3. Materials and Methods

3.1 Materials:

3.1.1 Plant material:

The plant material like leaves, buds, roots, petals and flowers of various medicinal plants were used for the study (Table 3.1). The plant material were cleaned and washed with sterile distilled water. They were kept for drying in the oven at 35-40 °C for 3-4 days till the weight became constant. They were regularly examined to check any fungal growth or rotting.

Table 3.1: Plants and their parts used in the study

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant name (common)</th>
<th>Botanical name</th>
<th>Plant part used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brahmi</td>
<td><em>Bacopa monnieri</em></td>
<td>leaves</td>
</tr>
<tr>
<td>2</td>
<td>Shyam Tulsi</td>
<td><em>Ocimum tenuiflorum</em></td>
<td>leaves</td>
</tr>
<tr>
<td>3</td>
<td>Neem</td>
<td><em>Azadirachta indica</em></td>
<td>leaves</td>
</tr>
<tr>
<td>4</td>
<td>Aloe vera</td>
<td><em>Aloe vera</em></td>
<td>leaves</td>
</tr>
<tr>
<td>5</td>
<td>Fumaria</td>
<td><em>Fumaria parviflora</em></td>
<td>leaves</td>
</tr>
<tr>
<td>6</td>
<td>Mint</td>
<td><em>Mentha arvensis</em></td>
<td>leaves</td>
</tr>
<tr>
<td>7</td>
<td>Rose</td>
<td><em>Rosa indica</em></td>
<td>petals and leaves</td>
</tr>
<tr>
<td>8</td>
<td>Amla</td>
<td><em>Emblica officinalis</em></td>
<td>pulp and seeds</td>
</tr>
<tr>
<td>9</td>
<td>Assam tea</td>
<td><em>Camellia sinensis assamica</em></td>
<td>leaves</td>
</tr>
<tr>
<td>10</td>
<td>Bryophyllum</td>
<td><em>Bryophyllum pinnatum</em></td>
<td>leaves</td>
</tr>
<tr>
<td>11</td>
<td>Curry</td>
<td><em>Murraya koenigii</em></td>
<td>leaves</td>
</tr>
<tr>
<td>12</td>
<td>Clove</td>
<td><em>Syzygium aromaticum</em></td>
<td>bud</td>
</tr>
<tr>
<td>13</td>
<td>Datura</td>
<td><em>Datura metel</em></td>
<td>fruit, bud, leaves</td>
</tr>
</tbody>
</table>
Figure 3.1: Plants used in the study (*Azadirachta indica* and *Aloe vera*).

Figure 3.2: Plants used in the study (*Mentha arvensis* and *Rosa indica*).
Figure 3.3: Plants used in the study (*Emblica officinalis* and *Syzygium aromaticum*).

Figure 3.4: Plants used in the study (*Bryophyllum pinnatum* and *Murraya koenigii*).
3.1.2 Bacterial strains:

3.1.2.1 *Helicobacter pylori* strains:

3.1.2.1.1 **Reference strains** procured from Bacteriology division, National Institute of Cholera and Enteric diseases (NICED) Kolkata, India:

- *H. pylori* 26695 ATCC 700392
- *H. pylori* J99 (Taxon identifier 85963)
- *H. pylori* SS1 (Taxon identifier 102617)
Figure 3.6: Single isolated colonies of *H. pylori* 26695 strain on a BHIA plate.

3.1.2.1.2 Clinical isolates procured from Bacteriology division, National Institute of Cholera and Enteric Diseases (NICED) Kolkata, India:

- *H. pylori* 154(1A)
- *H. pylori* 216(1A)
- *H. pylori* 225(1A)

3.1.2.1.3 Clinical isolates procured from Molecular Bacteriology division, Amity Institute of Biotechnology, Noida, India. Strains were isolated from patients of national capital region (NCR), Delhi:

- *H. pylori* 3B
- *H. pylori* 34A (clarithromycin resistant strain)
- *H. pylori* 39A (clarithromycin resistant strain)
- *H. pylori* 58A
- *H. pylori* 70A
- *H. pylori* 75A
3.1.2.2 Pathogenic strains were procured from NuLife Consultants and Distributors Pvt. Ltd., New Delhi, India:

- Methicillin resistant *Staphylococcus aureus* (*S. aureus*)
- *Pseudomonas aeruginosa* (*P. aeruginosa*)
- *Bacillus cereus* (*B. cereus*)
- *Bacillus subtilis* (*B. subtilis*)

Figure 3.7: Single isolated colonies of *B. cereus*, *B. subtilis*, *P. aeruginosa* and *S. aureus* strains.

