CHAPTER 4

MATERIALS AND METHODS

4.1 Ethnobotanical surveys

4.1.1 Description of study area: Tripura – North Eastern Region of India

Tripura is a small state in the North Eastern region of the country, the latitudinal and longitudinal extent range between 22.56 degrees North to 24.32 degree North and 92.21 degree East respectively. The state has a total geographical area of 10,491 sq. km. As per the Government statistics, 56.52 percent of the total geographical area of the state is forest [201]. In view of their abundance, about 50% of the total flora of India is found in this part of the country [202]. Hinduism is the majority religion in the state. 85.6% of the population following this religion. Muslims make up 8.0% of the population, Christians 3.2%, and Buddhists 3.1% [203]. Out of 32 lakhs people 31 percent belongs to the tribal communities.

Tribal communities have over the being referred to by a value-loaded and pejorative terms such as “primitive”, “savage”, “exotic”, “barbarian”, “naked”, uncivilized and so on. Today, however, there is growing realizations that people who are different from us are equally worthy of respect, and that such stereotype and preconceived notions of them should be avoided. The state Tripura has attracted towards itself not only by their curious eyes but also their culture, tradition, heritage and their food habit. Tribal plants showing good values in treating many diseases, they can save lives of many, particularly in the developing countries. Herbal drugs obtained from plants are believed to be much safer; this has been proven in the treatment of various ailments [204].
Now a day's scientific investigation of the tribal plants has been initiated because of their contribution to healthcare.


4.1.2 Survey of the study area and group discussion

A survey [184] was conducted randomly among the tribal families residing in and around Agartala, the capital of Tripura. The consultants were chosen according to their deep knowledge in nature and also the ability to speak and understand the different tribal and non tribal languages of Tripura. A general conversation also has been done with the tribal medicine men. Total sample was considered by approaching direct interviews and semi structured questionnaire (Appendix A) with the tribal people with an unbiased eventually prepared format and group discussing. Questionnaires were prepared in such a way to facilitate the outcome of list of the plants used as edible and also used in the treatment of stomach disorders/ulcer. Consultants were questioned about their diagnostic procedure, symptoms and the method of medication. The most common symptoms which were further characterized as ulceration in the stomach or risk factors for development of ulcer by the medicine men listed below : -

i) Acidity
ii) Feeling of a full stomach
iii) Excessive abdominal pain which relieves after food consumption
iv) Loss of appetite with abdominal pain
v) Belching with a sour taste

4.2 Selection and Collection of plant materials
Depending upon the field survey report, the plant *Paederia foetida* L. was chosen for current work. Plant materials (aerial parts) were collected from the surrounding tribes dominated market, where the tribal people come to earn money by exchanging their wild collections.

### 4.3 Authentication of plant materials

After the collection of the aerial part of the plant, the herbarium was prepared and submitted to the National Institute of Science Communication and Information Resources (NISCAIR) for authentication. The specimen is authenticated by botanist Dr. II. B. Singh, Scientist F and Head Raw Material Herbarium and Museum, NISCAIR, New Delhi. Authentication reference number is NISCAIR/RHMD/Consult/2010-11/1442/40.

### 4.4 Preparation of Extract

The collected plant material (leaf) gets free from extraneous material, dried in shade and subjected for size separation in a special herbal grinder avoiding the elevation of temperature more than 40°C. These powders were passed through mess 200. Soxhlet extraction was done for the leaf powered material (1.5 kg) with the solvent methanol (4.5 L). The solvents and other chemicals used were of the analytical grade.

### 4.5 Preliminary phytochemical screening

Phytochemical investigation was performed by doing different qualitative chemical tests [206] including tests for alkaloids, glycosides, tannins, carbohydrates, saponins, proteins and amino acids, phenolic compounds, flavonoids in the methanol extract of leaf of *P. foetida*.

#### 4.5.1 Test for tannins

About 1 g of the extract was dissolved in 20 ml of distilled water and filtered. Two to three drops of 10% of FeCl₃ was added to 2 ml of the filtrate. The development of blackish-blue or blackish-green coloration was indicating the presence of tannins. To another 2 ml of the filtrate 1 ml of bromine water was added. Development of precipitate was taken as positive for tannins.

#### 4.5.2 Test for flavonoids
Shinoda test: About 0.2 g of the extract was dissolved in 2 ml of methanol and heated. A chip of magnesium metal was added to the mixture, followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange coloration indicated the presence of flavonoids.

4.5.3 Test for saponin glycosides

Haemolytic test: Freshly prepared 7% blood agar medium was used and wells were made in it. The extract in methanol was applied with distilled water and methanol used as negative control while commercial saponin (BDH) solution was used as positive control. The plates were incubated at 35°C for 6 h. Complete haemolysis of the blood around the extract was the indication of the presence of saponin glycoside.

4.5.4 Test for anthraquinone glycosides

Borntrager's test: To the 3ml of extract, dilute H$_2$SO$_4$ was added and then boil and filtered. To the filtrate equal volume of benzene or chloroform was added. The mixture was shaken and separate the organic layer. To the organic layer ammonia was added. Pinkish red color of amonical layer indicated the presence of anthraquinone glycoside.

4.5.5 Test for cyanogenetic glycosides

The powder drug was moistened with water and taken in a conical flask and corked it. A filter paper was soaked in 10% picric acid followed by in 10% sodium carbonate. The moistened filter paper was placed above the conical flask. Brick red or maroon coloration of the filter paper indicates the presence of cyanogenetic glycosides.

