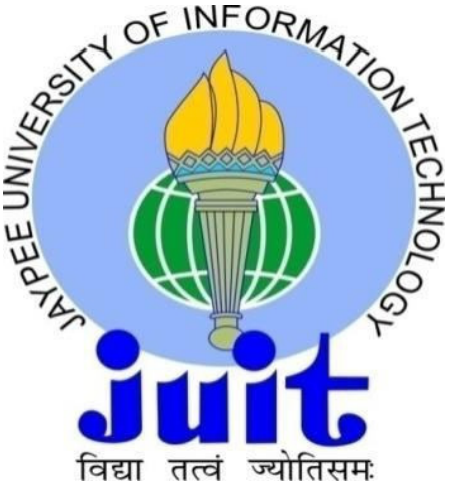
ANTI-ULCER POTENTIAL OF STANDARDIZED EXTRACT OF *PAEDERIA FOETIDA* Linn. : A TRADITIONAL MEDICINAL PLANT USED BY THE TRIBALS OF NORTHEAST INDIA

Thesis submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

by

SILPI CHANDA



Department of Pharmacy

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, SOLAN-173234, HIMACHAL PRADESH, INDIA

October 2014

Dedicated

• • • *To My Parents & Family*

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Anti-ulcer Potential of Standardized Extract of *Paederia foetida* Linn.: A Traditional Medicinal Plant Used by the Tribals of Northeast India"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Kuldeep Singh & Dr. Sayeed Ahmad**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

(Signature of the Scholar)

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Date: 26th September, 2014

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Anti-ulcer Potential of Standardized Extract of *Paederia foetida* Linn.: A Traditional Medicinal Plant Used by the Tribals of Northeast India"** submitted by **Silpi Chanda** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Acknowledgement

"Though gratification comes deep from the heart, if left unexpressed losses its memory, charm, and above all the biggest asset, its beauty"

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affection of my husband & in- laws and bhaiya-bhavi and it is to them I dedicated this thesis. The real source of inspiration comes due to their blessings only without which it would have been impossible for me to complete this study.
Last in the list but first in my heart I bend my head to almighty God. The feelings of his

continued presence surrounding me help to lead the life and showing me the path of life.

SILPI CHANDA

ABSTRACT

Development of management therapy for peptic ulcer disease (PUD) is one of the thrust area of research to the scientist. It is becoming the most important therapeutic areas to study due to its high mortality and morbidity. India rank 5th with a death rate of 12.37/lakh population where as in United States the direct/indirect treatment cost of PUD was estimated more than US$3.4 billion annually. As PUD is a multifactorial disease so there are several issues that need to be solved. The major issues remain to resolved immediately are the optimal way to eradicate *H. pylori*, solution of antibiotic resistance development, prevent ulcer recurrence in NSAIDs patients, minimizing the concomitant use of medicine etc. Due to the existing side effect/limitations of currently available first line treatment of PUD, Food and drug administration release safety alert regarding the long term use of the PPIs (omeprazole, lansoprazole, pantoprazole etc.) and H2 blocker (ranitidine, famotidine).

To resolve such crucial problem we focused our study to nature substances. According to WHO 80% of people rely on traditional medicine for their primary health care. Focusing on this we have done ethnobotanical field survey among the tribal community of Tripura to explore the richness of traditional claim.

Paederia foetida L. is a popular edible plant of tribes of Northeast India. Upon analyzing the survey outcome, *P. foetida* considered as a one of the potent plant having therapeutical interest related to PUD. The plant material (leaf) was collected from the tribal dominated market of Agartala, Tripura. Authentication of the plant was done from NISCAIR, New Delhi. Soxhlet methanolic extraction was yielded 36.21%w/w of extractive value.

The phytochemical analysis of the extract confirmed the presence of several important secondary metabolites like volatile oil, sterols, glycoside, alkaloid *etc. Qualitative estimation of* β-sitosterol was done by the HPTLC. Chemometric profiling of the leaf and stem hexane extract and hydrodistilled leaf oil were

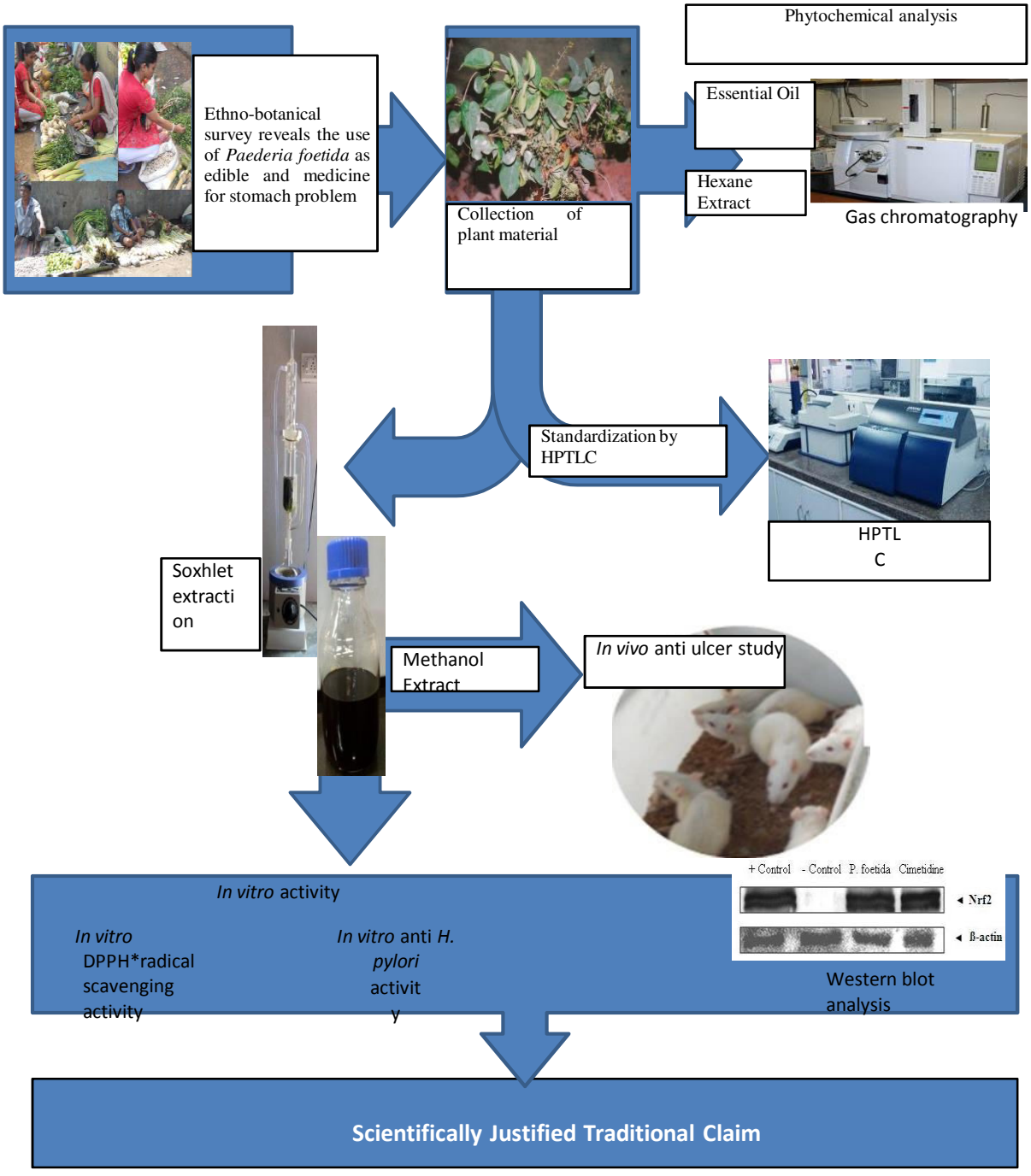
done by GCMS analysis. A total of 57 compounds were identified from the plant. Out of 57 compounds, 54 compounds were first time reported by our study.

The present work also described the development and validation of a new simple, rapid and reliable HPTLC method for asperuloside and paederoside in *P. foetida*. As the validation requirements vary according to the type of test and the technique employed. This section contains the text on analytical validation adopted by the ICH 1994. Quantitative estimation revealed 0.12%w/w asperuloside and 0.097%w/w paederoside present in the *P. foetida* extract.

The anti oxidant activity of the fresh and dried methanol extract was assessed by DPPH* radical scavenging activity. The IC_{50} values were found to be 40µg/ml fresh methanolic extract and 43µg/ml for dried methanolic extract.

In-vitro anti *H. pylori* activity of the methanol extract was studied on eight different clinical strains of *H. pylori*. The plant extract at several concentrations (25-3000 µg/ml) showed minimum 4mm zone of inhibition to maximum 11 mm of zone of inhibition.

The standardized methanol extract was screened for *in-vivo* anti ulcer activity by using four different animal models. In all model, the extract showed significant result (***P<0.001). *In-vivo* result was further supported by the western blot analysis of pylorus ligatured rats. *In-vitro* and *in-vivo* study revealed the ulcer protective activity of the *P. foetida* extract which may be obtained mainly due to the Nrf2 expression mediated anti oxidant and anti secretory effects.



Overview of the study

LIST OF ABBREVIATIONS

ABTS	*2,2′*-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
ARE	Anti oxidant response element
AQC	Analytical quality control
$BaCl_2$	Barium chloride
BHI	Brain heart infusion
Bw	Body weight
cAMP	Cyclic adenosine monophosphate
CDAD	Clostridium difficile associated diarrhea
cGMP	Cyclic guanosine monophosphate
$CHCl_3$	Chloroform
CFU	Colony forming unit
C_6H_6	Benzene
CO_2	Carbon di Oxide
$CuSO_4$	Copper sulphate
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-picryl-hydrazyl
ECL	Enterochromaffin like cells
ECD	Electron capture detector
EGF	Epidermal growth factor
EI	Electronic ionization
EU	Europe
eV	Electron volt
FDA	Food and drug administration
$FeCl_3$	Ferric chloride
GCMS	Gass chromatography and mass spectroscopy
GERD	Gastro-esophageal reflux disease
GIT	Gastrointestinal tract
GMPs	Good manufacturing practices

H. pylori	*Helicobacter pylori*
HCl	Hydrochloric acid
HPTLC	High performance thin layer chromatography
HPLC	High performance liquid chromatography
$HCO3^-$	Bicarbonate
H_2O	Water
Hr	Hour
H_2SO_4	Sulphuric acid
H2	Histamine
IBSD	Institute of bioresources and sustainable development
ICH	International conference on harmonization
IC_{50}	Half maximal (50%) inhibitory concentration
K	Potassium
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methaol
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
m1/m2	Mid region of Vac A gene
MPO	Myeloperoxidase
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NE	Northeast
NERD	Non-erosive reflux disease
NIST	National institute of standards and technology
N_2	Nitrogen
NF-*ţB*	Nuclear factor-kappa B
NISCAIR	National institute of science communication

NSAIDs	Nonsteroidal anti-inflammatory drugs
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2 related factor 2
O_2	Oxygen
OD	Optical density
OTC	Over the counter
PAF	Platelet activating factor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKG	Protein kinase G
PF	Paederia foetida
PGE_2	Prostaglandin E_2
p.o	Per oral
PPIs	Proton pump inhibitors
PUD	Peptic ulcer disease
R&D	Research and development
R_f	Retention factor
RP	Reverse Phase
Rpm	Rotation per minute
RSD	Relative standard deviation
s1/s2	Signal region of Vac A gene
Sc	Symbol colony
SC	Subcutaneous
SD	Standard deviation
SDSPAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error mean
SNI	Spared nerve injury
Temp	Temperature

TLC	Thin layer chromatography
UAN	Uric acid nephropathy
USP	United states of pharmacopoeia
UV	Ultra violet
WHO	World health organization

LIST OF SYMBOLS

ppm	Part per million
ʒ	Lamda
mM	Milli molar
w/v	Weight/volume
nm	Nanometer
µm	Micrometer
mm	Millimeter
ng	Nanogram
µg	Microgram
mg	Milligram
G	Gram
Kg	Kilogram
µl	Microliter
ml	Milliliter
L	Liter
min	Minute
% w	Percent
/w	Weight/weight
cm	Centimeter
°C	Degree centigrade
µM	Micro meter
M	Meter
nL/s	Nano litre per second
<	Less than
M	Male
Eq	Equivalent

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO PEPTIC ULCER DISEASE

Our gastrointestinal tract (GIT) is responsible to digest our food, absorb nutrients and excrete unabsorbed waste product. It is the organ that provides a continuous supply of water, electrolyte and nutrients to the whole part of the body. Peptic ulcer disease or PUD is a pathological condition of this organ and refers to the painful spot characterized by the presence of ulcer in any part of GIT which exposed to acid and pepsin. Mostly the organ stomach (gastric ulcer; Figure 1.1) and duodenum (duodenal ulcer; Figure 1.2) are affected by this disease. Benign ulcerative lesion of the stomach and duodenum are collectively called as peptic ulcer disease. The word ulcer derived from the Latin term *'ulcus'* which means painful spot, whereas the word peptic is derived from the Greek word „*peptikos*" means digestion [1]. PUD is formed due to the imbalance between the aggressive factors (gastric acid, gastrin, pepsin, *Helicobacter pylori*, NSAIDs, alcohol, bile, stress, etc.) and defensive factors (mucus, mucosal blood flow, prostaglandin, NO, $HCO3^-$ secretion etc.). PUD represents a worldwide health problem due to its high morbidity, mortality and economical factor.