3.2 Methods

3.2.1 Identification of *H. pylori*:

The biopsy tissue in the transport medium was vortexed for 1-2 min and then, 200 µl of the transport medium was streaked on an antibiotic supplemented brain heart
infusion agar (BHIA) plates. The plates were incubated for 3 to 5 days at 37 °C in a custom made incubator with the mixture of gases containing 5 % O₂, 10 % CO₂ and 85 % N₂ (microaerophilic condition). The incubated plates were periodically examined from day 3 onwards for the growth of *H. pylori*.

The identification of *H. pylori* was first done by the physical appearance of the typical colony morphology on selective BHIA medium. After 4-5 days of incubation of the primary plates under microaerophilic condition, *H. pylori* colonies were distinct enough to undergo further confirmation. *H. pylori* colonies appeared as transparent water droplets of 0.5 to 1.0 mm in size. Colonies were further confirmed by confirmatory tests as mentioned below.

### 3.2.1.1 Oxidase test:

A sterile filter paper strip (Whatmann No.1) was dipped in 1 % oxidase reagent and placed on a clean glass slide. A small amount of young culture was transferred using a sterile toothpick and placed on the filter paper previously soaked with the oxidase reagent. Since *H. pylori* are cytochrome oxidase positive, a deep purple colour was observed almost immediately. If there was no colour development within 30 s, the test was considered to be negative (Figure 3.8).

![Image of oxidase test result](image)

**Figure 3.8: Confirmation of the presence of *H. pylori* by oxidase test.**

### 3.2.1.2 Catalase test:

Using a sterile toothpick, a small portion of the young culture was transferred to a drop of 30 % hydrogen peroxide (H₂O₂) on a clean glass slide. The immediate formation of bubbles of nascent oxygen indicated strong catalase activity. Bubbles of
nascent oxygen were as a result of the decomposition of hydrogen peroxide into hydrogen and oxygen molecules. If there was an absence of formation of bubbles, the test was considered to be negative (Figure 3.9).

Figure 3.9: Confirmation of the presence of H. pylori by catalase test.

3.2.1.3 Rapid urease test (RUT):

Using a sterile toothpick, a small portion of the young culture was transferred to a RUT broth in a sterile eppendorf. A change in the colour of the broth from yellow to pink indicated the presence of H. pylori. The change in the colour was due to the breakage of urea into ammonia which resulted in the rise in pH and later to colour change (Figure 3.10).

Figure 3.10: Confirmation of the presence of H. pylori by rapid urease test (RUT). The presence of H. pylori resulted in colour change from yellow to pink.

After confirmatory identification of H. pylori, identical colonies were carefully picked from the primary plates using a sterile loop, sub-cultured on fresh BHIA plates for maintenance and growth of strains for future work.

3.2.2 Preservation of H. pylori strains:
Young cultures of all the *H. pylori* strains used in the study were preserved for future use as glycerol stock. 2-3 loopfuls of 48-72 h old culture was picked using a sterile loop and transferred in a 1.5 ml of cryovial containing 1 ml of sterile BHI broth supplemented with 20 % glycerol, which was used as a cryopreservative, and then stored at −80 °C. For reviving of the cultures, the cryovial tube was taken out and quickly thawed on ice. A small portion of the culture was aseptically taken out using a sterile loop and streaked on the BHIA plates supplemented with antibiotics and incubated in a microaerophillic environment (5 % O₂, 10 % CO₂ and 85 % N₂) at 37 °C with 60-70 % relative humidity, for 2–4 days. Subsequent analysis was performed with strains derived from the frozen stocks.

### 3.2.3 Growth of *H. pylori* strains:

*H. pylori* strains were grown on brain heart infusion medium with agar supplemented with 5 % Horse serum (inactivated at 50 °C for 30 min), 0.4 % isovitalex (Becton Dickinson Microbiology Systems, USA), polymyxin B (10000 U/ml of water) (Sigma), trimethoprim (2.5 mg/ml of alcohol) (Sigma), and vancomycin (5 mg/ml of water) (Sigma). The strains were revived, cultured in duplicates and incubated in a microaerophillic environment with same incubation conditions as mentioned above.