4.5.6 Test for cardiac glycoside

About 0.5 g of the extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 1% FeCl$_3$. This was underlaid with conc. H$_2$SO$_4$. A brown ring obtained at the interface indicated the presence of a deoxy sugar, characteristic of cardiac glycosides.

4.5.7 Test for iridoid glycosides

1gm of fresh sample was cut into small pieces and taken in a test tube and then added 5ml of 1% aqueous hydrochloric acid. After 3-6 hrs the extract was collected and Trim Hill reagent (made up from 10 ml acetic acid, 1 ml of 0.2% CuSO$_4$.5H$_2$O in water and 0.5ml of conc. Hydrochloric acid) was added. Development of the color indicates the presence of iridoids.

4.5.8 Test for alkaloid
To the alcoholic, aqueous and chloroform extract, dilute hydrochloric acid was added and filtered.

To the 2 ml of filtrate, Dragendorff’s reagent was added. The development of orange brown precipitate indicates the presence of alkaloids.

To the 2 ml of above filtrate, Hager’s reagent was added, formation of yellow precipitate indicate the presence of alkaloids.

4.5.9 Test for steroidal moiety

Salkowski method was used to test for steroids. About 0.5 g of the extract was dissolved in 3 ml of chloroform and filtered. To the filtrate concentrated H₂SO₄ was added to form a lower layer. A reddish brown colour was taken as positive for steroid ring.

4.5.10 Test for reducing sugars

One millilitre each of Fehling’s solutions I and II was added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2 – 5 min. The production of a brick red precipitate indicated the presence of reducing sugars.

4.5.11 Test for amino acids

To the 3 ml of test sample, 4% of sodium hydroxide and a few drops of 1% copper sulphate was added. Formation of violet or pink color indicates the presence of amino acids.

4.5.12 Qualitative HPTLC study of β-sitosterol

The presence of β-sitosterol was confirmed by performing the high performance thin layer chromatography (HPTLC). The methanol extract prepared in section 4.4 was used for qualitative estimation of β-sitosterol. The solvent system was used toluene: ethyl acetate: formic acid (8:2:0.1 v/v/v). Anisaldehyde sulfuric acid was used as spraying reagent to detect the spots. Standard β-sitosterol was used as reference standard. The Rf value was calculated by using the following formula:

\[ R_f = \frac{\text{Distance travel by solute}}{\text{Distance travel by solvent}} \]

4.6 GC-MS Analysis of volatile constituents of *P. foetida* L.

4.6.1 Preparation of extract/oil

4.6.1.1 n-Haxane leaf and stem extract
Leaf and stem were cut into small pieces and shade dried at room temperature for 10 days and then subjected to grinding mill. So obtained course powder of leaf and stem were used for solvent extraction and hydro-distillation as well. An about 20 gm of leaf and stem powder macerate with 200ml of HPLC grade n-hexane individually for overnight followed by sonication for 30 minutes. So obtained liquid extract was concentrated under the flow of nitrogen and kept in refrigerator for further use.

4.6.1.2 Steam distillation of leaf (oil sample)

A about 280gm of coarsely powdered leaves were hydro-distilled for 5 h in a Clavenger apparatus. The oil so obtained was dried over anhydrous sodium sulfate and filtered through 0.22-μM filter paper, and kept in a sealed vial in refrigerator (4°C) for further use. n-Hexane and all other chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.6.2 Preparation of sample for GC/MS analysis

The 5 mg of concentrated n-hexane, leaf and stem extracts was re-dissolved in the HPLC grade n-hexane, vortexes properly and filtered through 0.22 μM syringe filter (Millipore Corp., Bedford, MA, USA). One microlitre aliquot of the sample solution was injected into the GC/MS system for the requisite analysis.

1000μg/mL stock solution of hydrodistilled oil was prepared and then diluted to 100ppm. The prepared diluted oil was filtered through 0.22-μM filter paper. One microlitre aliquot of the sample solution was injected into the GC/MS system for the requisite analysis.

4.6.3 GC/MS Instrumentation and chromatographic conditions

The phytoconstituents present in the leaf, stem extract and hydrodistilled leaf oil were separated and identified by gas chromatography–mass spectrometry using an Agilent 7890A series (Germany) coupled to a Mass selective detector (ECD) acquired in electron ionization positive (EI) mode on 70eV and Agilent capillary column HP 5 MS (5% phenyl methyyl siloxane; 30 m X 250 μm X 0.25 μm film thickness). Helium was used as carrier gas, maintaining a constant flow of 1ml/min. Temperature programmed 65°C to 325°C, 65°C for 3 min with an increment of 2°C/min to 114°C then 4°C/min to 160°C and then 6°C/min to
302°C while the injector temperature kept at 260°C, detector and column temperature maintained 325°C. Injection volume was 2μL with a syringe size of 10μL and injection speed was maintained 50μl/s and split ratio was 1:100. A total of 62,667 min of the run time was attained. Spectra obtained over a range of 30 to 600m/z. Identification of phytoconstituents was made by comparison of their mass spectra with NIST 05 and Wiley 275 libraries mass spectra.

4.6.4 Identification of components

Interpretation of mass spectra of GC/MS was done using the NIST/EPA/NIH Mass Spectral Database (NIST11), with NIST MS search program v.2.0g [National Institute Standard Technology (NIST), Scientific Instrument Services, Inc., NJ, USA]. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST1 library.