Figure 1.1: Gastric ulcer

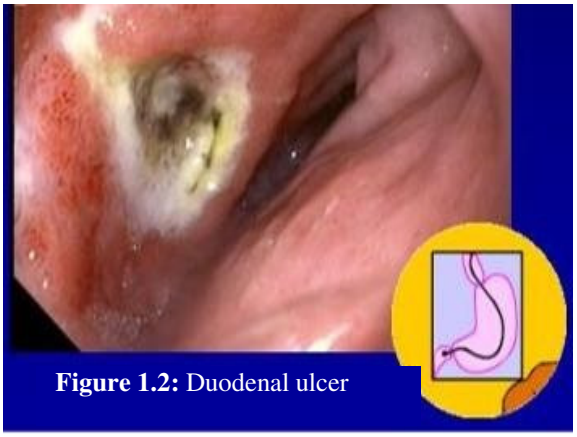


Figure 1.2: Duodenal ulcer

To fully understand the physiology of GIT and its pathological condition, it is necessary to acquire some knowledge on the following important headings given below:-

1.1.1 Layers and region of stomach

The stomach is divided into a total of five regions namely cardia, fundus, body, antrum and pylorus (Figure 1.3). Our stomach is made up of about four distinct layers of tissue (Figure 1.4). From the inner side to the outer side of the stomach these are mucosa, submucosa, muscularis and serosa. Mucosa is the inner lining of the stomach, which produces mucus. The injury or erosion to this layer leads to the development of the PUD. The adherent mucus layer provides a defensive barrier against self-digestion of stomach by gastric acid and pepsin [2,3]. The layer submucosa made up of connective tissue and produce digestive hormones where as the layer muscularis and serosa are the muscle and fibrous membrane respectively.

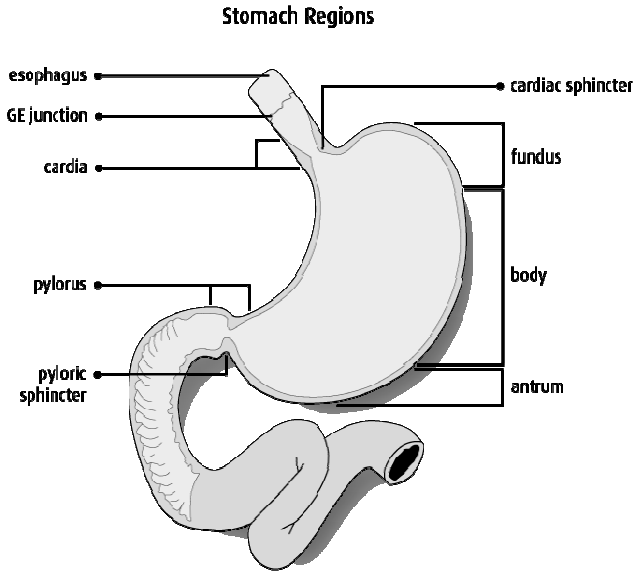


Figure 1.3: Different parts of stomach

1.1.2 Cells of stomach lumen

The stomach lumen consists of several different specialized cells from which various substances are released (Table 1.1). These secretory substances are taking part either in the pathogenesis of the disease or in the management of disease.

Table 1.1: Secretory substance of stomach

Cell types	Substance secreted
Mucus neck cell	Mucus (protects lining) Bicarbonate
Parietal cells	Gastric acid (HCl) Intrinsic factor (Ca^{++}, Vit B12 absorption)
Enterochromaffin like cells (ECL)	Histamine (stimulate acid secretion)
Chief cells	Pepsinogen (Pepsin) Gastric lipase
D cells	Somatostatin (inhibits acids)
G cells	Gastrin (stimulate acid secretion)

1.1.3 Gastric acid secretion and regulation

There are three phases of gastric acid secretion, the cephalic phase, gastric phase, and intestinal phase (Figure 1.5-1.8). In the epithelium, gastric glands are present, which secretes acid. During each phase, the secretion of gastric juice can be stimulated or inhibited.

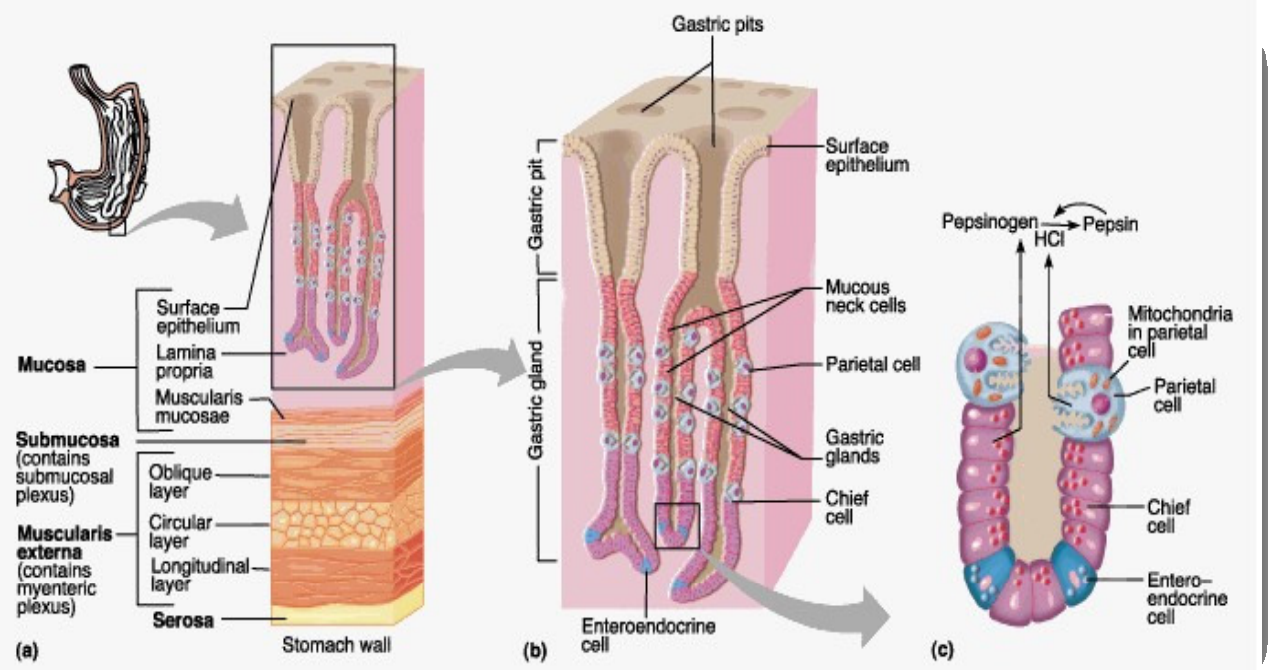


Figure1.4: Layers and cells of stomach and their secretory substances (Copyright ©2001 Benjamin Cummings, an imprint of Addison Wesley Longman, Inc.) In the epithelium, gastric pits lead to gastric glands that secrete gastric juice. (One gastric glands is enlarged & shown on the right) (a) Different stomach layers; (b) Mucus layer of stomach; (c) Gastric gland

1.1.3.1 Cephalic phase: When we see any food the sensory stimulation trigger the parasympathetic out load via the glossopharyngeal nerve stimulation, i.e. secretion of saliva and vagus nerve stimulation, which initiate gastric and pancreatic secretion.

1.1.3.2 Gastric phase: It starts when food enters to stomach and provide chemical and

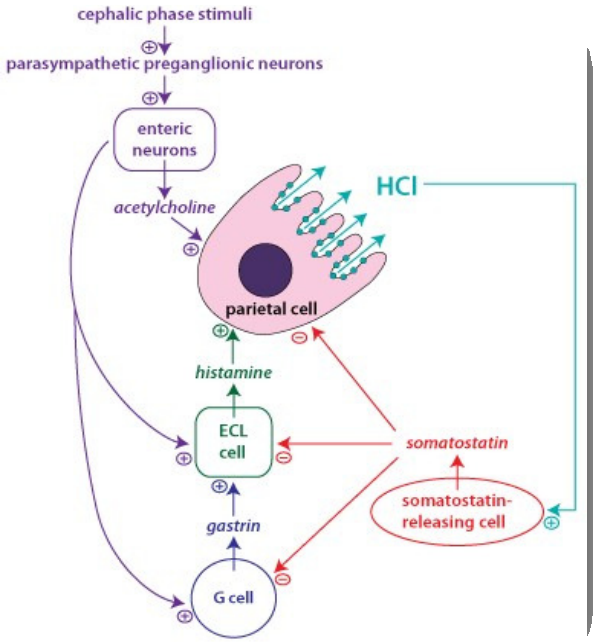


Figure 1.5: Cephalic phase [5]

mechanical stimulation by the distention in the stomach lumen which initiates enteric neuron

to release acetylcholine. Acetylcholine act on parietal cell to release hydrochloric acid (purple color). In gastric phase, there is a stimulation of stress receptor proteins by the smaller peptides and amino acids. Amino acids stimulate G cell to secrete gastrin which act on ECL (enterochromafin like cell) to release histamine and further activate parietal cell to release acid (blue color) [4]. Presence of food in the stomach also increases pH, which prevents the stimulation of somatostatin (Green color).

1.1.3.3 Intestinal phase:

Chemical stimuli present in the duodenum initiate this phase. The presence of proton, high osmolarity, nutrient in intestinal lumen, fats in duodenum stimulate three regulatory pathways namely neural, hormonal and paracrine which in turn increases pancreatic secretion and decreases gastric secretion.

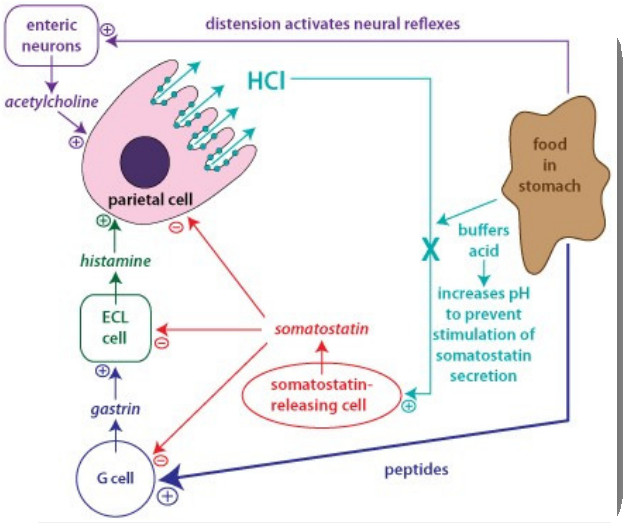
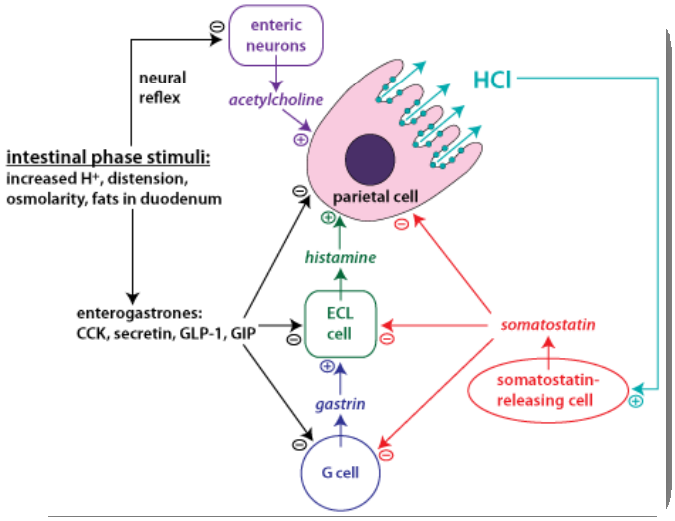


Figure 1.6: Gastric phase [5]



ure 1.7: Intestinal phase [5]

1.1.3.4 Cellular mechanism:

Acetylcholine, gastrin and histamine are the three agonists for the physiology of gastric acid secretion and our parietal cells are having three different receptors for these three agonists for e.g. muscarnic receptor (M3) for acetylcholine, cholecystokine receptor for gastrin and histamine receptor for histamine. Acetylcholine is released by cholinergic terminals, whereas gastrin from G cell and histamine from ECL. Both acetylcholine and gastrin activate second messenger to release calcium ions. Elevated cAMP level and intracellular

calcium, enhance hydrochloric acid secretion by activating Na^+/K^+ pump, H^+/K^+ ATPase and chloride channels in apical membrane. Acetylcholine is having both the excitatory effect on G cell and inhibitory effect on D cell. Gastrin increases the secretion of somatostatin, which release from the D cell act as a regulatory molecule that inhibits acid secretion. It reduced the cAMP level, therefore, inhibit acid release. On the other hand somatostatin also blocks gastrin receptor to release gastrin as a result no further acid secretion.

1.1.4 Pathogenesis of peptic ulcer disease

There are several factors that are responsible for the pathogenesis of PUD (Figure 1.9).The factors may be environmental, microbial, pharmacological, psychological, genetical etc. [6-9]. The risk factors for PUD are *Helicobacter pylori* infection, use of non-steroidal anti-inflammatory drugs, stress, smoking, alcohol etc. These factors are either increase gastric acid secretion or impaired mucosal barrier protection. Brief mechanisms of individual risk factors involved in the pathogenesis of PUD are described in figure no.1.10 – 1.14.

