment was run in triplicate.

**ELISA protocol for assaying**

IFN-\gamma cytokine induction

100\mu l anti rat IFN-\gamma antibody in each well and overnight incubation at room temp.

Each well was aspirated and washed with washing buffer (300 \mu l) 3 times

Plate was blocked by 300 \mu l of block buffer at room temp. for 2 hours

Three washing with washing buffer
100 µl of culture supernatant/standard was added and incubated at room temp. for 2 hours

Four washing with washing buffer

100 µl of anti rat IFN-γ biotin conjugated (detection antibody) was added and incubated at room temp. for 2 hours

Six washing with washing buffer

100 µl of streptavidine – HRP added to the well and incubated for 30 minutes at room temp. in dark

Seven washing with washing buffer

100 µl of substrate (TMB) added in each well and kept in dark

50 µl of stop solution was added

Determination of OD at 450 – 570 nm immediately

Calculation of IFN-γ concentration by standard curve

3.9.2 Quantitation of cytokines (IL-10) using sandwich ELISA

Experimental plan was made as given in table-4. Cytokine analysis was done by ELISA method. Different concentrations of IL-10 std. 1-1000 pg/ml, std. 2-500 pg/ml, std. 3-250 pg/ml, std. 4-125 pg/ml, std. 5-62.5 pg/ml and 31.25 pg/ml were made. The collected supernatant from Con A activated spleen cells cultures were thawed and used for ELISA as per format given below. Optical density was measured at dual wavelength (450-570 nm). Each was run in triplicate.
ELISA protocol for assaying IL-10 cytokine induction

Coat the plate by 100 µl/well anti rat IL-10 antibody and overnight incubation at 4°C

Each well was aspirated and washed with washing buffer (400 µl) one times

Plate was blocked by 300 µl of assay buffer at room temp. for 1 hours

Aspirate, invert and tap on absorbent paper to remove excess liquid.

100 µl of culture supernatant/standard was added and incubated at room temp. for 1 hour and 30 minutes.

Aspirate and five washing with washing buffer

100 µl of detection antibody (anti rat IL-10 biotin conjugated) was added and incubated at room temp. for 1 hours.

Aspirate and five washing with wash buffer

100 µl of streptavidine – HRP added to the well and incubated for 45 minutes at room temp. in dark.

Aspirate and five washing with washing buffer

100 µl of substrate (TMB) added in each well and kept in dark for 30 minutes

100 µl of stop solution was added

Determination of OD at 450 – 570 nm immediately

Calculation of IL-10 concentration by standard curve
Table 7: Experiment design for *in vitro* Sandwich ELISA test for IFN-γ / IL-10 cytokine

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<tr>
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<td>Blank reagent</td>
<td>Std.-1</td>
<td>Std.-2</td>
<td>Std.-3</td>
<td>Std.-4</td>
<td>Std.-5</td>
<td>Std.-6</td>
<td>Std.-7</td>
<td></td>
</tr>
<tr>
<td><strong>Con-A Control</strong></td>
<td>Con A Control + 125 µg HAEP</td>
<td>Con A Control + 125 µg HAEP</td>
<td>Con A Control + 125 µg HAEP</td>
<td>Con A Control + 125 µg HAEP</td>
<td>Con A Control + 125 µg HAEP</td>
<td>Con A Control + 125 µg HAEP</td>
<td>Con A Control + 125 µg HAEP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Con-A Control</strong></td>
<td>Con A Control + 250 µg HAEP</td>
<td>Con A Control + 250 µg HAEP</td>
<td>Con A Control + 250 µg HAEP</td>
<td>Con A Control + 250 µg HAEP</td>
<td>Con A Control + 250 µg HAEP</td>
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<td>Con A Control + 250 µg HAEP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Con-A Control</strong></td>
<td>Con A Control + 500 µg HAEP</td>
<td>Con A Control + 500 µg HAEP</td>
<td>Con A Control + 500 µg HAEP</td>
<td>Con A Control + 500 µg HAEP</td>
<td>Con A Control + 500 µg HAEP</td>
<td>Con A Control + 500 µg HAEP</td>
<td>Con A Control + 500 µg HAEP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.10 *In vitro* antibacterial effect of HAEP, HALE and HMEP of *C. fistula* against *Staphylococcus aureus*, *Escherichia coli* and *Pasteurella multocida*

Bacterial isolates obtained were characterized and confirmed by morphological cultural and biochemical tests as described by CruickShank, (1975).