4.7 Standardization of P. foetida L. extract

4.7.1 Optimization of Thin Layer chromatography solvent system

Different solvent systems were tried for developing a TLC system for identification of constituents in the extract based on the literature survey and keeping in mind the chemical nature of the constituents and the one showing maximum separation was selected as mobile phase for the study.

*The following solvents were used for the development of the TLC system:*

1. Chloroform : methanol (95:5)
2. Ethyl acetate : methanol : acetic acid (18:5:0.2)
3. Chloroform : Methanol (8:2)
4. Toluene : Ethyl acetate (9:1)
5. Toluene : Ethyl acetate (8:2)
6. Toluene : Ethyl acetate : Formic acid (8:2:0.1)
7. Toluene : Ethyl acetate (7:3)
8. Ethyl acetate : ethanol : water (6.1.3.1)
10. Chloroform : Methanol : Formic acid (18:5:2)
11. Chloroform : Methanol : Formic acid (8:1.5:0.1)
12. Chloroform : Methanol : Formic acid (8:2.5:0.1)
13. Chloroform : Methanol : Formic acid (7:2:20.1)
14. Chloroform : Methanol : Formic acid (18:5:2)
15. Chloroform : Methanol : Formic acid (7.5:1.5:0.1)

4.7.1.1 Procedure
The extract was dissolved in methanol and filtered, then spotted on the pre-coated silica gel G 254 plates with the help of capillary tubes. TLC plates were developed and scanned at 234 and 366 nm. Different reagents were then sprayed on the chromatogram to observe the separation. The two solvent system viz. toluene : ethylacetate (8:2) and toluene: ethyl acetate: formic acid (8:2:0.1) were found to be the most suitable solvent system given maximum band and better separation. Whereas in case of the solvent system chloroform: methanol: formic acid (8:2.5:0.1), the movement of reference standards (asperuloside and paederoside) were taken place in a better way and visibility was better in both the case i.e. in standard reference track and as well as in mother extract track. Hence the later one was chosen for standardization purpose.

4.7.2 Development and validation of HPTLC method for simultaneous estimation of asperuloside and paederoside

4.7.2.1 Chromatographic condition
The following HPTLC condition maintained throughout the experiment.
System : Camag Linomat V HPTLC (Switzerland) equipped with 100 µl Camag syringe and scanner III.
Stationary phase : Precoated silica gel aluminium plate 60F-254 (20 x 10 cm with 0.2 mm of thickness, E. Merck, Germany)
Mobile Phase : Chloroform: Methanol: Formic acid (8:2.5:0.1)
Detector : UV Detector.
Scanning Wavelength : 200 to 400 nm
Maximum absorption wavelength : 245nm
Scanning speed : 20 mm/s
Study parameter: Mean, Retention factor, Peak area, Peak height, Standard deviation,
Relative standard deviation, Relative retention time,
Theoretical plate

Sample application /Flow rate: 150 nL/s
Sample volume : 8μL
Data acquisition : Microsoft Excel and winCATS software

4.7.2.2 Sample preparation

The extract was prepared by taking 500 mg of leaf powder in 50ml of conical flask and
defatted with petroleum ether. The petroleum ether extract was collected and discarded. The
marc obtained from petroleum wash was macerated with 5 ml of methanol for overnight,
followed by 30 min of sonication. The extract obtained was filtered and dried using nitrogen
gas and was dissolved in 1 ml of HPLC grade methanol and subjected to 0.22 μm syringe
filter (Millipore Corp., Bedford, MA, USA). The concentration of the sample was obtained
500μg/μL.

4.7.2.3 Reference Standard Preparation

Asperuloside and paederoside standards were procured from WUXI App Tech Co Ltd.
China. The purity of asperuloside and paederoside was 98.47% and 98.12% respectively.
The stock standard solution of asperuloside and paederoside were prepared individually by
dissolving the same in HPLC grade methanol to obtain 1000μg/ml concentration and was
used to prepare other working standards.

4.7.2.4 Solvent system preparation

Solvent system was selected on the basis of its capability to give maximum bands, well
resolved spots and better movement of reference standards along with the visibility of
standards in extract too. Chloroform, methanol and formic acid were used as the solvent
system at a ratio of 8:2.5:0.1 (v/v/v) throughout the experiment.

4.7.2.5 Saturation of solvent system

At about 30 mL of solvent system was poured into Camag Twin trough glass chamber which
was lined with filter paper. A total of 20 min of time was allowed for saturation.

4.7.2.6 Application of sample and development of chromatogram

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The sample was applied to precoated silica gel aluminium plate 60F-254 (20 cmX10cm with 0.2 mm of thickness, E. Merck, Germany) by using with Camag microlitre syringe with a constant flow of nitrogen. The HPTLC fingerprint of the extract was established by developing the solvent system by adopting ascending thin layer chromatography technique. Chromatogram was developed up to the length of 80 mm. The developed TLC plates were dried in current of hot air.

4.7.2.7 Densitometry

Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 245 nm and operated by winCATS planar chromatography version 1.1.3.0. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. An evaluation was done via peak areas with linear regression. The whole analysis was performed in an air-conditioned room (temp. 22± 2°C and RH 55 ±5%). Absorption/remission was the measurement mode at a scan speed of 20 mm/s. Spots of asperuloside and paederoside were scanned from 200 to 400 nm to record their UV spectrum and to obtain their wavelengths of maximum absorption. HPTLC densitogram was recorded for whole extract and also for the reference standards at 243nm. The calibration curve for asperuloside and paederoside was investigated over a wide concentration range. The data of peak areas plotted against the corresponding concentrations were treated by least-square regression analysis.