1.1.5 Symptoms of PUD

- Burning pain in the middle or upper stomach
- Bloating
- Dyspepsia
- Heartburn
- Coffee ground emesis
- Nausea or vomiting
- Vomiting blood
- Melena
- Weight loss

1.1.6 Diagnosis of PUD

- Test for *H. pylori*
- Endoscopy
- X-ray
- Biopsy

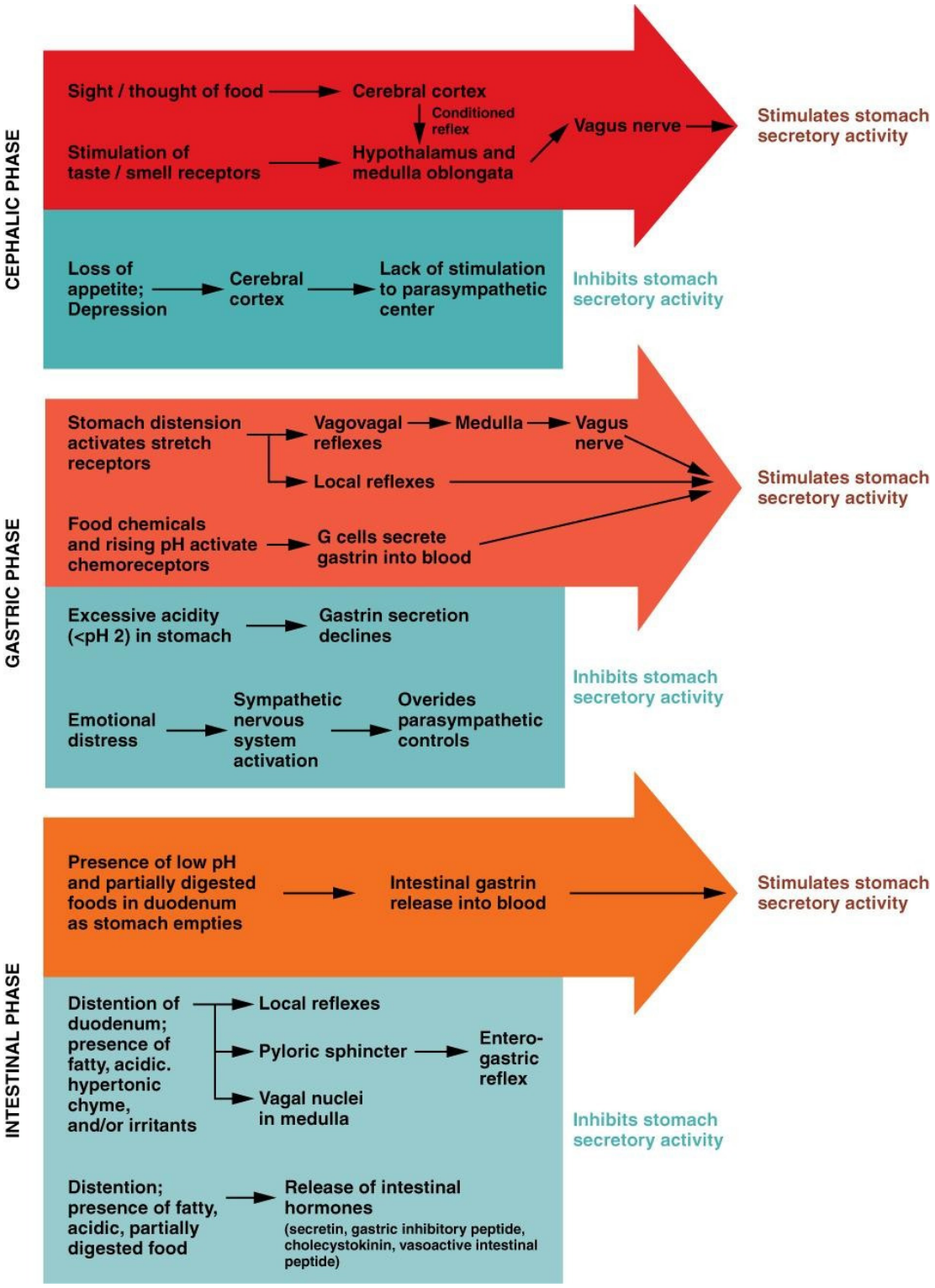


Figure 1.8: Phases of gastric secretion. During each phase, the secretion of gastric juice can be stimulated or inhibited [10].

1.1.7 Treatment available

In the later stage of the 20^{th} century, PUD was treated with antacid, anti secretory agents, proton pump inhibitors and antibiotics. But in the 21^{st} century, when the role of stress and diet in the pathogenesis of PUD is understood, there is a revolution in the treatment of PUD with hospitalization, bed rest and proper diets. By the 1950s, antacid therapy had become the treatment of choice for PUD with 80% of respondents in duodenal ulcer healing after 4 weeks of therapy. The introduction of the histamine (H2) receptor antagonist (cimetidine) in 1977 became choice of treatment with a good ulcer healing rate (80% to 95%), after 6 to 8 weeks of therapy. The other H2 blockers available in the market are Ranitidine, Famotidine, Nizatidine. Probably the chord of success in ulcer healing was achieved with the availability of proton pump inhibitors (PPIs) (Omeprazole, Lansoprazole, Pantoprazole, Rabeprazole, Lansoprazole, Dex Lansoprazole) in the 1980s. The PPIs decrease gastric acid secretion through inhibition of H^+/K^+-ATPase, the proton pump of the parietal cell. In the 20th century PUD was considered as a chronic, incurable disorder due to its recurrences on discontinuation of treatment. There was another fruitful success in the management of PUD after knowing the relationship between *H. pylori* and PUD by Marshall and Warren in the year 1982. Antimicrobials available for the treatment of *H. pylori* are Clarithromycin, Metronidazole, Amoxicillin, Tetracycline etc. Apart from the above described treatment, mucous protective agents (sucralfate, Bismuth salts), prostaglandin analogs are also available.