3.10.1 Preparation of McFarland’s nephelometer for determination of bacterial concentration

McFarland’s nephelometer for determination of bacterial concentration was prepared as described by Heneric et al., (1956). 1% solution of H₂SO₄ and BaCl₂ were prepared. 10 cleaned standard test tubes of uniform diameter were set up in a rack. 0.1 ml of 1% BaCl₂ solution was added to the first tube and 1% BaCl₂ solution to remaining 9 tubes were added increasing the amount by 0.1 ml for each succeeding tube and thus the 10th tube contained 1 ml of 1% BaCl₂ solution. Enough amount of 1% solution of H₂SO₄ was added to each tube to bring the total volume of all
tubes to 10 ml. The tubes were then sealed with paraffin wax to prevent evaporation and avoiding errors in turbidity.

The density of the suspension increased from the tube 1 to tube 10 corresponds with the approximate number of bacteria / ml in saline suspension.

Table 8: McFarland's Nephelometer

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1% H₂SO₄ (ml)</th>
<th>1% BaCl₂ (ml)</th>
<th>Approximate number of bacterial cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.9</td>
<td>0.1</td>
<td>3.0 x 10⁸</td>
</tr>
<tr>
<td>2</td>
<td>9.8</td>
<td>0.2</td>
<td>6.0 x 10⁸</td>
</tr>
<tr>
<td>3</td>
<td>9.7</td>
<td>0.3</td>
<td>9.0 x 10⁸</td>
</tr>
<tr>
<td>4</td>
<td>9.6</td>
<td>0.4</td>
<td>1.2 x 10⁸</td>
</tr>
<tr>
<td>5</td>
<td>9.5</td>
<td>0.5</td>
<td>1.5 x 10⁸</td>
</tr>
<tr>
<td>6</td>
<td>9.4</td>
<td>0.6</td>
<td>1.8 x 10⁸</td>
</tr>
<tr>
<td>7</td>
<td>9.3</td>
<td>0.7</td>
<td>2.1 x 10⁸</td>
</tr>
<tr>
<td>8</td>
<td>9.2</td>
<td>0.8</td>
<td>2.4 x 10⁸</td>
</tr>
<tr>
<td>9</td>
<td>9.1</td>
<td>0.9</td>
<td>2.7 x 10⁸</td>
</tr>
<tr>
<td>10</td>
<td>9.0</td>
<td>1.0</td>
<td>3.0 x 10⁸</td>
</tr>
</tbody>
</table>

3.10.2 Preparation of inoculum and determination of bacterial concentration

Small amount of characterized bacterial culture from the stock culture was taken and inoculated into 5 ml of nutrient broth and incubated for 6 hours at 37°C. The broth culture was centrifuged at 3000 rpm for 10 minutes to collect the bacterial pellet that was then washed twice with normal saline. After washing, the pellet was suspended in 5 ml of normal saline. Density of microbial suspension was adjusted equal to that of 5x10⁶CFU/ml (standardized 0.5McFarland standard) and used for performing anti bacterial activity.

3.10.3 Preparation of herbal discs

Discs were prepared by the method as described by Bauer et al; (1966). Discs of 6 mm diameter were prepared from what man's filter paper
no-1 and sterilized by hot air oven at 160°C for 90 minutes and then dipped in solution of different concentrations of HAEP/HAEI of C. fistula. Discs were then allowed to dry and used as herbal discs. Discs containing 0.625 mg/1.25 mg/2.5 mg/5 mg/10 mg of HAEP/HAEI were used to study the antimicrobial activity against S. aureus while disc containing 1.25 mg/2.5 mg/5 mg/10 mg/20 mg of HAEP/HAEI were used to study E. coli and Past. multocida.