4.7.3 Method validation

Method validation was carried out to confirm that the analytical method employed for the specific analysis is suitable for its intended use. Results from the method validation can be used to check its quality, reliability and consistency. The developed method was validated as per the ICH guidelines (ICH, 1997). An analytical method that to be validated can be done by any of the categories mentioned below:

a) Identification test
b) Quantitative estimation of actives.
c) Quantitative estimation of impurities

Estimation of marker compounds in *Paederia foetida* L. extract is classified under category i.e. quantification method. The analytes to be estimated were asperuloside and paederoside. The characteristics required for its validation are listed below:
1. Linearity
2. Specificity
3. Accuracy
4. Range
5. Precision
6. Repeatability
7. Intermediate precision
8. Robustness
9. Limit of Detection
10. Limit of Quantification

4.7.3.1 Validation of linearity

Linearity was determined by applying a series of standard at about seven different concentrations of three replicates that span between 50-200% of the expected working range. The Chromatogram was recorded to determine the peak area, % peak area, peak height.

The stock solution of asperuloside and paederoside were prepared in HPLC grade methanol by dissolving 1 mg of individual sample in 1ml of HPLC grade methanol. Further dilution was made to obtain a concentrate of the range of 400 to 3000ng/mL of concentration. The calibration graphs were plotted peak areas vs concentration. For assessing the linearity, the least square regression equation correlation coefficients were determined.

8 μL of standard (asperuloside and paederoside) of different concentrations (400 - 3000ng/ml) were spotted. It was repeated for 3 times for all concentration. The peak areas were recorded and calibration curve was drawn.

Acceptance criteria: Coefficient of correlation should be less than 0.99 $r^2$

4.7.3.2 Validation of specificity

The specificity of the analyte was checked by checking the peak purity or by checking the resolution when mixed with the other related compounds. The relevant chromatogram
spectra were recorded to show the specificity. The peaks of asperuloside and paederoside were confirmed by comparing the $R_f$ and the spectra of the peaks with that of the standard.

**Acceptance criteria**: - Resolution $> 2\%$.

### 4.7.3.3 Validation of accuracy

The accuracy of an analytical method was determined by adding a known amount of analyte to the extract and calculated the spike recovery. The concentration range of sample solution was prepared which was approximately spaced and span 50\% (lowest concentration), to 200\% (highest concentration), of the expected operating range. Accuracy was determined by spike recovery method. The pre-analyzed samples were spiked with standard at four different concentration levels, i.e. 0\%, 50\%, 100\% and 150\% and the sample was re-analyzed using the same method, which was to be validated. The sample was analyzed according to the method and the assay value, % recoveries were reported. The different batches of *P. foetida* L. extract was analyzed to find out the actual content of asperuloside and paederoside.

**Acceptance criteria**:  
- Assay $> 10\%$, $< 95$; recovery should be 95 to 110\%  
- Assay $> 0.5\%$, $< 7.5$; recovery should be 85 to 120\%

### 4.7.3.4 Validation of Range

The specific range derived from the linearity studies. The range was calculated from the linear graph, i.e. the lower to higher concentration between which the response is linear, accurate and precise.

**Acceptance criteria**: RSD $< 2.5$

### 4.7.3.5 Validation of Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous sample under the similar conditions. The precision of the proposed method was obtained by repeatability and intermediate precision. Inter-day and intra-day precisions were done by preparing and applying three different concentrations of samples (in triplicate) in the same day and in three
different days, respectively. Inter system and inter analyst precision were carried out by repeating the same procedure by using different systems of same lab and by a different analyst respectively. The method precision and intermediate precisions were determined and reported in terms of % RSD

4.7.3.6 Validation of Robustness

Robustness of the analytical procedure are a measure of its capacity to remain ineffective by small, but deliberate, variations in the method parameters and provide an indication of its reliability during normal usage. Robustness of the method was carried out by introducing small changes in the composition of the mobile phase and detection wavelength, the effect on the result was examined as % RSD.

4.7.3.7 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD is defined as the lowest concentration of analyte in a sample that can be detected and LOQ is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The concentration of the sample giving the signal to noise ratio of three was fixed in the LOD. The concentration of the sample giving the signal to noise ratio of ten was fixed as LOQ.

4.8 Evaluation of in-vitro antioxidant activity

4.8.1 Chemicals

1,1-Diphenyl-picryl-hydrazyl (DPPH) was obtained from the Sigma Aldrich Co., St. Louis, USA. Rutin (Ozone, Mumbai) was used as a standard drug. All other chemicals/solvent used was of analytical grade.

4.8.2 Preparation of Extract

Extracts were prepared for both the fresh and dried sample. Fresh leaf material was collected and reduced its size by scalpel. The size reduced material (50gm, leaf) was subjected to Soxhlet extraction with methanol (750 ml). The methanolic extract, evaporated on a rotary evaporator till dryness. The obtained extract of fresh sample was used for evaluation of
DPPH* radical scavenging activity. Methanolic extract of dried sample obtained in section 4.4 was also used as a sample.