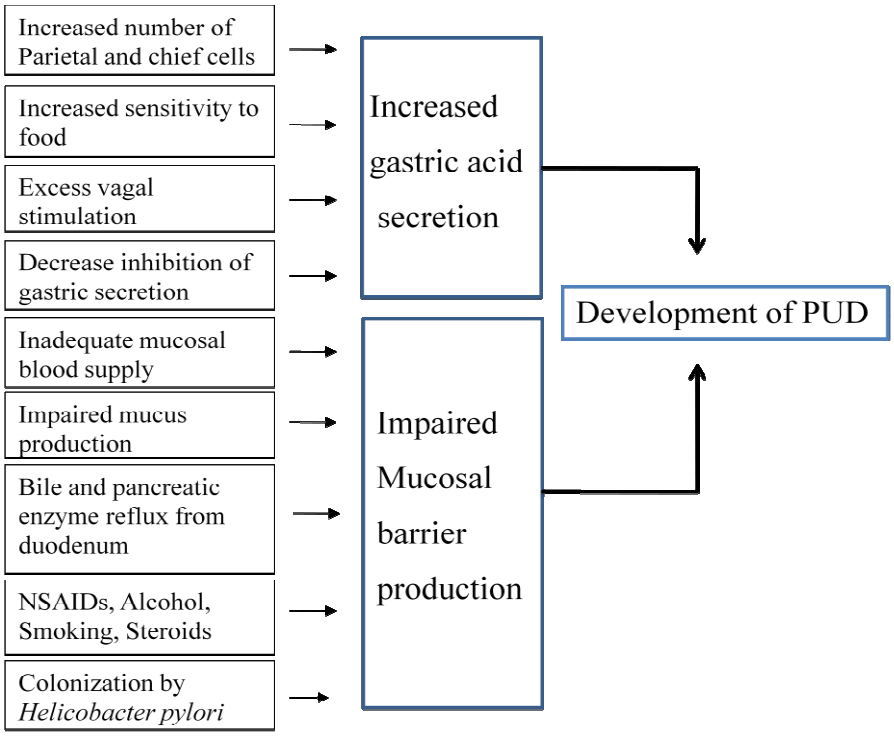


Figure 1.9: Pathogenesis of Peptic Ulcer Disease

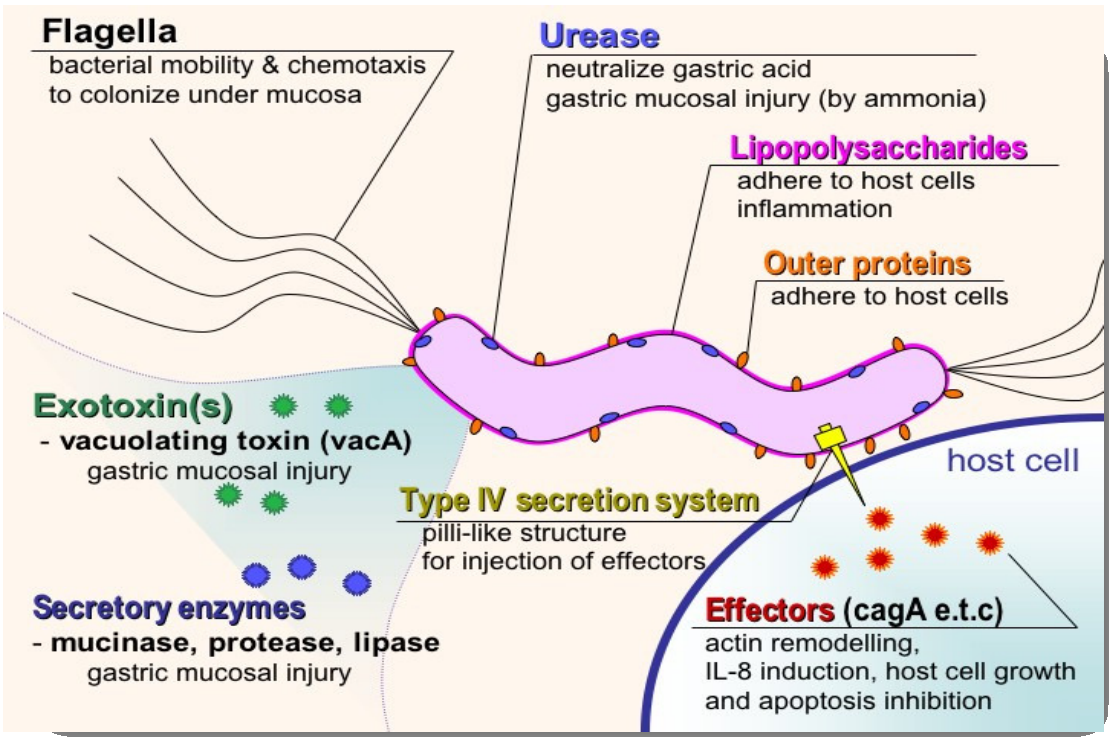


Figure 1.10: Pathogenesis of PUD due to the attack of *H. pylori*

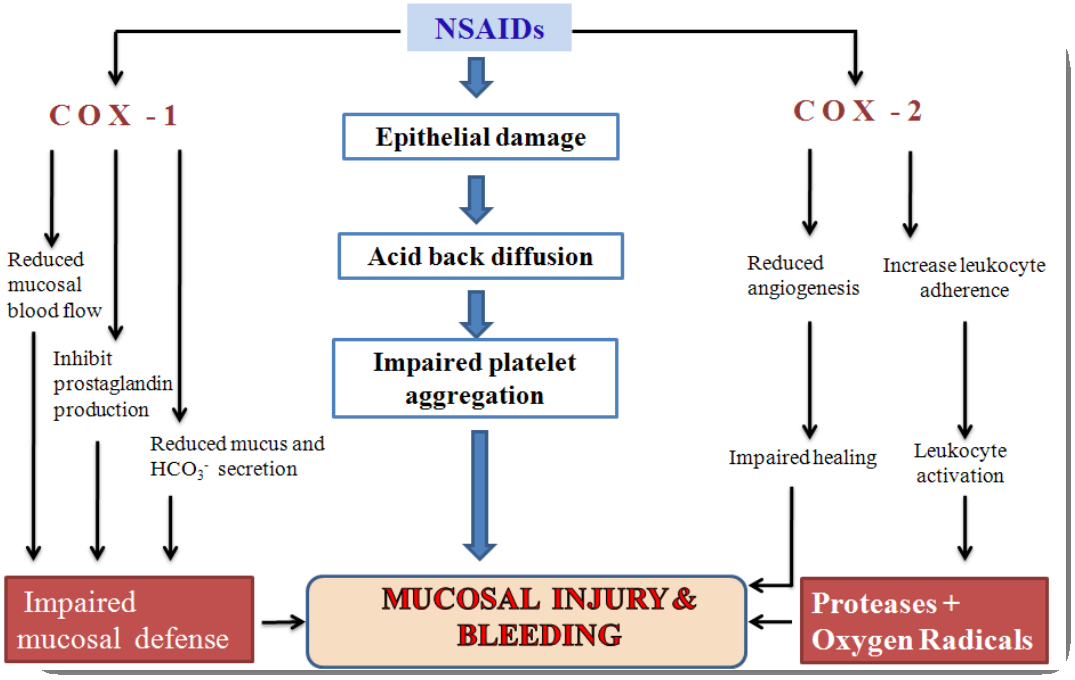


Figure 1.11: Mechanism involved in pathogenesis of PUD by NSAIDs

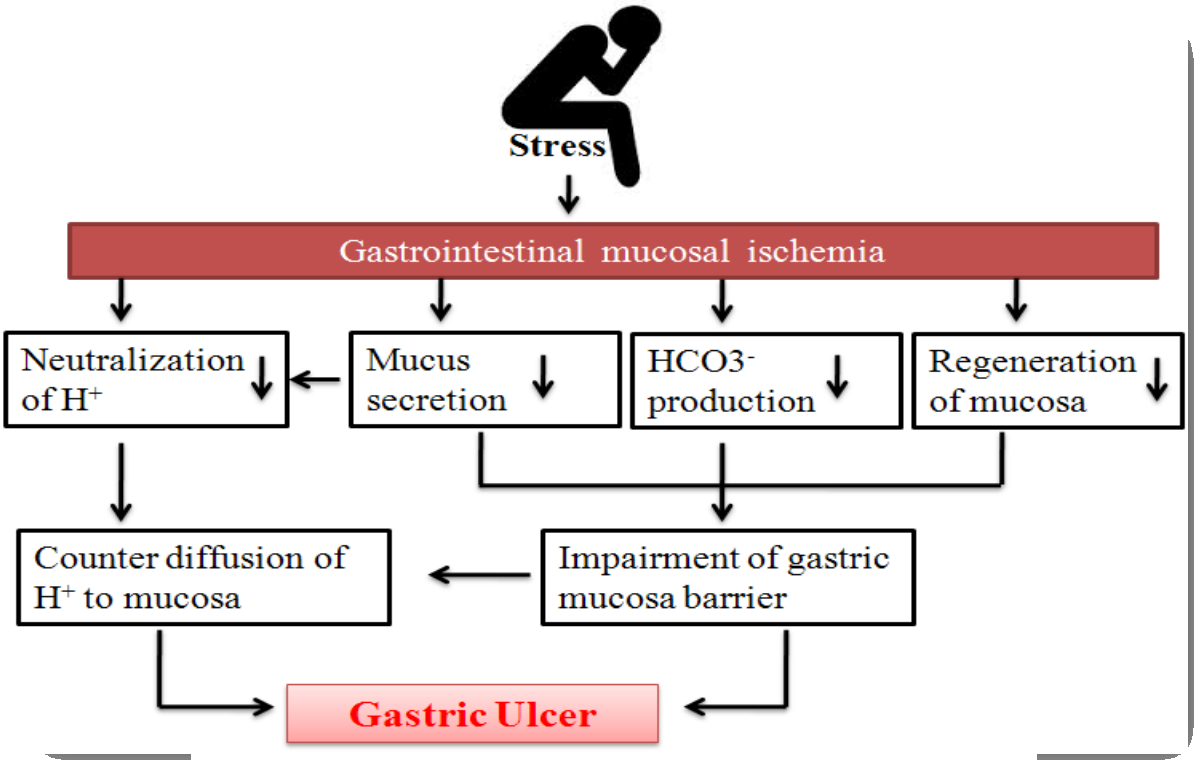


Figure 1.12: Mechanism involved in stress ulceration

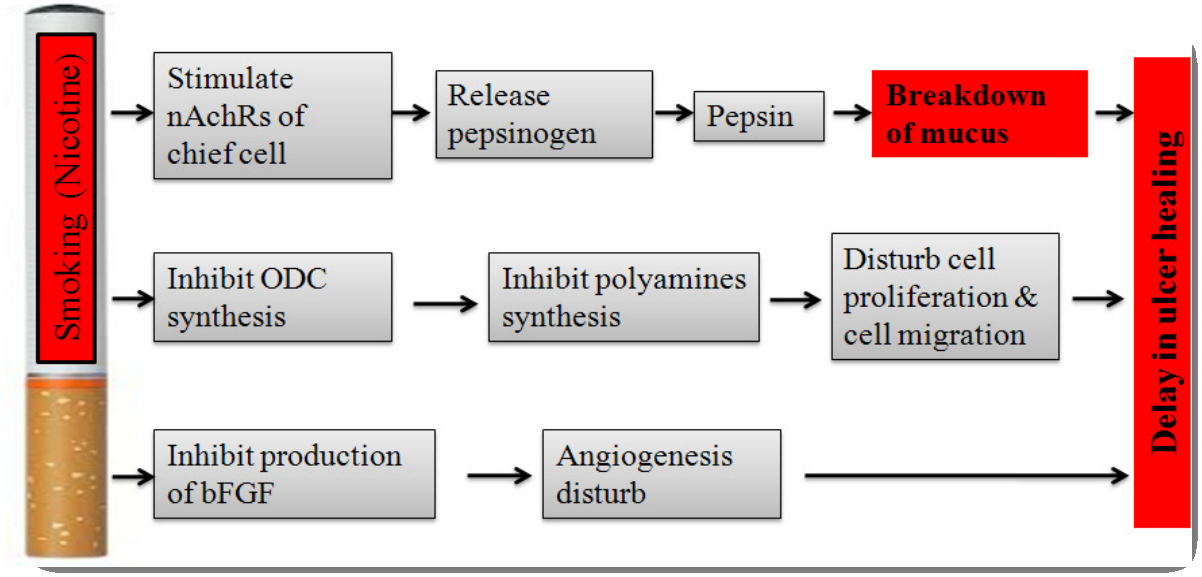


Figure 1.13: Mechanism involved in smoking ulceration

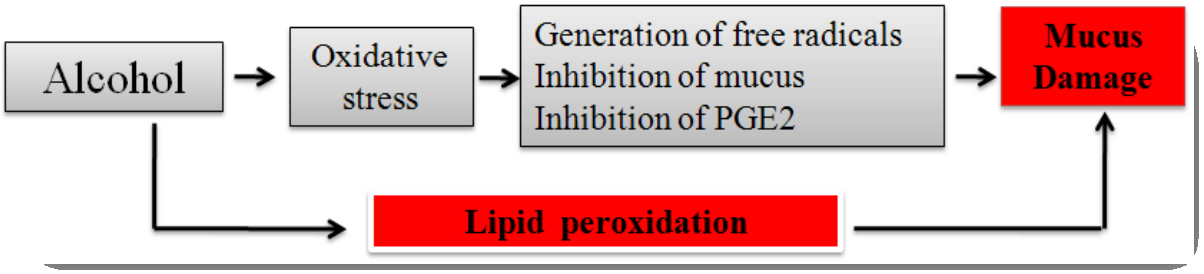


Figure 1.14: Mechanism involved in alcohol ulceration

1.2 INTRODUCTION TO PLANT

Paederia foetida L. known as Prasarani in Sanskrit belongs to the family Rubiaceae. It is an extensive smelling perennial climber. Profile [11-13] of *P. foetida* L. is described below:

1.2.1 Synonyms

Paederia chinensis Hance

Paederia scandans (Lour.) Merr

Paederia tomentosa Blume

Figure 1.15(a): *Paederia foetida* L. leaf

Figure 1.15(b): *Paederia foetida* L. dried leaf

Figure 1.16: *Paederia foetida* L. dried stem & seed

1.2.3 Plant Part used

Leaf, root, bark, fruit

1.2.4 Ayurvedic Properties and Action:

Rasa - Tikta (bitter) *Guna - Guru (heavy)*

Veerya - Ushna (hot) *Vipaka -Katu (pungent)*

Karma - Vedanasthapana, Shothahara, Stabdhatanashaka, Nadibalya, Vatanulomana, Raktaprasadana, Mootrala, Ashmaribhedana, Vrishya, Balya, Sandhaneeya, Saraka.

1.2.5 Vernacular Names

English : Skunk vine, Stinkvine, Chinese fever vine, King`s Tonic

Sans :Prasarni, Sarani, Prasarani, Gandhapatra

Hindi : Gandhali

Beng : Gandha bhaduli, Gandhal

Mar. : Hiranvel

Guj : Gandhana

Tel. : Savirela

Tam. : Penarisangai

Kan. : Hesarane

Mal. : Talanili

Oriya : Gandali

Assam : Bedoli sutta, Bhedilata, paduri-lata

1.2.6 Macroscopical and Microscopical Character

Leaf - Simple, petiolate, stipulate; 10-15 cm long, 5-6 cm broad; somewhat glabrous; ovate, entire, apex acute or cuspidate.

Midrib - composed of single layered epidermis covered with cuticle; ground tissue, consisting of 2-5 layered of collenchyma towards the upper and lower side and the rest are parenchyma; crescent-shaped vascular bundle present with xylem towards upper side and phloem towards lower side.

Lamina - shows a dorsiventral structure; epidermis single layered covered with striated cuticle; uniseriate covering trichomes and paracytic stomata present; mesophyll composed of single layered palisade cells and 3-4 layered spongy tissue; vein islet number 5-10 per sq. mm, palisade ratio 6.75-14.2.

Leaf- Petiole - shows a similar structure as midrib but differs in trichomes, comparatively smaller, starch grains, oil globules and raphides of the calcium oxalate present

Root - Tap root 2-4 cm long, 0.5-2 cm thick, cylindrical or sub cylindrical, tortuous, dark brown; odour disagreeable and foetid. Mature root shows 6-13 layers of cork, secondary cortex 5-16 layers of thin-walled, phloem appears as wedge-shaped, cambium 1-3 layered,

starch grains, oil globules and raphides of calcium oxalate present in a few cells of secondary cortex, phloem, xylem and medullary rays are present.

Stem - Slender, sub-erect with diffuse branching, up to 4 cm thick; longitudinal anastomosing wrinkles, ridges and a few transverse cracks and circular lenticels, fracture, fibrous; odour- foetid, 7-11 layers of cork composed of rectangular cells, secondary cortex 6-9 layers, pericyclic fibres present in singles or in groups, cambium 1-2 layers, pith, secondary cortex, phloem, xylem and medullary rays contain starch grains, oil globules and raphides of calcium oxalate.

Flower - Violet to pink; bracteate, pedicellate, bisexual, calyx campanulate, corolla funnel-shaped, usually pubescent, ovary turbinate, two celled containing one ovule.

Fruit - Berry, orbicular, ellipsoid, five lines on each side, two seeded,

Seed - Compressed, smooth,

1.2.7 Chemical Constituents

Asperuloside (Figure 1.17a), deacetylasperulside, paederosidic acid, paederoside (Figure 1.17b), scandoside (Figure 1.17c), (iridoid glycosides), ceryl alcohol, hentriacontane, hentriacontanol, palmitic acid, methyl mercaptan, campesterol (Figure 1.17d), β-sitosterol (Figure 1.17e), stigmasterol (Figure 1.17f), ursolic acid, carotene, vitamin C, protein, amino acids: arginine, cystine, histidine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, epifridelinol, friedelan-3-one etc. are present in *P.foetida*.

1.2.8 Pharmacological activities

Antiinflammatory, antispasmodic, anticancer, anthelmintic, hepatoprotective.

1.2.9 Formulations and preparations

Prasarini taila, Dashamoolarishta, Prasarini leha, Kubja prasarini taila, Narayana taila, Pushparaja prasarini taila, Trishatiprasarini taila, Saptashatika prasarini taila, Ekadashashatika prasarini taila, Maharaja prasarini taila,Mahamasha taila. Rheu Capsule (Ban Lab, Bombay)

1.2.10 Safety aspects

Considered as safe due to its traditional use.