3.10.4 Antibacterial activity by disc diffusion method (Bauer’s method)

0.5 ml of respective bacterial culture, containing approximately $5 \times 10^6$ CFU/ml was swabbed on the top of the Muller Hinton Agar (MHA) medium. Disc containing different concentrations 0.625 mg/1.25 mg/2.5 mg/5 mg/10 mg/20 mg of HAEP/HAEI were placed at even distances on test culture seeded plates. Discs dipped in respective solvent were used as negative control. The culture plates with disc were incubated at 37°C for 24-48 hours. The antimicrobial activity of extract marked by the zone of inhibition around the disc, was measured at 24 and 48 hours intervals. Each experiment was repeated three times.

3.11  In vitro antifungal effect of HAEP, HAEI and HMEP of C. fistula against Candida albicans

Candida albicans used as test fungi was procured from Department of Microbiology and Immunology, DUVASU, Mathura. C. albicans was cultured on Sabouraud dextrose agar (SDA) at 30°C for 24 hours and characterized on the basis of morphological cultural characteristics, and sugar fermentation as described by Bhavan et al; (2010).

3.11.1 Preparation of inoculums and preparation of fungal concentration

Small amount of C. albicans taken from stock culture was inoculated in to 5 ml Sabouraud’s broth and incubated at 37°C for 6 hours. The broth culture was then centrifuged at 3000 rpm for 10 min. Pellet was washed twice with normal saline. Finally, the pellet was suspended in 5 ml of normal
saline and stored at 4°C for further use. The final concentration of culture used in the study was $5 \times 10^6$ CFU/ml

3.11.2 Disc diffusion method

Discs of 2.5, 5, 10 and 20 mg of each extracts were prepared as per method described above. 0.5 ml of respective bacterial culture, containing approximately $5 \times 10^6$ CFU/ml inoculums was swabbed on the top of the SDA medium. Disc containing different concentrations 0.625 mg/1.25 mg/2.5 mg/5 mg/10 mg/20 mg disc of each extract were placed at even distances on test culture seeded plates. Discs dipped in respective solvent were used as negative control. The culture plates with disc were incubated at 37°C for 24-48 hours and examined for the zone of inhibition around the disc measured at 24 and 48 hours interval.

3.12 Determination of \textit{In vitro} antiviral effect of HAEP and HAEL of \textit{C. fistula} against IBR virus using MDBK cell line

Madin-Darby Bovine Kidney (MDBK) cell line was obtained from the Department of Epidemiology, DUVASU, Mathura and maintained in Dulbecco’s Minimum Essential Medium (DMEM). Confluent monolayer of MDBK cells were grown in cell culture flasks using DMEM growth medium supplemented with 10% fetal calf serum, antibiotic antimycotic solution, sodium pyruvate and sodium bicarbonate. The cells were sub cultured twice a week. The preparation MDBK monolayer is given below.

\begin{center}
\textbf{Flow chart for subculture of MDBK cell line}
\end{center}

- MDBK cell line
- Discard the growth media from cell culture flask
- Add DMEM without serum and shake gently to remove dead cells. After shaking discard the media
- Add 1 ml trypsin-EDTA solution (0.25% trypsin and 0.22% EDTA in PBS) and keep it for 1 min.
Aspirate trypsin-EDTA solution with sterilized pipette

Keep the cell culture flask in CO2 incubator maintained at 37°C, 5% CO2 and 80% relative humidity for 3-7 min.

When cells become round, give gentle thrush to detach the cells from flask

Add 15 ml growth media with 10% fetal bovine serum.

Incubate flask in CO2 incubator maintained at 37°C, 5% CO2 and 80% relative humidity

Counting of cells by haemocytometer

Adjustment of cells $2 \times 10^5$ cells/ml and used for

- Determination of TCID$_{50}$
- Determination of MNTD
- Antiviral assay

### 3.12.1 Preparation of different concentrations of plant extract for antiviral screening assay

For antiviral screening assay, 1gm of HAEP/HAEI of *C. fistula* was dissolved in 5 ml PBS which were filtered by 0.45 µm membrane filter. Then 2 fold dilution of the each of filtered extract was prepared. For this 0.5 ml PBS was added in 10 small extract preparing vials. Then 0.5 ml plant extract was added in $1^{st}$ vial and then it was transferred to the II vial and subsequently up to $10^{th}$ vial. Thus different concentrations of plant extracts were prepared.