4.8.3 DPPH* radical scavenging activity

The free radical-scavenging activity of fresh and dried extract of *P. foetida* L. was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH. The lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. DPPH assay was carried out as per the method of Jain et al [207]. In brief, a 250µl total reaction volume contains 10µl of DPPH solution; various concentrations of test solution and sufficient volume of methanol to make 250µl. The reaction mixture was mixed and incubated at 25°C for 20 min following which the absorbance was read at 510nm using microwell plate reader. A control sample was prepared containing the same volume without any extract and reference standard. Methanol (95%) and rutin were used as blank and standard respectively. The IC₅₀ values were obtained by calculating percent scavenging activity of the DPPH free radical using the following equation:

Calculation

\[
\% \text{ inhibition} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100
\]

Where, \(A_0\) was the absorbance of the control (blank, without extract) and \(A_1\) was the absorbance of the extract or standard. The 50% inhibitory dose (IC₅₀ value) was found by interpolation by using graph pad (prism 6 software) and compared with standard [208].

4.9 Assessment of *in-vitro* anti *Helicobacter pylori* activity

4.9.1 *H. pylori* strains and culture

Patients were enrolled in this study according to the following inclusion and exclusion criteria. Inclusion criteria included being aged between 18 and 80 years with symptoms of dyspepsia, and no previous antimicrobial therapy to eradicate *H. pylori* infection. Exclusion criteria included previous gastric surgery, any use of bismuth, antimicrobial agents, H2-receptor antagonists, proton pump inhibitors within 4 weeks prior to endoscopic examination; or any of several concomitant medical illnesses including cardiac, respiratory,
renal and liver diseases. The details are given in table no. 4.1. The study was approved by the ethical committee at Yashoda superspeciality hospital, Ghaziabad, Uttarpradesh, India. *H. pylori* strains were isolated from antral mucosal biopsy specimens of patients suffering from gastro-duodenal diseases. The strains were identified on the basis of colony appearance, gram staining, and positive reactions in biochemical tests (catalase, urease and oxidase). *H. pylori* strains were revived and cultured on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) supplemented with 5% horse serum (Invitrogen, NY), 0.4% IsovitaleX (Becton Dickinson, MD), trimethoprim (5μg/ml), vancomycin (8μg/ml), and polymixin B (10μg/ml). The plates were incubated at 37°C in a microaerophilic atmosphere (5% O₂, 10% CO₂, 85% N₂) (Double gas incubator, Hera cell 150i) for 3 to 6 days. Stock cultures were maintained until use at – 70°C in Brain heart infusion broth with 20% glycerol.

**Table 4.1**: Details of *H. Pylori* strain isolated from patients following inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Strain No</th>
<th>Patient suffered from</th>
<th>Genotype</th>
<th>Gender (patient)</th>
<th>Age of patient</th>
<th>Metronidazole (MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>340Asc</td>
<td>NERD</td>
<td>s1m1, Cag (+)</td>
<td>M</td>
<td>29</td>
<td>0.2μg/ml</td>
</tr>
<tr>
<td>2</td>
<td>347Asc</td>
<td>NERD</td>
<td>s2m2, Cag (-)</td>
<td>M</td>
<td>47</td>
<td>&lt;8 μg/ml</td>
</tr>
<tr>
<td>3</td>
<td>339Asc</td>
<td>GERD</td>
<td>s1m1, Cag (+)</td>
<td>M</td>
<td>59</td>
<td>&lt;8 μg/ml</td>
</tr>
<tr>
<td>4</td>
<td>354Asc</td>
<td>Duodenal ulcer</td>
<td>s2m2, Cag (-)</td>
<td>M</td>
<td>20</td>
<td>&lt;8 μg/ml</td>
</tr>
<tr>
<td>5</td>
<td>137Asc</td>
<td>GERD</td>
<td>s2m2, Cag (-)</td>
<td>M</td>
<td>30</td>
<td>1.5 μg/ml</td>
</tr>
<tr>
<td>6</td>
<td>122Asc</td>
<td>GERD</td>
<td>s2m2, Cag (-)</td>
<td>M</td>
<td>28</td>
<td>1.5 μg/ml</td>
</tr>
<tr>
<td>7</td>
<td>383Asc</td>
<td>GERD</td>
<td>S2m2, Cag (-)</td>
<td>M</td>
<td>60</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>8</td>
<td>399Asc</td>
<td>NERD</td>
<td>s1m1, Cag (+)</td>
<td>M</td>
<td>50</td>
<td>64 μg/ml</td>
</tr>
</tbody>
</table>

GERD = Gastro-Esophageal reflux Disease; NERD = Non-Erosive Reflux Disease; M = male; MIC = Minimum inhibitory concentration. Sc= symbol colony; Strains considered resistant if the MIC is > 8 μg/ml (because that concentration is cell toxic and harmful for human. Therefore the strain 399Asc is metronidazole resistant (MIC = 64 μg/ml) while others are metronidazole sensitive i.e. MIC is less than 8 μg/ml [709]).

4.9.2 Suspension Preparation
The bacterial suspension was prepared by the direct colony method [210]. The colonies were taken directly from the plate and were suspended in 5 mL of sterile 0.85% phosphate buffer saline (PBS). The turbidity of the initial suspension was adjusted by comparing with McFarland’s standard number 4 (0.4 mL 1% w/v BaCl₂ × 2H₂O + 99.6 mL 1% w/v H₂SO₄). When adjusted to the turbidity of the McFarland’s standard no. 4, the bacterium suspension contains about 12 X 10⁸ colony forming unit (CFU)/mL.