### 3.2.4 Stock culture preparation and condition of the test strains:

Stock cultures of the test organisms (*B. cereus*, *B. subtilis*, *P. aeruginosa* and *S. aureus*) were maintained in Luria bertani (LB) broth supplemented with 50 % glycerol (cryoprotectant) stored at -80 °C. Subsequent analysis was performed with strains derived from the frozen stocks. Stock culture were prepared by thawing the test organisms at 4 °C and grown separately on LB agar plate on an orbital shaker at 37 °C for 24 h. Cultures were streaked out on LB agar media and incubated at 37 °C for 18-24 h. All cultures were maintained on LB agar media at 4 °C and transferred weekly to maintain viability. Purity of the cultures were checked at regular intervals as described by Acheampong *et al.*, (1988).

### 3.2.5 Extract preparation (Maceration):
Solvent used for extraction was on the basis of low to high polarity (hexane, dichloromethane and ethanol). Ten g of the dried plant material was crushed using mortar pestel and then, soaked in 40 ml of hexane. It was kept in a 250 ml sterile conical flask plugged with a cotton plug and placed in 37 °C incubator with continuous shaking at 120 rpm for 24 h. The content was filtered using Whatman filter paper No. 1 and the plant material was re-soaked in 40 ml of dichloromethane for next 24 h and later in 40 ml of ethanol for next 72 h. Each of the filtrates was concentrated to dryness, weight was taken, dissolved in 4 ml of respective solvents and then stored in a sterile glass vial at 4 °C until use. This method was adopted from Rangasamy et al., (2007) with slight modifications. The antimicrobial activity of all the three different plant extracts were checked and compared. Based on the antimicrobial activity result, finally experiments were performed with ethanol as the solvent.

3.2.6 Antibacterial activity of the crude extracts of plants against *H. pylori* and other pathogenic strains:

Kirby Bauer method was used for preliminary screening of plant extracts active against *H. pylori* strains (Bauer et al., 1966). For assay against *H. pylori*, bacterial suspension was adjusted to yield approximately 12X10⁸ cfu/ml, McFarland 4 turbidity standard (McNulty et al., 2002) plated with a sterile spreader on media plates (brain heart infusion agar media). Whatman paper disks (6 mm in diameter) were soaked with 50 µl (65 µg/µl) of the plant extract and placed on the bacterial inoculated plates. 70 % ethanol was used as a negative control and clarithromycin as positive control. The *H. pylori* plates were kept for the observation of results for 2 days at 37 °C under microaerophillic conditions (5 % O₂, 10 % CO₂ and 85 % N₂) and high humidity (60-70 %). Antimicrobial activity of the extract was determined by measuring the diameter of zone of inhibition in millimeter produced against the bacterial strains. The experiment was done thrice and the mean values were calculated.

The agar well diffusion method was used for the primary screening of the plant extracts against the pathogenic strains as described by Perez et al., (1990) with slight modifications. Standardized inoculums (100 µl) of 0.5 McFarland turbidity standard, that is, equivalent to 5X10⁸ cfu/ml (Lopez-Brea et al., 2008) of each test bacterial
strains was spread using a sterile glass spreader onto sterile LB solid media plates so as to achieve even growth. The plates were allowed to dry and then a sterile cork borer (8.0 mm diameter) was used to bore wells in the agar plates. The extracts 50 μl (65 μg/μl) were loaded in the wells and 70 % ethanol (50 μl/well) was taken as negative control. The plates were then incubated at 37 °C for 24 h. Antimicrobial activity of the extracts was determined by measuring the diameter of inhibition zone in millimeter produced against the pathogens. The experiment was done three times and the mean values were calculated.

3.2.7 Minimum inhibitory concentration (MIC) of the plant extracts against *H. pylori* strains:

Based on the initial screening, the ethanolic extracts that were identified to have good antibacterial activity were selected for MIC. The MIC was determined against the *H. pylori* strains 26695, SS1, 154(1A), 225(1A) and 216(1A). Stock solutions of the crude extracts were prepared and each disc was loaded with different concentrations of the extract. The MIC was determined as the lowest concentration of the extract inhibiting visible growth of the bacterium on the agar plate. The experiment was repeated thrice.

3.2.8 Phytochemical estimation in the plant extracts:

The crude plant extracts were checked for the presence of phytochemicals- phenolics and flavonoids were estimated with respect to the standards.