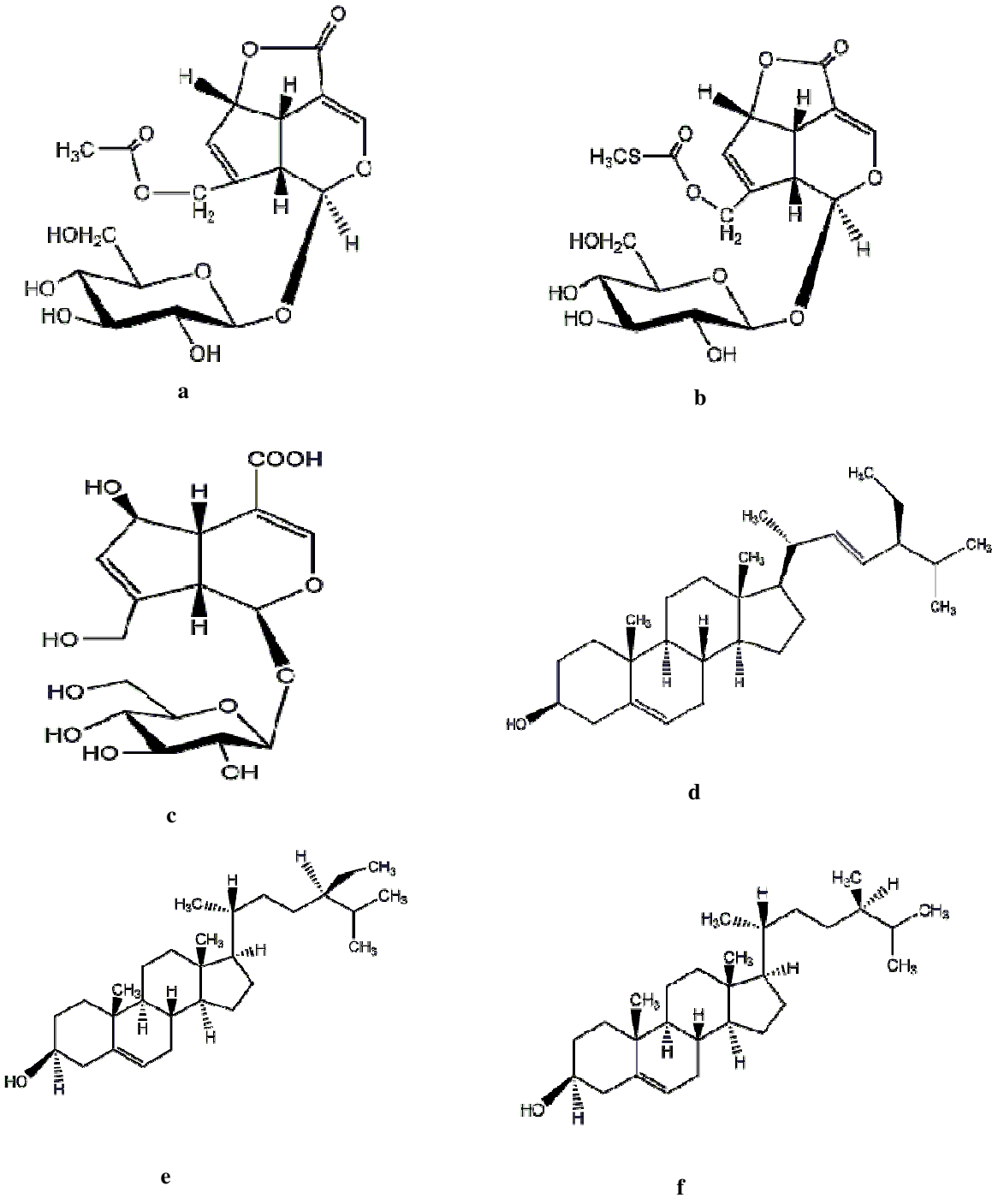


Figure 1.17: (a) Chemical Structure of Asperuloside, (b) Structure of Paederoside, (c) Structure of Scandoside, (d) Structure of Campesterol, (e) Structure of β-sitosterol, (f) Structure of Stigmasterol

1.3 INTRODUCTION TO STANDARDIZATION AND VALIDATION

Medicinal plants play a major source of therapeutic agents for the alleviation of human diseases since our ancestor times. India is considered as "Botanical Garden of the world."

Indian flora and fauna consist of more than 2200 species of medicinal and aromatic plants. Due to the increase in the interest of use of medicinal plants throughout the world, there is a manifold increase of medicinal plants based industries, which are growing at a rate of 7-15% annually. Though, India has the largest number medicinal and aromatic plant producer, but the contribution in herbal industry less than 12%. In India most of the traditional knowledge about medicinal plants was in the form of oral knowledge that has been eroded or distorted due to the persistent invasions and cultural adaptations.

1.3.1 Features that restricting development of herbal medicine in India

- Lack of standardization
- Lack of validation of standardized method
- Deficient of market contacts
- Marketing is inefficient, imperfect, selective and opportunistic

Due to these reasons, there is a drastic reduction in the monograph on crude drugs and plant products in Indian Pharmacopoeia. There is a prevalence of using plants and plant based products in various contemporary and traditional systems of medicine, without any written documentation or regulation. To meet the demand of international market it is mandatory to standardize the product or method and validate it in writing document form. Now days R&D thrust in the pharmaceutical sector focuses on the standardization and validation of the developed standardized techniques.

1.3.2 Standardization

Standardization is a system that ensures a predefined amount of quality, quantity and therapeutic effect of the ingredients present in each dose [14]. It is an important aspect of maintaining and assessing the quality, purity and safety of herbal product/extract/raw

material/ formulation to attain the desired therapeutic effect [15]. A standardized herbal extract means a measurable marker substance or substances present, which is extracted from the herb. The markers present in an extract may be active or inactive. The first Indian National Health Policy 1983 claims that India is the richest source of herbs and the drugs should be standardized [16].

1.3.3 Need of Standardization

The revival of interest in natural drugs and the herbal drugs started in the last decade, mainly because of the wide spread and belief that green medicine is healthier than synthetic one. This leads to the rapid spurt of demand for health products of traditional medicine, which leads to the market of medicinal plants for commercialization. To fulfill this need there is a practice of indiscriminate and unscientific collection practices, without any consideration for the quality control of the material. Thus decreases in the therapeutic efficacy and quality of the final product. Hence standardization plays an important role in the quality assurance of herbal drugs and their products. Raw plant material contains various chemical constituents, thus sometimes standardizing the herbal drug require more than one analytical technique [17].

1.3.4 What is Validation

Validation is a concept that has been evolving continuously its first formal appearance in the United States in 1978 [18]. The validation of an analytical procedure is the process of confirming that the analytical procedure employed for a test of pharmaceuticals is suitable for its intended use. In other word, the validation of an analytical procedure requires to demonstrate scientifically that risks of errors in different analytical steps are acceptably small [19]. Validation is a basic requirement to ensure quality and reliability of the results for all analytical applications. The object of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose, determine by means of well-documented experimental studies [20].

1.3.5 Reasons for validation

There are two important reasons for validating assays in the pharmaceutical industry. The first, and by to the most important, is that assay validation is an integral part of the quality-control system. The second is that current good manufacturing practice regulation requires assay validation [21]. In industry, it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for the validation program in pharmaceutical analysis.

1.3.6 Benefits of method validation [17]

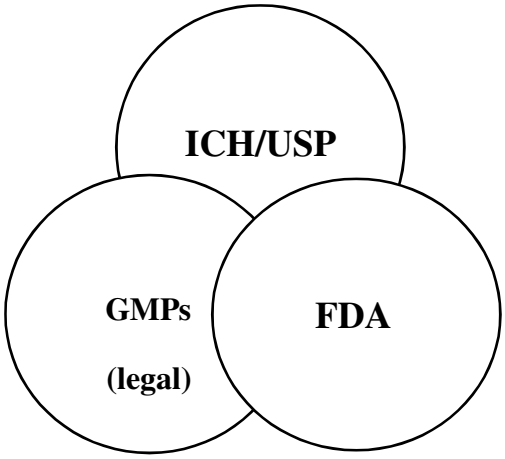
- A fully validated process may require less in-process control and end−product testing.
- It deepens the understanding of processes, decrease the risks of processing problems, and thus assure the smooth running of the process**.**

1.3.7 Steps in method validation

- Develop a validation protocol, or operation procedure for the validation.
- Define the application, purpose and scope of the method.
- Define the performance parameter and acceptance criteria.
- Define validation experiments.
- Verify relevant performance characteristic of equipment.
- Quality materials (for e.g. standard and reagents).
- Perform pre validation experiments.
- Adjust method parameter or/and acceptance criteria if necessary.
- Performed full internal and external validation equipments.
- Developed SOP for executing the method in the routines.
- Define criteria for revalidation.

- Define the type and frequency of system for suitability test and/or analytical quality control (AQC) for the routine.
- Document validation experiment and result in the validation report.

1.3.8 Today's validation requirements



The objectives of ICH as laid down in their terms of reference in their early years were:

- To provide a forum for constructive dialog between and among regulatory authorities and the pharmaceutical industry on the real and perceived differences in the technical requirements for product registration in the EU, United States, and Japan
- To identify areas in which modification in technical requirements or greater mutual acceptance of R&D procedure could lead to a more economical use of human, animal and material resources without compromising safety
- To make recommendations on practical ways to achieve greater harmonization in the interpretation and application of technical guidelines and requirements for registration [20].

There are two guidelines of validation issued by the US (FDA), one for the applicant, the other for inspecting and reviewing. The first one is also intended to ensure that the analytical

procedure can be applied in an FDA laboratory and therefore requires a detailed description of the procedure, reference material as well as a discussion of the potential impurities etc. The second guidelines focus on RP- chromatography and provides a lot of details with regard to critical methodological issues as well as some indication of acceptability of results [20].

There are specific guidelines given by ICH, FDA, and USP. There are different validation characteristics normally evaluated for the different types of test procedure: -

- Specificity
- Linearity
- Range
- Accuracy
- Precision (Repeatability, Intermediate precision, Reproducibility)
- Limit of detection
- Limit of quantification

CHAPTER 2

REVIEW OF LITERATURE

2.1 REVIEWS ON PEPTIC ULCER DISEASE

Approximately 15% of world populations are suffering from PUD [22] whereas the mortality rate is 5% to 25%. Mortality is mostly due to perforated peptic ulcer and bleeding and rising up to 50% with age [23-25]. PUD is also accounting for about 35-50% of non-variceal hemorrhage [26-29]. In the United Kingdom the incidence varies, approximately 50-150 cases per 100 000 per year and an about 15000 hospitalizations per year [29]. In the United States about 15 million people are having peptic ulcer disease [30]. Approximately 5million adults suffer annually and 500000 new cases and 4 million recurrences. It causes an estimated 1 million hospitalizations and 6500 deaths per year [31]. In the current scenario, there is a great change in epidemiology of PUD in India due to a vital improvement in hygiene, better lifestyle, availability of potent histamine blockers and as well as anti ulcerogenic drugs [32]. An endoscopic and epidemiological study were conducted by Institute of Medical Sciences, India, where the point prevalence of PUD was reported 4.72% and the lifetime prevalence was 11.22% [33] whereas in another study reported on an average, around 8.0 per 100,000 [34]. Peptic ulcer disease has been reported to occur more frequently in men as compared to women [35-38] but several other studies also reveals that the development of the PUD is not gender specific [39,40]. Throughout the life span approximately 25 million Indians are suffering from the PUD. Duodenal ulcers are 5 to 10 times more common than gastric ulcers. The male and female ratio is 3:1. The peak incidence of duodenal ulcer in between the age of 30 to 60 whereas gastric ulcer over the age of 50.

2.1.1 *Helicobacter pylori* & Peptic ulcer disease

More than 50% of the world population are infected by *H. pylori* [41]. In India prevalence is more than 80% [42]. There are several reports for the association of *H. pylori* in gastric ulcer and gastric cancer, whereas there is some report which claim there is no association between stomach cancer and *H. pylori* infection [43-48]. About 75% of the population in

developing country and 40% in developed countries being infected by this organism [49]. The risk factors for the development of PUD by *H. pylori* are mainly associated with the lower economic status, consumption of restaurant food, meat, fish, family history and non filtered water [50]. The anti microbial used for *H. pylori* eradication therapy is amoxicillin, bismuth compounds, clarithromycin, metronidazole, tetracycline etc. But in all the cases the development of resistance has been reported (51-60). As a result, resistance development is frequently associated with eradication failure [61, 62]. Some studies also revealed 100% resistance rate for clarithromycin and metronidazole [53, 54]. The majority of investigators believes that as soon as the infection is detected, it should be eradicated [63]. But after eradication also there are several reports from India, which reports 5 to 14% of recurrence [64, 65].

2.1.2 Nonsteroidal anti-inflammatory drugs (NSAIDs) & Peptic ulcer disease

NSAIDs are most widely prescribed drugs used as analgesic and anti-inflammatory agents worldwide. NSAIDs account for 15-30% of PUD [66]. In the US, 50% of the population over the age of 50 uses NSAIDs [67]. About 70 million are consuming NSAIDs as prescription drugs whereas 30 million used as over the counter drug [68]. It is accounting for 5% of hospitalization. There is a fourfold increase in the risk of ulcer with NSAIDs [69, 70]. The pathogenesis of NSAID-induced ulceration was explained in figure no 1.11.

2.1.3 Stress & Peptic ulcer disease

There are 75 to 100% of incidence of stress ulceration depending upon the condition of patient disease and severity of illness [71,72] whereas 1.5% clinical report for gastrointestinal bleeding [73]. The general principle of stress induced ulceration is acute loss of mucosal barrier protection.

2.1.4 Smoking (Nicotine) & Peptic ulcer disease

Smoking also enhances the stress levels. It has been shown that there is a decline in stress level after smoking cessation [74]. PUD is more common in smokers than nonsmokers. Moreover, in the case of smokers there is a delay in ulcer healing [75]. Nicotine stimulates the central nervous system and chronic nicotine intake also boost the mucosal damages due to stress [76,77]. The interaction between stress and nicotine has been reported to work

synergistically [78, 79]. Cigarette smoking significantly initiate the formation of gastric mucosal lesions and also enhance the gastric mucosal MPO activity [80]. Tobacco smoking has been reported to suppress nitric oxide (NO) release [81-83]. Nicotine elevates the endogenous level by cAMP-protein kinase A signaling system as a result elevates the endogenous vasopressin level. Vasopressin has an aggressive role in the development of gastroduodenal ulcer. The production of platelet activating factor (PAF) and endothelin is increasing due to smoking. Both the endothelin and PAF act as potent ulcerogens. Nicotine also reduces the level of circulating epidermal growth factor (EGF) as a result, there is a disturbance of gastric mucosal cell renewal [84].