### 4.9.3 Determination of antimicrobial susceptibility and resistance

*H. pylori* cells growing exponentially on antibiotic free BHI agar were suspended in Phosphate buffered saline (PBS), a series of 10-fold dilutions of these cell suspensions was prepared, and 10 μl of each dilution was spotted on freshly prepared BHI agar containing various concentrations of different antibiotics (μg/ml) viz. Amoxicillin (0.125, 0.25, 1, 2), Clarithromycin (0.125, 0.25, 1, 2), Metronidazole (0.2, 0.5, 1.5, 3, 8, 16, 32, 64), Furazolidone (0.2, 0.5, 1, 2), Tetracycline (1, 2, 3, 4).

### 4.9.4 Minimum Inhibitory concentration (MIC)

After 72 h incubation under microaerophilic conditions, the minimal inhibitory concentration was recorded as the lowest concentration that inhibited visible growth of organisms. Minimal inhibitory concentration (MIC) for different antibiotics was defined as Metronidazole (> 8 μg/ml), Clarithromycin (>2 μg/ml), Amoxicillin (>8μg/ml), Furazolidone (>2 μg/ml), Tetracycline (>2 μg/ml).

### 4.9.5 DNA extraction from *H. pylori* culture

The C-TAB method of Murray and Thompson [211] was used for DNA isolation and PCR analysis was done by Multiplex PCR.

### 4.9.6 Amplification of DNA by Polymerase Chain reaction by Multiplex PCR

PCR amplification of *H. pylori* genes was performed for cag A, vacA s1/s2, vacA m1/m2 and cagA typing PCR was performed in 25-μl volumes containing 2.5 pmol of primers VAG-F and VAG-R, 25 pmol of primers VA1-F and VA1-R, 10 pmol of primers cag5c-F
and cag3c-R, 0.25 mM of each deoxynucleoside triphosphate, 0.9 U of Taq DNA polymerase, and 1.5 mM of MgCl₂ and were amplified under the following conditions: 3 min at 94°C for initial denaturation followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of 10 min at 72°C, in 96 plate thermal cycler (24) and the PCR product was finally stored at 4°C. 3% agarose gel electrophoresis was used to examine the product under gel documentation system.

4.9.7 Anti H. pylori assay

Sterile Whatman paper disks (6mm in diameter) were soaked with different concentration of samples and placed on the inoculated plates with 1.2 X 10⁹ colony forming unit (CFU) of H. pylori. The plates were kept under observation for 2 days at 37°C under microaerophilic conditions (5% O₂, 10% CO₂ and 85% N₂). All experiments were performed in triplicates. Pure methanol was used as a negative control.

4.10 Evaluation of in-vivo anti-ulcer activity

4.10.1 Animals

Albino rats weighing 150 to 180 g of both sexes were used in the current study. They were procured from Regional Institute of Medical Sciences (RIMS), Imphal. The rats were acclimatized for one week under laboratory conditions. They were housed in polypropylene cages and maintained at 25°C ± 2°C temperatures and 12 hr dark / light cycle. They were fed with soya bean bori, Gram or standard pellet food once in a day and water ad libitum. The litter in the cages was renewed daily to ensure hygienic condition and maximum comfort for animals. The different capacity of specially designed iron cages was used to house the rats. Animals of different groups were placed in separate cages to facilitate experimental work and accurate evaluation.

4.10.2 Ethical Approval

Ethical clearance for handling and experimentation on animals was obtained from the Institutional Animal Ethical Committee (IAEC), IBSD, Imphal with approval No.-IBSD/IAEC/Ext. Inst./PC/4 (1) prior to the beginning of the experimental works.

Ulceration was induced by using the following method:
1. Indomethacin-pylorus ligation-induced ulcer
2. Alcohol induced gastric ulcer
3. Water immersion stress induced ulcer (WISIU)
4. Western blot analysis of pylorus ligatures rats

The overview of all the methods is given in table no. 4.2

4.10.3 Indomethacin-pylorus ligation-induced ulcer

Albino rats were randomly divided into five groups, each consisted of 5 animals. The normal control (Group 1) and ulcer control (Group 2) groups received vehicle (distilled water; 5ml/kg, p.o.) throughout the course of the experiment. The treatment groups received different doses of methanol extract of *P. foetida* (100 and 200 mg/kg b.w./day; Group 3 & 4) and ranitidine (10mg/kg, p.o./day; Group 5) for a period of three days. All the animals, other than those in the normal group, were administrated indomethacin suspension (25mg/kg, s.c.) once daily for three days as a ulcerogenic agent, 30 minutes after the administration of vehicle or test or standard drugs.

4.10.3.1 Surgical procedure

On the 4th day 30 minutes after the treatment, surgical procedure was done as per Goel et al. [212]. The rats were fasted for 24 hours before starting of surgical procedure. They were provided free access to water during this period. Each rat was anaesthetized with ether and the abdomen was opened through a midline incision. The pylorus located and ligated tightly with silk suture. Both the muscular layers and skin were then stitched with the help of suture and ligature separately. Four hours after pylorus-ligation, the animals were sacrificed by giving over dosage of ether. Stomach of all sacrificed rats was examined under microscope. The gastric damages in the glandular regions were located in the gastric mucosa as elongated black-red lines parallel to the long axis of stomachs.