3.2.8.1 Determination of total phenolic content:

Total phenolic content was determined by the Folin–Ciocalteu method. 150 μl of appropriately diluted extract solution was added to 2400 μl of distilled water and 150 μl of 0.25 N Folin-Ciocalteu reagent. The mixture was made to react for 3 min; 300 μl of 1 N Na₂CO₃ solution was then added and mixed well following incubation in dark for 2 h at room temperature. Using a spectrophotometer, the absorbance was measured at 725 nm. The results were expressed in mg of gallic acid equivalents per g of dry weight of extract since, gallic acid was used as a standard compound for the quantification of total phenolic content (Thaipong et al., 2006).

3.2.8.2 Determination of total flavonoid content:
1 ml aliquot of appropriately diluted extract solution was added with 2 ml of distilled water and 0.15 ml of a 5 % NaNO₂ solution. After 6 min incubation at room temperature, 0.15 ml of 10 % AlCl₃ solution was added and left for 6 min, then 2 ml of 4% NaOH solution was added to the obtained mixture. The mixture was mixed well and allowed to again stand for another 15 min. Absorbance of the mixture was measured at 510 nm using a spectrophotometer. Rutin was used as standard compound for the quantification of total flavonoid content and thus, the result was expressed as mg of rutin equivalents per g of dry weight of extract (Yanping et al., 2004).

3.2.9 Antioxidant activity estimation of the plant extracts:

The antioxidant activity in the crude plant extracts were determined by the estimation of the total reducing power content, antioxidant activity by FRAP assay, DPPH and ABTS assay.

3.2.9.1 Determination of total reducing power content:

1 ml of the diluted extract solution was added to 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. They were incubated at 50 °C for 20 min. 2.5 ml of 10 % trichloroacetic acid was then added, and given centrifugation at 3000 rpm for 10 min. 2.5 ml of the upper layer was taken and added with 2.5 ml of distilled water and 0.5 ml of 0.1 % FeCl₃. The mixture was thoroughly mixed. Using a spectrophotometer, the absorbance of the mixture was measured at 700 nm. Since, vitamin-C was used as standard compound for the quantification of total reducing power, the results were expressed as mg of vitamin-C equivalents per g of dry weight of extract (Yanping et al., 2004).

3.2.9.2 Determination of antioxidant activity by FRAP assay (ferric reducing antioxidant power):

The stock solution of the FRAP assay included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl₃.6H₂O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml FeCl₃.6H₂O solution. The mixture was shortly warmed at 37 °C before using. 150 µl of the diluted extract solution was made to react with 2850 µl of the FRAP solution for 30 min in dark condition. The
coloured product (ferrous tripyridyltriazine complex) was obtained and the absorbance was then measured at 593 nm. Since, trolox was used as a standard compound for the quantification of the antioxidant power by FRAP assay, the results were expressed in mg of trolox equivalents per g of dry weight of extract (Benzie et al., 1996).

3.2.9.3 Determination of antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay:

DPPH assay stock solution was prepared by adding 24 mg of DPPH to 100 ml methanol and then stored at -20 °C until needed. The working solution was made by mixing 10 ml stock solution with 45 ml methanol to obtain an absorbance of 1.1 units at 515 nm. 150 µl of the extract was added to 2850 µl of the DPPH solution and allowed to react for 2 h in the dark. The absorbance was taken spectrophotometrically at 515 nm. Since, trolox was used as a standard compound for the quantification of the antioxidant power by DPPH assay, the final results were evaluated in mg of trolox equivalents per g of dry weight of extract (Thaipong et al., 2006).

3.2.9.4 Determination of antioxidant activity by ABTS (2,2’-azino-bis(3-ethyl benzo thiazoline-6-sulphonic acid) assay:

The stock solution was made by adding 7.4 mM ABTS and 2.6 mM potassium persulfate solution. The working solution was made by mixing the two stock solutions equally leaving them to react for 12 h at room temperature in dark. The solution was diluted then by adding 1 ml ABTS+ solution and 60 ml of methanol to reach an absorbance of 1.1 units at 734 nm. 150 µl of the diluted extract was added to 2850 µl of the ABTS+ solution for 2 h in a dark condition and made to react. The absorbance was measured at 734 nm using spectrophotometer. Since, trolox was used as standard compound for the quantification of the antioxidant power by ABTS assay, the results were evaluated in mg of trolox equivalents per g of dry weight of extract (Re et al., 1999).