2.1.5 Alcohol & Peptic ulcer disease

Though *H. pylori* is one of the major issues in the development of the PUD, but after successful eradication of the bacteria the recurrence rate was 3.02%. The recurrence rate was significantly higher in patients who consume alcohol. Recurrence rates as high as 83.9% who smoked, consumed alcohol, and used NSAIDs [85]. There is a generation of superoxide anion and hydroperoxy free radicals upon metabolism of alcohol which leads to further lipid peroxidation [86]. Alcohol causes ulceration by necrosis and apoptosis. It reduces bicarbonate secretion and mucus production [87].

2.1.6 Challenges to treatments

On March 2011 US FDA (Food & Drug Administration) issuing drug safety communication regarding the lower serum magnesium levels, which may result due to the long time use of PPIs. The worst thing is that the low level of magnesium cannot improved by magnesium supplement. The major alarming sign for low level of magnesium are the development of tetany, arrhythmias, and seizures. FDA is revising the prescription and over-the-counter (OTC) labels for proton pump inhibitors and included new safety information about the risk factors of PPIs and H2 blocker such as fractures of the hip, wrist, and spine [88]. There was also a safety announcement in August 2012 by the FDA regarding the risk of *Clostridium difficile*–associated diarrhea (CDAD) which is associated with the prolong use of PPIs [89]. There are several other side effects of long term use of PPIs like acute interstitial nephritis,

acute renal failure, osteoporosis, hepatitis, visual disturbance etc. [90-92]. Each and every side effect itself acts as a threat to human health.

2.2 REVIEWS ON NATURAL APPROACHES

Considering the several side effects described above *viz.* hip fracture, acute interstitial nephritis, acute renal failure, osteoporosis, hepatitis, and visual disturbance of currently available medicine for PUD [90-92], indigenous drugs possessing fewer side effects and should be looked for as a better alternative for the treatment of peptic ulcer disease. Traditionally, various medicinal plants are used in the treatment of PUD. They exhibit individual and multiple mechanism of action like antioxidant, cytoprotective, antisecretory action, etc. in the management of PUD. Table 2.1 listed the names of the plant showing anti ulcer activity with their mechanism. There are several reports for the drug/chemicals derived from botanical possess anti ulcer activity [129-131].

Table 2.1: Plants used in peptic ulcer disease

Plant name	**Plant part**	**Extract**	**Mechanism involved**	**References**
Jasminum grandiflorum L	Leaf	70% ethanol extract	Anti-secretory & antioxidant activity	93
Anogeissus latifolia (Roxb. ex DC.) Wall. ex Guill	Bark	50% aqueous alcohol extract	Histamine antagonistic, anticholinergic, anti-secretory and antioxidant effect	94
Alchornea castaneaefolia A. Juss.	Leaves and bark	Hydro-ethanol extract	Gastro-protective	95
Utleria salicifolia Bedd. Ex. Hook. F	Rhizome	50% Ethanol extract	Antioxidant activity	96
Solanum nigrum Linn	Fruit	Methanol extract	Inhibition of H (+) K (+) ATPase & decrease gastrin secretion.	97

Ocimum sanctum Linn	Leaf	Ethanol extract	Anti-secretory or cytoprotective or both	98
Scoparia dulcis L.	Aerial part	Aqueous freeze-dried extract	Inhibition of the $H^+ K^+$ ATPase enzyme	99
Byrsonima crassa Niedenzu	Leaf	Hydromethanol, methanol and chloroform extracts	Scavenging of the reactive oxygen species	100
Asparagus racemosus	Root	Methanol extract	Mucosal defensive, antioxidant activity.	101
Centaurea solstitialis L.	Flower	Chloroform extract	Cytoprotective	102
Anacardium occidentale L.	Leaf	Hydroethanol extract	Free radical scavengers, Anti *H. pylori*	103, 104
Calophyllum brasiliense Camb	Stem bark	Dichloromethane fraction of hexane extract	Anti-secretory property, stimulation of gastric mucus	105
Rhizophora mangle L.	Bark	Aqueous extract	Antioxidant	106
Larrea divaricata Cav	Leaf	Methanol extract	Antioxidant activity	107
Hemidesmus indicus var.	Root	Aqueous ethanol extracts	Gastroprotective activity	108
Spartium junceum L	Flower	Methanolic extract	Cytoprotective	109
Amomum subulatum Roxb.	Fruit	Crude methanol extract	Cytoprotective	110
Terminalia chebula Retz.	Fruit	Hydroalcohol extract	Anti-secretory activity	111
Aspilia Africana Pers.	Leaf	Water extract	Free radical scavenging	112
Picrorhiza kurroa	Rhizome	Methanol extract	Reducing oxidative stress, promoting mucin secretion, prostaglandin synthesis and augmenting expressions of	113

			cyclooxygenase enzymes and growth factors.	
Cedrus deodara Roxb. Loud.	Wood	Steam distillate	Anti-secretory	114
Azadirachta indica NLEa & NLEa	Bark	Aqueous extract	Inhibition of H (+) K (+) ATPase, preventing oxidative damage & apoptosis	115
Zingiber officinale Rosc.	Rhizome	Ethanol extract	Anti inflammatory	116
Glycyrhiza glabra	Rhizome	Extract	Free radical scavenzing	117
Desmodium gangeticum DC	Whole plant	Ethanol extract	Cytoprotective, Antisecretory	118
Allophylus serratus Kurz	Leaf	Ethanol extract	Anti secretory, cytoprotective	119
Terminalia palida Brandis	Fruit	Ethanol extract	Decrease acid secretion, potent antioxidant	120
Centella asiatica Linn.	Whole plant	Juice	Increase mucin secretion	121
Bacopa monniera Linn.	Whole plant	Fresh juice	Increase mucin secretion	122
Musa sapientum L.	Unripe fruit	Powder	Increase mucin secretion	123
Bidens pilosa L.	Aerial part	Ethanol extract	Anti-secretory, cytoprotective	124
Eugenia jambolana Lam.	Seed	Ethanol extract	Decrease acid, pepsin secretion	125
Ziziphus mauritiana Lam	Stem bark	Methanol	Anti oxidant	126
Toona ciliate Roemer	Heart wood	Ethanol extract	Anti secretory	127

Excoecaria agallocha L.	Bark	Water extract	Free radical scavenging activity, stimulating the contraction of the wound	128

2.3 REVIEWS ON PAEDERIA FOETIDA

2.3.1 Phytochemistry

Tripathi et al (1974) isolated and characterized friedelan-3-one and β-sitosterol from petroleum ether and benzene extract [132].

Shukla et al (1976) investigated the presence of different iridoids such as asperuloside (0.08%), paederoside (0.084) and scandoside (0.064%) in the leaf and stem of the plant [133].

These iridoids are found in a wide variety of plants as glycosides. They are optically active cyclopentanoid monoterpenes biosynthesized from isoprene. They often act as an intermediate in the biosynthesis of alkaloids. They are volatile and therefore occasionally reported as components of essential oils.

Iridoids are biosynthesized in plants from geranlpyrophosphate via hydroxynerol and then iridodal. The later exists in equilibrium with its hemiacetal form. A cleavage of the cyclopentane ring at the bond between C7 and C8 yield secoiridoids. Variations in the iridoid structures are attained due to the direct modification of the iridane skeleton such as loss of carbons at the 4 and/or 8 positions. Variation in the structure also occurs due to the oxidation of the skeleton to aldehyde or acid and epoxidation with specific stereostructure.

Apart from these three iridoids Shukla et al also reported the presence of methyl mercaptan, hentriacontane, ceryl alcohol, hentriacontanol, palmitic acid, sitosterol, stigmasterol, campesterol and ursolic acid [133].

De et al (1993) reported the presence of fatty acids in the petroleum ether extract of leaves of Paederia. A total of thirteen fatty acids of which six are

identified as nonanoic acid (0.33%), capric acid (2.44%), lauric acid (1.22%), myristic acid (1.66%), arachidic acid (65.05%) and palmitic acid (13.78%). [134]

Steam volatile constituents of the aerial parts of Paederia were studied by Wong et al (1994) [135]. GC-MS analysis of the oil of leaf, stem and flower reveals the presence of oxygen-containing monoterpene as main chemical class of compounds. Among the all monoterpenes linalool, α-terpineol and geraniol are the most abundant compounds.

Ghisalberti (1998) studied that the biological activity of iridoids is mainly due to the presence of aglycons, which readily transform into reactive intermediates in the presence of β-glucosidase or acid. Iridoids with a glucosidic or acetal ester linkage at C-1 are less active than the free hydroxyl at C-1. [136]

2.3.2 Therapeutic Properties and Mechanism of Action

The anti inflammatory activity was studied by Chaturvedi and Singh (1965 & 1966) [137, 138].

Roychoudhury et al (1970) investigated the anthelmintic efficacy of the liquid extract of *P. foetida*. The study revealed 100% effectiveness against *Strongyloides sp., Trichostrongylus sp.* and *Haemonchus sp.* and 50-70% effectiveness against *Bunostomum sp.* and *Monezia sp.*[139].

The anti-inflammatory activity was also studied by Srivastava et al in the year 1973 [140]. The authors investigated that the alcohol fraction of the plant is stronger than acetylsalicylic acid but weaker than hydrocortisone.

Inouye et al (1974) studied the purgative activity of asperuloside and its acetyl compounds *viz*. deacetylasperulosidic acid and methyl deacetylasperulosidate on mice [141].

The hepatoprotective activity of methanol extract of Paederia was studied by De et al (1993) [142]

The same action of the plant was also confirmed by another study done by De et al (1994) [143].

Kapadia et al (1996) [144] studied the anti-virus, anti-tumor, anti-inflammatory and anti-microbial activity of asperuloside, paederoside and scandoside. The author reported the

highest order of anti tumor promoting activity of paederoside among other 15 iridoids. The order of activity paederoside > genipin > kutkoside > scandoside hexaacetate methyl ester > monotropein > aucubin > deacetylasperulosidic acid > deoxyloganic acid > catalposide > asperuloside > gentiopicroside > picroside-I > secologanin > amaro-gentin > deoxyloganin.

Miura et al (1996) studied deacetylasperulosidic acid for its activity in lowering blood glucose level in normal mice [145].

Kim et al (2005) reported that deacetylasperulosidic acid (63.8 ± 1.5%) and scandoside (62.2 ± 1.6%) inhibited LDL-oxidation, at a concentration of 20 μg/ml [146].

Wang et al (2005) [147] studied the anti-virus, anti-tumor, anti-inflammatory and anti-microbial activity of asperuloside, paederoside and scandoside.

Afroz et al (2006) studied anti diarrhoel activity of ethanol extract of *P. foetida* by using castor oil and magnesium sulphate-induced diarrhoea models in mice. The extract showed promising result by the reduction in gastrointestinal motility and significantly increased the latent period of diarrhoea [148].

Bin et al (2006) reported the presence of iridoid glycoside - asperuloside and deacetyl asperuloside from the plant *Lasianthus acuminatissimus* Merr. In this study asperuloside was reported for its use in the treatment of rheumatoid arthritis [149].

Hossain et al (2006) evaluated the analgesic activity by using acetic acid induced writhing inhibition method. Hexane and methanol extract of whole plant showed significant antinociceptive activity by inhibition in the number of writhing, which supports its peripheral mechanism [150].

Nosalova et al (2007) evaluated the antitussive activity of the ethanol extract of Paederia on cats by mechanical stimulation of laryngopharyngeal (LP) and tracheobronchial (TB) mucous areas of airways. The plant showed cough-suppressive effect. The activity compared with narcotic and non-narcotic antitussive. The extract showed similar action as compared to non-narcotic antitussive (dropropizine) but lesser to that of narcotic antitussive (codeine) [151].

Yu-Feng Chen et al (2007) reported the isolation of paederoside, asperuloside, paederosidic acid and scandoside from the n-butanol fraction of the whole plant of *P. scandens*. These iridoids represented about 2.17% of the n-butanol fraction. Out of 2.17%, paederoside

constitute 1.23%, asperuloside represented 0.068%, whereas scandoside and paederosidic acid account for 0.18% and 0.7% respectively. The authors also suggested that the anti-nociceptive activity of the n-butanol fraction of methanol extract of the plant due to the presence of above mention iridoids [152].

Author Li B. (2007) reported the activity of asperuloside in inhibition of TNF-α, decreased IL-1β production, reduced formation of PGE2, and also successfully treated for rheumatoid arthritis [153].

Olga Tzakoua et al reported the insecticidal properties of scandoside in the year 2007 [154].

Osman et al (2009) also studied and confirmed the presence of phenolic content of the plant and investigated the antioxidant activity of the fresh and dried plant by β-carotene and ABTS method. Fresh leaves showed 78.13% and 75.38% antioxidant activity by β-carotene and ABTS method respectively, whereas, dried leaves showed 66.67% and 67.74% of the antioxidant activity [155].

P. foetida protects liver against hepatotoxin-induce oxidative damage studied by Uddin et al (2011) [156].

Zhu et al (2012) studied anti-inflammatory and immunomodulatory activity of iridoid glycosides (asperuloside, paederoside and scandoside) of *Paederia scandens.* These iridoids exhibit protective effect against renal injury in uric acid nephropathy rats. UAN (uric acid nephropathy) rats, possesses anti-inflammatory and immunomodulatory effects by inactivating NF-κBp65 pathway transmembrane signal transduction, down regulating the expression of monocyte chemoattractant protein-1 and α-smooth muscle actin to modulate pro-inflammatory mediator production in nephropathy tissue to improve renal fibrosis in UAN rats [157].