4.10.3.2 Calculation of ulcer index [213]

Ulcer index = \((UN + US + UP) \times 10^{-1}\); Where UN = Average of number of ulcer per animal
US = Average of severity score, UP = Percentage of animals with ulcers.
The percentage of ulcer protection was calculated by using the following formula:-

\[
\text{% of ulcer Protection} = \left( \frac{\text{Control mean ulcer index} - \text{test mean ulcer ind}}{\text{Control mean ulcer index}} \right) \times 100
\]

4.10.3.3 Determination of gastric volumes, pH and acid outputs

The stomachs of rats were excised out under ether anesthesia exactly after 4 hours of pylorus ligation and the gastric contents were collected. The stomachs were washed with luke warm sterile water. Both the washing and gastric contents were collected and centrifuged together at 4000 RPM for 10 minutes. The volumes of all the supernatants of gastric contents were measured and other gastric secretion studies, like pH, acid output, acidity, ulcer index/ ulcer score was also done [214, 215]. The pH of all supernatants was measured and their acidities were determined by titration to pH 7 with 0.1 N NaOH solution.

The acid outputs were calculated by following equation:

\[
\text{Acid output (μEq/hr)} = \text{Acidity (mEq/l)} \times \frac{\text{Vol. of gastric juice(ml)}}{4(\text{hr})}
\]

\[
\text{Acidity} = \frac{\text{Titre value of NaOH } \times \text{Actual normality of NaOH}}{\text{Assumed normality of NaOH}} \times 100
\]

4.10.4 Alcohol induced gastric ulcer

The animals were divided into five groups, each consisting of five rats. In this case ulcer was induced by administering 70% ethanol (10ml/kg, p.o.). All animals were getting fasted for 36 hours before administration of alcohol. Group 1 receiving distilled water (5 ml/kg, p.o.). Group 2 receiving 70% ethanol to induce gastric ulcer and 5ml distilled water/kg p.o. as treatment, Group 3 & 4 received methanol extracts of \textit{P. foetida} (100 and 200mg/Kg, p.o.) one hour befor administration of 70% ethanol. Sucralfate was administered to fifth group as reference standard drug at the dose100mg/kg, p.o. one hour before administration of ethanol. They were kept in specially constructed cages to prevent coprophagia during and after the experiment. The animals were anaesthetized 4 hours later with anesthetic ether and stomach was incised along the greater curvature and ulcer score was recorded [216].
For normal stomach score is 0, for red coloration 0.5 while for spot ulcer 1.0, for streak, ulcer and perforation score 1.5, 2.0 and 3.0 respectively.

### 4.10.5 Water immersion stress induced ulcer (WISIU)

Group 1 and 2 were treated as normal control and ulcer control respectively. Group 3 and 4 received methanol extracts at the dose 100 & 200 mg/kg b.w. respectively and group 5 received Lansoprazole (8mg/kg, p.o.). After treatment animals were allowed to swim in a glass cylinder having water (25°C) [216] for 3 hours. Animals are killed by high dose of anesthetic ether and ulcer score was recorded.

**Table 4.2:** Animal models used to evaluate anti ulcerogenic activity

<table>
<thead>
<tr>
<th>Model</th>
<th>Total Group</th>
<th>N</th>
<th>Plant extract dose</th>
<th>Standard dose</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>5</td>
<td>5</td>
<td>100mg/kg body weight &amp; 200 mg/kg body weight</td>
<td>Ranitidine 10mg/kg</td>
<td>Volume of gastric juice, pH, acid output, ulcer index</td>
</tr>
<tr>
<td>Model 2</td>
<td>5</td>
<td>5</td>
<td>100mg/kg body weight &amp; 200 mg/kg body weight</td>
<td>Sucralfate 100mg/kg body weight</td>
<td>Ulcer score</td>
</tr>
<tr>
<td>Model 3</td>
<td>5</td>
<td>5</td>
<td>100mg/kg body weight &amp; 200 mg/kg body weight</td>
<td>Lansoprazole 8mg/kg body weight</td>
<td>Ulcer score</td>
</tr>
<tr>
<td>Model 4</td>
<td>4</td>
<td>6</td>
<td>150mg/kg body weight</td>
<td>Cimetidine 8mg/kg body weight</td>
<td>Volume of gastric secretion, pH, free acidity, total acidity, ulcer index</td>
</tr>
</tbody>
</table>

### 4.10.6 Western blot analysis of *in-vivo* stomach tissue from pylorus ligatures rats

Animals were divided into four groups, each containing six and were starved for 24 hours before experimentation. Group 1 and 2 served as normal control, and ulcer control, respectively, and administrated water (5ml/kg b.w., p.o). Group 3 and 4 received *P. foetida* L. methanol extract (150 mg/kg, p.o.) and cimetidine (8mg/kg, p.o.) respectively. After 30 minutes of treatment the pylorus located and ligated tightly with silk suture as described
above paragraph (4.10.3.1). The number of either erosions or ulcers was determined under the magnifying glass.

4.10.6.1 Western blot Analysis

Western blotting, also known as immunoblotting or protein blotting, is a core technique in cell and molecular biology. In most basic terms it is used to detect the presence of a specific protein in a complex mixture extracted from cells. Western blotting procedure relies upon three key elements to accomplish the task. These are the separation of protein mixtures by size using gel electrophoresis, the efficient transfer of separated proteins to a solid support, and the specific detection of a target protein by appropriate matched antibodies. One of the critical features of any successful western blot is the highly specific interaction between an antibody and an antigen. The antigen, usually a protein or peptide, is the target of the antibody. A typical western blot relies upon a purified, semi purified or crude extract of cellular proteins containing a target protein that can be detected by antibodies. The three key preparative stages are:

Sample preparation by lysis of homogenization to solubilize and release cellular proteins.