3.2.10 Stability of plant extracts with antimicrobial activity at different temperature and pH:
3.2.10.1 Effect of temperature on the stability of the plant extracts with antimicrobial activity:

Plants extracts tested were *A. indica* leaves, *A. vera* leaves, *R. indica* (petals and leaves), *E. officinalis* pulp, *C. sinensis assamica* leaves, *S. aromaticum* buds and *D. metel* (fruit, bud, leave and flower) extracts. They were given temperature treatment at 4 °C, 25 °C, 60 °C and 100 °C for an hour in a heating block. Bioactivity of the treated plant extracts was checked by agar cup and disc diffusion assay. A control tube was also included in the experiment which contained untreated plant extract. Results were compared with the control and zone of inhibition was measured in millimeter.

3.2.10.2 Effect of pH on the stability of the plant extracts with antimicrobial activity:

Plants extracts tested were *A. indica* leaves, *A. vera* leaves, *E. officinalis* pulp, *C. sinensis assamica* leaves and *S. aromaticum* buds. The plant extract separated by TLC (thin layer chromatography) strips were treated separately with 100 µl of the citrate buffers of pH 2, pH 7 and pH 8 for an hour and one untreated TLC strip (control) was also included in the experiment. The pH stability of the treated plant extracts was compared with the control by contact bioautography.

3.2.11 Chemical characterization:

3.2.11.1 Separation of compounds by thin layer chromatography and identification of the bioactive spots by contact bioautography:

The plant extracts 50 µl (65 µg/µl) was spotted on precoated silica gel 60 F$_{254}$ sheets (Merck, Darmstadt, Germany). Toluene: chloroform: acetone (40: 25: 35) was taken for 1-dimensional run and methanol: formic acid (1:1) was taken for 2-dimensional run. After drying, for contact bioautography, the chromatogram was placed with face down onto the inoculated agar plate to enable diffusion and the agar plate was incubated at 37 °C for 2 days under microaerophilic conditions (5 % O$_2$, 10 % CO$_2$ and 85 % N$_2$) and high humidity (60-70 %) for observation of the bioactive spots against *H. pylori* strains and the TLC plate was separately bioassayed against the pathogenic strains and was incubated at 37 °C for 24 h for the observation of results. Rf values of the bioactive spots were measured.
3.2.11.2 Chemical characterization of the bioactive TLC spots:

Chemical characterization of the bioactive spots was performed to determine the chemical nature as mentioned by Fiedler, (1993). After running and drying of the TLC sheets, they were sprayed with different chemical sprays. Phosphomolybdic acid reagent was used for the presence of essential oils, dragendorff reagent was used for the presence of alkaloids and 3 % FeCl₃ was used for the presence of tannins / phenolics. The sheets were observed in the visible wavelength for the colour change and the observation of the results.

Table 3.2: Spray reagents for study of chemical nature of bioactive spots

<table>
<thead>
<tr>
<th>Spray Reagent</th>
<th>Detection of</th>
<th>Wavelength</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphomolybdic acid</td>
<td>Essential oils</td>
<td>Visible</td>
<td>Blue</td>
</tr>
<tr>
<td>Dragendorff reagent</td>
<td>Alkaloids</td>
<td>Visible</td>
<td>Orange</td>
</tr>
<tr>
<td>Ferric Chloride (3 %)</td>
<td>Tannins/Phenolics</td>
<td>Visible</td>
<td>Red brown zones/ Dark zones</td>
</tr>
</tbody>
</table>

3.2.11.3 Elution of the bioactive spots:

The bioactive spots after TLC run and contact bioautography were scrapped and eluted in ethanol by keeping on the rocker for 24 h at 45 movements per min. The samples were filtered using Whatmann filter paper No.1 and stored in sterile glassvials at 4 °C until use. For bioactivity, disc diffusion assay was done in which the eluted bioactive spots (50 µl) were loaded on sterile filter paper disc and checked against the *H. pylori* strains (26695, J99 and SS1). Plant extracts tested were *R. indica* (petals and leaves) and *D. metel* (fruit, bud, leaves and flower).