Fujikawa et al (2012) reported the anti obesity and anti metabolic activity of the asperuloside [158].

Asperuloside, paederoside and scandoside also possesses the antinociceptive effect, which is related to the inhibition of nitric oxide (NO) / cyclic guanosine monophosphate (cGMP) / and protein kinase G type I (PKG) signaling pathway in the rat spared nerve injury (SNI) model of neuropathic pain reported by me et al (2012) [159].

Antioxidant activity also investigated by the author Srianta et al (2012) [160].

Kumar et al (2014) investigated the anti-hyperglycemic activity which is due to pancreatic and extra pancreatic mechanism. They also studied the anti-hyperlipidemic activity of the methanol extract which lower the higher level of lipid profile and decreasing the intercalated disc space in the heart [161].

There are several methods for isolation and quantitative analysis of iridoids from plant source. Mostly different spectroscopic and chromatographic (GC, HPLC) method is used for quantification [162-168].

2.3.3 Traditional / folkloric uses in food and medicine

Traditionally Paederia mainly used by the tribal people of the Northeast as vegetables reported by Das P. in the year 1997 [169].

The Boro tribe of Goalpara district of Assam uses the juice of the leaf in diarrhoea and dysentery reported by Basumatary in the year 2004 [170].

Apatani tribes of the Eastern Himalayan region of India use stem in Gastritis, diarrhea, stomach disorder reported by Chandraprakash Kala in the year 2005 [171].

Borah et al (2006) reported that apart from the Boro tribal, people of upper Assam also use the aerial part in diarrhoea and dysentery [172].

Kalita et al (2006) studied that the people of Lakhimpur district of the Brahmaputra valley, Assam use the leaf in case of allergy [173].

Purkayastha et al (2006) reported the oral use of leaf in gastralgia and in postnatal pain and also in bleeding [174].

Majumder et al (2007) also reported the activity of the leaves in rheumatic pain, particularly during eclipse by the Tripuri tribal of Tripura [175].

Barua et al (2007) reported the use of the leaves by the Majuli Island and Darrang districts of Assam as a vegetable and also reported the use of leaf for dysentery [176].

Reddy et al (2007) also reported the use of the plant as vegetables by the tribal people of Andra pradesh [177].

Kar et al (2008) reported the use of the leaf in gastritis by the people of Karbi-Anglong district of Assam [178].

Hynniewta et al (2008) reported the use of the leaf as antidote in case of snake bite by the Khasi tribes of Meghalaya [179].

The Chakma community residing in the Northwestern periphery of the Namdapha National Park in Arunachal Pradesh used the whole plant extract for the treatment of urinary disorders, kidney stone and digestive problems reported by Sarmah et al (2008) [180].

Kumar et al (2009) also reported the use of leaves and twigs by Sonitpur people of Assam in dysentery and in stomach problem [181].

Kadir et al (2009) studied the use of the leaves of Paederia as a vegetable by the Garo tribe inhabiting the Madhupur forest region of Bangladesh and also use as medicine in different kinds of stomach ailments [182].

Rethy et al (2010) reported the use of the plant as vegetables and also as medicine [183].

Chanda et al (2010) reported the use of the plant as a vegetable and also as a diuretic, in diarrhea and also in infection [184].

Kagyung et al (2010) also reported the use of the leaf as vegetables by the Adi tribes of Dehang-Debang Biosphere Reserve in Arunachal Pradesh. They also use this plant in diarrhoea, dysentery and in indigestion [185].

Biswas et al reported the use of leaf juice by the tribal people in the Chittagong Hill tracts, Bangladesh for dysentery in the year 2010 [186].

Srivastava et al (2010) studied the use of juices of leaves for gastric trouble, clean stomach, diarrhea and stomach swelling by an Apatani tribe of Arunachal Pradesh [187].

Singh et al (2010) reported that the tribal communities of Mayurbhanj district, Orissa use the whole plant in different kind of joint diseases like rheumatism, arthritis, gout and lumbago etc. [188].

Tai Ahom of Dibrugarh district of Assam use the leaf, stem bark in case of abscess reported by Kalita et al (2010) [189].

Rahmatullah et al (2010) reported that the folk medicinal practitioners Natore and Rajshahi districts, Bangladesh use the whole plant in rheumatoid arthritis, as a tonic and in various colic [190].

Sumpam et al (2011) reported the activity of leaf against gastritis and loose motion. The Eastern Himalayan zone of Arunachal Pradesh uses leaf water decoction to treat such diseases [191].

Nimachow et al (2011) studied the use of the leaf and stem in fire and hot and even in water burns by the Aka tribe of Arunachal Pradesh [192].

Panda et al (2011) studied the use of the plant in fever, gout, rheumatism by the tribal communities of Mayurbhanj district, Orissa [193],

Sahu et al (2011) reported the use of the leaf paste in mucostool by the people of the coastal districts of Odisha [194].

Rahmatullah et al (2011) also reported that the folk medicinal practitioners of Noakhali and Feni district of Bangladesh use this plant in Cancer [195].

People of Arambagh sub-division of Hooghly district of West Bengal use the leaves as a vegetable and also as medicine for fever, dysentery and diarrhea reported by Biswas et al (2012) [196].

Pfoze et al (2012) reported the activity of leaf and roots in stomachache, diarrhea, dysentery, gastritis, body ache, joint dislocation and bone fracture by the tribal people of Senapati District of Manipur State [197].

CHAPTER 3

RATIONALE OF THE STUDY AND OBJECTIVES

3.1 Rationale of the study

At present, the pharmaceutical prescription market to treat GI disorders is valued at just over $20 billion, reflecting that it is one of the largest and most important therapeutic areas. 5 - 15% of the adult population of the world are suffering with peptic ulcer disease [22]. India has 5th rank in peptic ulcer disease with a death rate of 12.37/Lakh population. Though peptic ulcer of the stomach and duodenum was known in the 1700s but there is revolution of understanding and management of PUD after the discovery of *Helicobacter pylori.* Based on the type of mechanism of action, a number of treatments are available for peptic ulcer. For example, use of antacids, H2 blockers, proton pump inhibitors, antibiotic, and combination therapy. But each category is having unavoidable limitations, for example, high doses and long term use of H2 blockers and proton pump inhibitors (PPIs) cause hip fracture, [93] carry a possible increased risk of bone fractures [89]. Although PPIs are well-tolerated, but there have been reports for acute interstitial nephritis (AIN) and progression to acute renal failure (ARF) [91] due to long term use of PPIs. The other alarming side effects of PPIs are osteoporosis, hepatitis, visual disturbance [91] which cannot be overlooked. FDA approved double, triple & quadruple therapies are not also resulting 100% eradication of *H. pylori* which causes treatment failure. The main reason of treatment failure is patient noncompliance due to concomitant use of medicine. Patients have to follow the combination therapy, as PUD is a multifactorial disease and each category of medicine is having a different mechanism of action. Hence a single drug treatment fails to meet the need. For the cheaper option if any, category is removed from combination therapy, it leads to reduction in efficacy. Development of antibiotic resistance is one of the most challenging issues in the management of PUD. In this aspect, herbal approach would be a better option as it might act through multiple mechanisms of action.

Paederia foetida L. (Rubiaceae) is an extensive foetid climber found in the Himalayas up to an altitude of 1800 m [198]. There are many scientific and traditional claims to the benefits

of *P. foetida* especially concern to the gastrointestinal tract (GIT) disorders such as anti diarrhoeal, loose motion, clean stomach, against stomach swelling, piles, abdominal pain, carminative, spasms, in gastralgia, flatulence or other gastric trouble like gastritis and ulcer [199,172,180,181,185,192]. It has been observed that from Northeast to South part of India, different community of tribal people uses this plant as vegetables and also in different ailment which is seems to be peptic ulcer and also in other gastric trouble [176,177,183,186,197]. In spite of the miles of distance, the common use of the plant by different community of people concrete the hypothesis that the plant must contain some potent anti ulcer agent which need to be evaluated, standardize and commercialize.

There are several research reports which support that concomitant use of NSAIDs in case of arthritic patients increase the risk of gastric ulcer 3 to 4 fold [70,71] which is becoming a crucial issue for the recurrence of ulceration. It is anticipated that a standardized extract of *Paederia foetida* L. not only beneficial for the ulcer patient, but also could effective for arthritic patients too as clinical study reveals that *P. foetida* extract is potent against the rheumatoid arthritis [200]. So it seems to be saying kill two birds with one stone. Rheu capsule for arthritic patient manufacture by Ban Lab, Mumbai contain *P. foetida* as one of the ingredients.

There is no systemic study has been done in the area of standardization of the plant extract with respect to markers. We hypothesize that standardization and evaluation of *P. foetida* not only having a unique benefit to medicinal prevention, but also it sheds light on the nutritional importance and value of the wild products used as food and medicine. Evaluation and analysis of tribal plant offer a new platform for development of new drug and can serve as a key role in the safety and cost effective.

3.2 Objectives

The current study has the following objectives:-

3.2.1 Field study to assess the richness of ethno botanical plant species used by the tribes of north east Tripura

3.2.2 Phytochemical analysis and standardization of *P. foetida* extract

3.2.2.1 Qualitative phytochemical analysis

3.2.2.2 GC-MS analysis of volatile constituents of *P. foetida*

3.2.2.3 Standardization of *P. foetida* extract

3.2.3 Evaluation of anti ulcer activity (*in vivo & in vitro*) of *P. foetida* extract

3.2.3.1 Evaluation of *in-vitro* antioxidant activity

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

3.2.3.3 Evaluation of *in-vitro* anti ulcer activity

3.2.3.4 Western blot analysis

CHAPTER 4

MATERIALS AND METHODS

4.1Ethnobotanical 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.

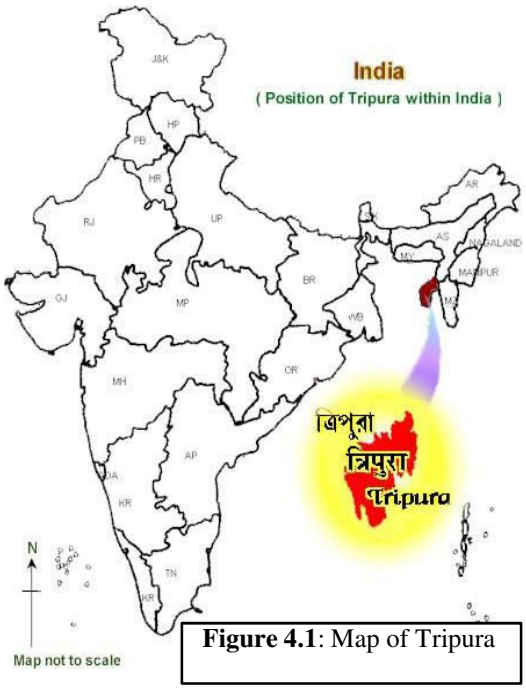


Figure 4.1: Map of Tripura

Tribal communities have over the being referred to by a value-loaded and pejorative terms such as “primitive”, “savage”, “exotic”, “barbarior”, “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.

There are as many as 19 scheduled tribes in Tripura. They are: (1) Tripuri or Tripura, (2) Riang, (3) Jamatia, (4) Chakma, (5) Halam, (6) Noatia, (7) Mog, (8) Kuki, (9) Garo, (10) Munda, (11) Orang, (12) Lushai, (13) Santal, (14) Uchai, (15) Khasia, (16) Bhil, (17) Lepcha, (18) Bhutia and (19) Chaimal [205]. Tribal communities of Tripura, depend on plant resources not only to fulfill the need of stomach, but also as herbal medicines, construction of dwellings, making household implements, sleeping mats, and for fire and shade forage.

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. H. 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 $FeCl_3$ 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_2SO_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_2SO_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% $CuSO_4$.5H2O 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 $H_2SO_4$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 R_f value was calculated by using the following formula:

$$Rf = \frac{\text{Distance travel by solute}}{\text{Distance travel by solvent}}$$

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 methuyl siloxane; 30 m X 250 µ m X 0.25 µ m 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 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 62.667min 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 NIST 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)
9. Ethyl acetate : ethanol : water: ammonia (15:7:2:1)

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 × 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 500µ g/µ
L.

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

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 245nm. 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
5. Intermediate precision
6. Robustness
7. Limit of Detection
8. 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 measurement 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 IC_{50} values were obtained by calculating percent scavenging activity of the DPPH free radical using the following equation:

Calculation

% inhibition= $[(A_0-A_1/A_0)]$ X 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 (IC_{50} 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 (5μ g/ml), vancomycin (8μ g/ml),
and polymixin B (10μ g/ml). The plates were incubated at 37° C in a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) (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

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

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 μ g/ml (because that concentration is cell toxic and harmful for human. Therefore the strain 399Asc is metronidazole resistance (MIC = 64 μ g/ml) while others are metronidazole sensitive i.e. MIC is less than 8 μ g/ml [209]).

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 $BaCl_2 \times 2H_2O$ + 99.6 mL 1% w/v H_2SO_4). When adjusted to the turbidity of the McFarland's standard no. 4, the bacterium suspension contains about 12 X 10^8 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 µ g/ml), Clarithromycin (>2 µ g/ml), Amoxicillin (>8µ g/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 $MgCl_2$ 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 10^9 colony forming unit (CFU) of *H. pylori*. The plates were kept under observation for 2 days at 37° C under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). 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 4^{th} 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) X 10^{-1}; Where UN = Average of number of ulcer per animal

US = Average of severity score, UP = Percentage of animals with ulcers.