Separation of protein mixture using gel electrophoresis

Transfer of separated proteins to a blotting membrane which can be manipulated more easily than a gel.

4.10.6.1.1 Sample preparation

Crude cellular lysates are the most common direct source of starting material used in Western blotting. The stomachs of rats were removed and opened along the greater curvature and then scratched with the help of a scalpel, washed with ice cold phosphate buffer solutions and homogenized with ice cold whole cell lysis buffer. Once the tissue has been homogenized and lysed, the solubilized cellular components were clarified by centrifugation and estimated the protein concentration. Mixtures were centrifuged at 14000 RPM, 4 °C for 10 minutes (5430R, Eppendorf). All the steps are carried out on ice, which minimized the chances of proteolysis, dephosphorylation, and denaturation. The supernatants containing 1:100 protease inhibitor cocktail (P8340, Sigma-Aldrich) were kept in -80 °C until used.
4.10.6.1.2 Determination of protein concentration (Bradford protein estimation)

Protein concentration was estimated by Bradford assay. In brief, initially Bradford dye (Bio-Rad) diluted with distilled water (1ml:4.5ml), protein sample diluted with water (1.5 μl : 73.5μl) and prepared bovine serum albumin (BSA) solution of different known concentration (20, 40, 80 μg/ml) with water as standard were prepared. The 96 wells microplate was used for protein estimation, where 200 μL diluted Bradford dye taken in each well, Read O.D. at 590 nm in microplate reader pre-programmed with protein estimation protocol (Molecular Devise, USA Spectra Max 5e).

4.10.6.1.3 Loading buffer

After knowing the concentration of protein, the samples were diluted in gel loading buffer. This buffer contains glycerol so that samples sink easily into the wells of the gel and a tracking dye (bromophenol blue) which migrates through the gel first to indicate how far the separation has progressed. To fully denature the protein and remove all the higher order structure, sodium dodecyl sulfate and reducing agent was added to the loading buffer. Samples were heated for 10 minutes at 70°C to aid denaturation. The samples were placed at 4°C.

**Composition of loading buffer**

<table>
<thead>
<tr>
<th>Composition</th>
<th>Pipetted volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>0.493g</td>
</tr>
<tr>
<td>SDS</td>
<td>1.0g</td>
</tr>
<tr>
<td>Bromo Phenol Blue (BPB) 0.1%</td>
<td>5mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5.75mL</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>2.5mL</td>
</tr>
<tr>
<td>Water q.s to make total volume and adjust pH 6.8 with dilute HCL</td>
<td>50mL</td>
</tr>
</tbody>
</table>

4.10.6.1.4 Gel electrophoresis
The proteins were separated by size using SDS-PAGE (Sodium dodecy sulfate polyacrylamide gel electrophoresis). A 10% of SDS-PAGE was used to separate the proteins. Since the samples had been denatured in gel loading buffer containing SDS detergent, the protein was uniformly negatively charged and migrated in electric field through the gel and towards the positive electrode.

When the gel sets, it was placed into the running apparatus. About 20μl volume of protein sample dissolved in loading buffer was added to each individual well. The gel was then connected to a power supply and allowed to run for a few hours in a buffer tank to separate the proteins.

**Composition of Gel Electrophoresis Running Buffer**

25mM Tris base

190 mM Glycine

0.1% SDS

**Composition of Transfer Buffer**

50mM Tris base

380mM Glycine

0.1% SDS

20% Methanol

**4.10.6.1.5 Blotting**

After the gel electrophoresis, the separated protein mixtures were transferred to a solid support, i.e. polyvinylidene difluoride (PVDF) membranes (Merck- Millipore), by means of trans blot turbo unit (BIO RAD). The air bubbles were removed by rolling with a pipet. It is important that no air bubbles are allowed to remain between the gel surface and the blotting membrane, since air bubbles will disrupt the transfer of any proteins in that area distorting the results.

**4.10.6.1.6 Blocking**
Blocking is a very important step in the immune-detection phase of western blotting because it prevents binding of antibody to the non-specific protein present in the blotting membrane. 5% fat free mild (Bio Rad) solution in wash buffer was used as blocking solution.

4.10.6.1.7 Antibody incubation and Visualization

After the transferred proteins from gel, membrane were incubated with primary antibodies (Anti-Nr2f2; Ab1) produced in rabbit and anti-Nf-Kb p65 antibody produced in rabbit for 15 minutes, then washed three times in one minute interval, thereafter incubated with biotin conjugated secondary antibodies, rewashed in similar way as done before SNAP-ID instrument (Merck-Millipore) and then visualized by chemluminesance ECL solution (chromogenic method and taken image in Bio-Rad gel Doc system.

4.10.7 Statistical Analysis

Data were expressed as mean ± Standard Error Mean (SEM). Differences were considered significant at **P<0.001, or *P<0.01 or *P<0.05 when compared test group vs control (–ve) group. For numerical results, one-way analysis of variance (ANOVA) with Dunnett test (compare all vs. control) was performed using GraphPad InStat Version 3 (GraphPad Software).