3.2.12 Isolation of bioactive molecules with anti-*H. pylori* activity from *Datura metel* leaves extract by column chromatography:
The *D. metel* leaves extract was run in silica column to isolate the bioactive compound from it. The glass column was washed with acetone and allowed to dry completely. Silica gel (80 g) 100-200 mesh size was activated for an hour at 120 °C and soaked in hexane (250 ml) overnight at room temperature. The glass column was packed by pouring the slurry (activated silica gel and hexane) and then, allowed the column to stand overnight. 500 g of the crude plant extract was prepared in 2 litres of hexane. The plant extract after concentration was dissolved in 25 ml of hexane and added slowly to the packed column for the run. The column was run in various solvent systems using hexane, ethyl acetate and methanol. A gradient of non-polar to polar solvents was prepared by mixing them in different proportions. 100 ml of the respective solvents were used for the run and 100 ml of 10 fractions were collected from each solvent system. Total 10 solvent systems were run and 100 fractions were collected.

The collected fractions (1-100) were checked for similar profile by running them in TLC with 5 % methanol in chloroform. The isolated column fractions were also checked for their bioactivity against the *H. pylori* strain SS1 by disc diffusion assay in which 50 µl (16 µg/µl) of the isolated fraction was loaded on a disc of 6 millimeter diameter.

3.2.13 Estimation of phytochemicals and antioxidant activity in the isolated fraction of *Datura metel*:

The isolated fraction no.18 was checked for the phytochemicals and antioxidant activity by the method as described in 3.2.8 and 3.2.9.

3.2.14 Identification and purification of the isolated bioactive fraction by HPLC (High performance liquid chromatography):

HPLC was performed in a C-18 reverse phase column (Varian A6000250X046). 100 µl (16 µg/µl) of column purified fraction was evaporated to dryness and then suspended in mobile phase methanol with 5 % acetic acid (27:73). The flow rate was adjusted to 0.200 ml/min. The sample was loaded. Purified fractions were collected (100 µl). The fractions collected showed absorbance (mAU). The retention time of the collected fractions were noted. The collected fractions were completely dried in
laminar air flow and finally reconstituted in 50 µl of ethanol and checked for antimicrobial activity against *H. pylori*.

### 3.2.15 Scanning electron microscopic (SEM) study of the *H. pylori* cells treated with the isolated bioactive fraction:

The *H. pylori* SS1 strain was grown till OD (optical density) 0.8 in brain heart infusion liquid broth. The culture (2 ml) was transferred in two autoclaved test tubes. One being the control with 30 µl of 70 % ethanol and the other being the sample treated with 30 µl of the isolated fraction no.18 for 2 h at 37 °C in microaerophillic incubator. After treatment, the content was transferred in a microfuge tube and centrifuged at 6000 rpm for 10 min at 4 °C. The supernatant was discarded; pellet formed was washed with phosphate buffered saline (PBS) thrice. It was resuspended in 0.1 M sodium phosphate buffer (900 µl) and 25 % glutaraldehyde (100 µl). The sample was incubated at 4 °C overnight. After 24 h, it was centrifuged again, the supernatant was discarded and 1 ml PBS was added. The sample was dehydrated serially with 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % and 100 % ethanol and in between centrifuged at 6000 rpm for 10 min. Finally 200 µl of 100 % ethanol was added and the sample was analyzed by SEM and compared with the control.

### 3.2.16 Characterization of the isolated bioactive compound by HPTLC (High performance thin layer chromatography):

The isolated bioactive compound was characterized using HPTLC. Standards like, hisperidine (0.5 mg/ml) and quercetin (1 mg/ml) were used in this study.

HPTLC was done on 20 cm X 10 cm precoated silica gel 60 F_{254} (Merck, Darmstadt, Germany) TLC sheets. Samples were applied as 4 mm width bands using Camag 100 µl sample syringe (Hamilton, Switzerland) with a Camag Linomat 5 applicator (Camag, Switzerland). For the calibration curve, 20 µl of standard solution was applied.

The application rate of 150 nl/s was used which was constant. Linear ascending development with mobile phase chloroform: ethyl acetate: formic acid= 7.5: 6: 0.5 for quercetin and ethyl acetate: methanol: water = 10: 1.7: 1.3 for hisperidine was
carried out in a twin trough glass chamber (Camag) (20 x 10 cm) which was saturated with the mobile phase vapour for 20 min at room temperature (25 °C). After development, the plates were air dried. The scanning was then done using Camag TLC scanner 3 at their respective wavelength in the absorbance mode and operated by winCATS software (version 1.4.1). The source of radiation during HPTLC procedure was a deuterium lamp emitting a continuous UV spectrum in the range 190–400 nm. The slit dimensions were 3X0.45 mm and the scanning speed was 100 mm/s.