### 3.12.2 Determination of maximum non toxic dose (MNTD)

MDBK cells ($2 \times 10^5$ cells/well) were seeded in 96 well tissue culture plate in DMEM media to form monolayer. After incubation of 24 hours 100 µl of each concentration i.e. 40, 20, 10, 5, 2.5, 1.25, 0.625 mg/ml of
HAEP/HAEL extract was added to MDBK monolayer using 3 wells per concentration and incubated at 37°C in humified atmosphere of 5% CO\textsubscript{2} for 72 hours. The cytotoxicity was determined by microscopic examination of cell morphology (CPE) and change in pH in medium extract treated and untreated cultures and cytotoxicity was also determined by the MTT dye uptake assay (Lader et al., 1989). CPE included rounding of cells, degeneration and death of cells. 10µl of MTT dye (5 mg/ml) was added to each well and microtitre plates were further incubated for 4 hours at 37°C. After removal of supernatant the dye utilized by the viable cells was extracted with 100 µl DMSO by shaking 15 min. Optical density was measured at dual wave length 560 nm – 670 nm by ELISA reader concentrations showing minimum on CPE was taken as non toxic dose.

3.12.3 Virus titration in MDBK cells

MDBK cells (2x10\textsuperscript{5}/well) were seeded in 96 well plate in 0.1 ml DMEM medium and incubated overnight at 37°C in growth media for 24 hours. Supernatant was removed from each well and monolayer were infected with 10 fold dilutions of cell free IBR virus in 0.1 ml of DMEM containing 5% new born calf serum. The cultures were incubated for 5 days at 37°C. Optical density was also measured at dual wave length 560 nm – 670 nm by ELISA reader. The virus dose required to cause 50% death MDBK cells was calculated from dose response curve and virus titer was expressed as 50% tissue culture infection doses (TCID\textsubscript{50}). The cellular toxicity on MDBK cells were assessed by observing two methods. By cytopathic effect such as rounding of cell, degeneration and death of cells occurred. The number of viable cells was determined by using the MTT dye uptake assay as mentioned earlier.

3.12.4 Antiviral assay

To investigate the effect of HAEP/HAEL against IBR virus, 24 Hours old monolayers (2 x10\textsuperscript{5} cells/well) of MDBK cells in 96 well microtitre plate were taken and the plates were incubated at 37°C in the presence of 5% CO\textsubscript{2} until the cells become confluent. There after supernatant was removed
from each well and virus suspension containing 10 TCID$_{50}$ in 0.1 ml of DMEM with serum were mixed and appropriate concentrations of the extract from minimal to maximal non toxic concentrations were added to each well based serial dilution. For the virus control 0.1 ml of virus suspension and 0.1 ml of culture medial containing 5% serum without extract were used. For the cell control 0.1ml of culture medium without extract were used. Both were added simultaneously and the infected cell cultures incubated at 37°C in a unified CO$_{2}$ atmosphere (5% CO$_{2}$). All assays were run in triplicate. Every 24 hours observations were made and cytopathic effect was recorded by light microscopy for the appearance of cytopathogenic effect (CPE). The absence of CPE at the concentration of extract was considered to the indicative of antiviral activity. Cell viability was evaluated by MTT dye uptake assay. Antiviral activity of C. fistula was determined by the reduction in the CPE in extract treated group as compared to virus control group. The percentage of antiviral activity (in terms of CPE reduction) was calculated by the following formula

\[
\% \text{ of antiviral activity} = \frac{(OD_{T})_{V} - (OD_{c})_{V}}{(OD_{c})_{m} - (OD_{c})_{V}} \times 100
\]

Where \((OD_{T})_{V} = \text{virus infected cells with plant extract}\)
\((OD_{c})_{V} = \text{virus infected cells without plant extract.}\)
\((OD_{c})_{m} = \text{Cells without virus infection and plant extract}\)