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 3hours. 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

Model	Total Group	N	Plant extract dose	Standard dose	Parameter
Model 1	5	5	100mg/kg body weight & 200 mg/kg body weight	Ranitidine 10mg/kg	Volume of gastric juice, pH, acid output, ulcer index
Model 2	5	5	100mg/kg body weight & 200 mg/kg body weight	Sucralfate 100mg/kg body weight	Ulcer score
Model 3	5	5	100mg/kg body weight & 200 mg/kg body weight	Lansoprozole 8mg/kg body weight	Ulcer score
Model 4	4	6	150mg/kg body weight	Cimetidine 8mg/kg body weight	Volume of gastric secretion, pH, free acidity, total acidity, ulcer index

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. Group1 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 cimeditine (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 (Brad ford 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 µ l :
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 4.3: Composition of loading buffer

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

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).

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1Ethnobotanical survey and selection of plant

The ethnobotanical survey revealed the use of six edible plants (Table 5.1) by the tribal people to treat various ailments of the stomach. In our current study, we mainly focused the leaf portion of the plant on the basis of the outcome of the ethno-botanical field survey report [184] which revealed that leaf is only the part showing dual use as edible and as well as medicine by the tribal people of Tripura. Secondly, extensive review of literature (2004 – 2012) concern to only the ethnobotanical survey report (n=24), a total of 79.17 % of articles claimed the use of leaf as ethnomedicine whereas 12.5 % (n=3) claimed use of whole plant, only 8.33% (n=2) stated the use of root and twig and 4.17% article claimed the use of stem, stem bark, aerial part and fruit individually. So in the present study, we focused to evaluate the activity of Paederia leaf as antiulcer agent on animal models. Figure 5.1 explained the percentage of use of different part of the plant.

Table 5.1: List of plants used by tribes of Tripura to treat various stomach disorders

SN.	Plant	Family	Regional name	Parts used	Other uses
1.	*Alpinia allughas* Rose	Zingiberaceae	Tharai	Stem and Flower	As liver tonic
2.	*Cissus quadrangularis* L.	Vitacea	Naljora/ Mukhram Yathek	Shoot	In bone fracture, analgesic, as liver tonic, inflammation.
3.	*Dioscorea glabra* Roxb	Dioscoreaceae	Thabarchuk	Tuber	As a laxative
4.	*Leucaena leucocephala* Lamk	Mimosaceae	Subabul	Tuber, Fruit, and Seed	As a laxative & in liver tonic
5.	*Paederia foetida* L.	Rubiaceae	Duk Pui	Leaf	Diuretic, in diarrhoea, & in infection
6.	*Polycarpon prostratum* Forssk	Caryophyllaceae	Bukhate	Leaf	In menstrual pain & in fever

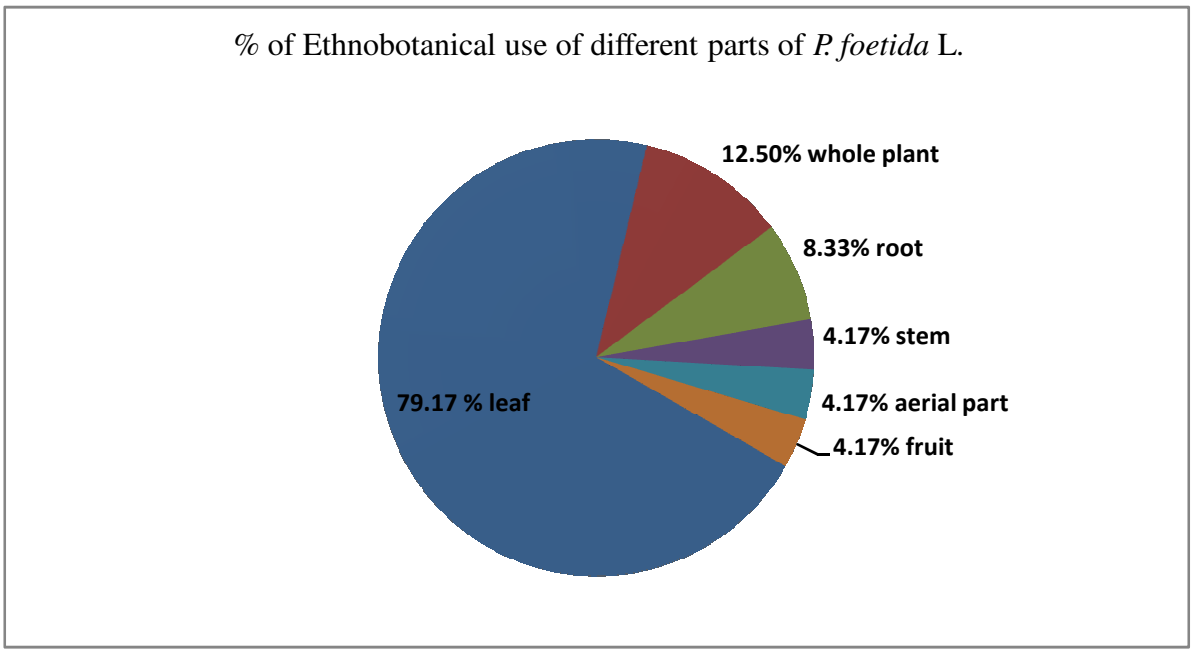


Figure 5.1: Percentage of Ethnobotanical use of different parts of *P. foetida* L.

5.2 Collection of plant material

The aerial part of the plant was collected from the local tribal dominated market of Agartala, Tripura.

5.3 Authentication of plant

The plant was authenticated from National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. The received authentication reference number is NISCAIR/RHMD/Consult/2010-11/1442/40.

5.4 Preparation of Extract

Methanol extract was prepared for phytochemical analysis and biological evaluation. The percentage yield of solvent free extract was 36.21%w/w.

5.5 Preliminary Phytochemical screening

Preliminary phytochemical analysis was done for investigation of different kinds of secondary metabolites. As secondary metabolites are directly related to the therapeutic responses of any drug, it is becoming very important to analyze the metabolites present in an extract. The chemical tests were included tests for tannins, glycosides, alkaloids, steroidal moiety, reducing sugar, amino acids etc. in methanol extract of leaf of *P. foetida.* The secondary metabolites present in the methanol extract are tabulated in table no.5.2.

Table 5.2: Secondary metabolites present in methanol extract of *P. foetida* L.

Sl. No.	Secondary Metabolites	Result
1	Glycoside	Present
2	Alkaloid	Present
3	Sterols	Present
4	Volatile oil	Present
5	Tannins	Present
6	Triterpenoids	Present

5.5.1 Qualitative HPTLC study of β-sitosterol

Literature supports the anti-inflammatory properties [216] and gastroprotective activity [217-220] of β-sitosterol. As in the current study anti ulcer activity of the plant was targeted so it is becoming worthwhile to detect whether the plant extract is having β-sitosterol or not. As the presence of β-sitosterol in methanol extract may contribute a vital role in ulcer protective effect.

The presence of β-sitosterol was confirmed by performing the HPTLC of methanol extract of leaf of *P. foetida* L. along with standard β-sitosterol by using toluene: ethyl acetate: formic acid (8:2:0.1 v/v/v) as mobile phase and anisaldehyde sulfuric acid was used as spraying reagent. The system produces a compact spot of standard β-sitosterol with R_f value 0.35.

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

A total of 57 constituents are identified and characterized from the plant of *P. foetida* L. Out of 57 compounds 54 compounds are reported first time. Table 5.3 listed all the identified chemicals along with their peak area percentage. GC/MS chromatograms of n-hexane stem (Figure 5.2), leaf extract (Figure 5.3) and hydrodistilled oil of leaf (Figure 5.4) of *P. foetida* L. showed various peaks indicating the presence of a wide range of volatile principles. Components were identified by using the combination of retention index value and mass spectral matching against library standards.

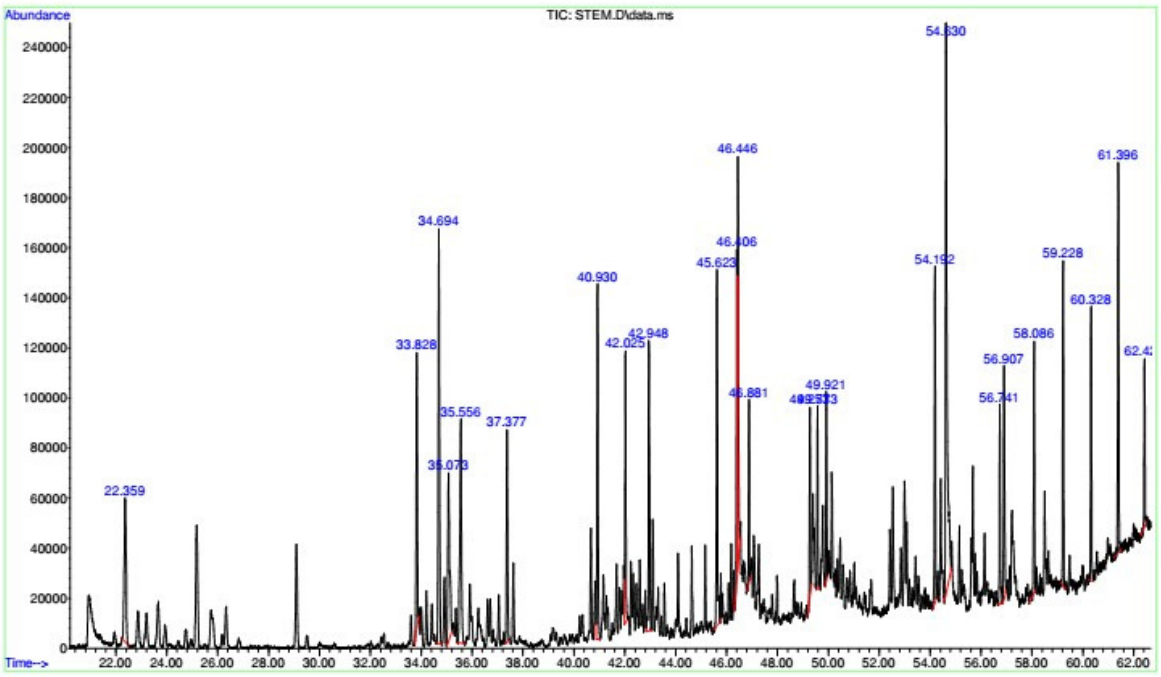


Figure 5.2: GC/MS chromatogram of n-hexane stem extract of *P. foetida* L.

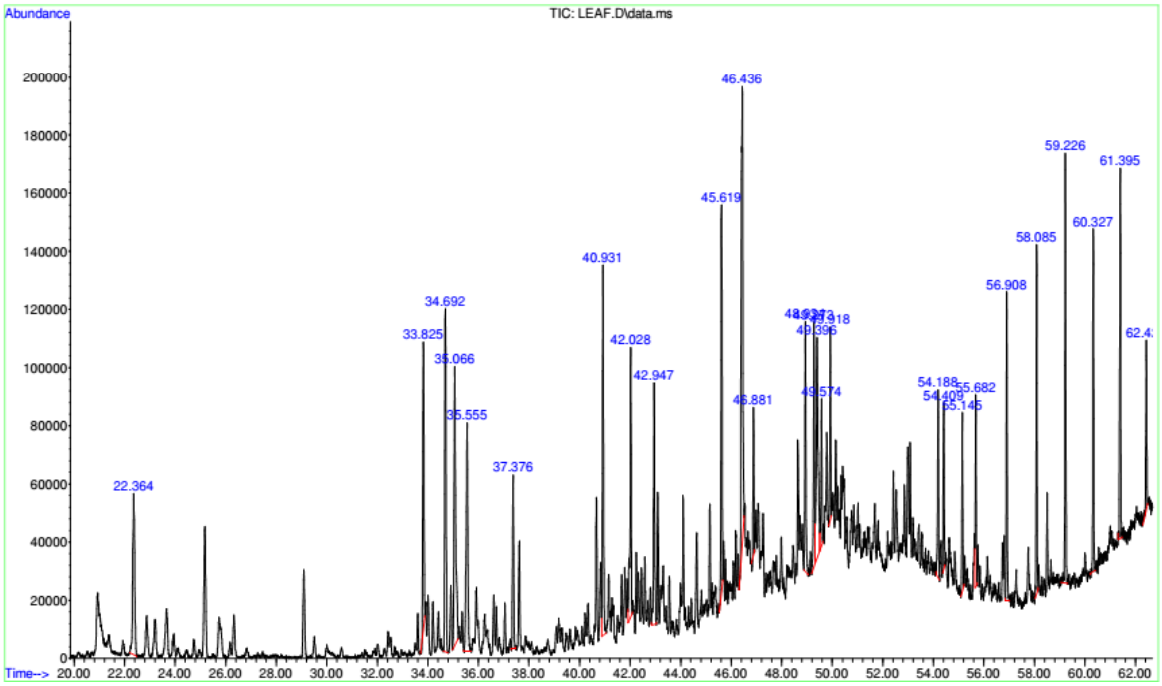


Figure 5.3: GC/MS chromatogram of n-hexane leaf extract of *P. foetida* L.

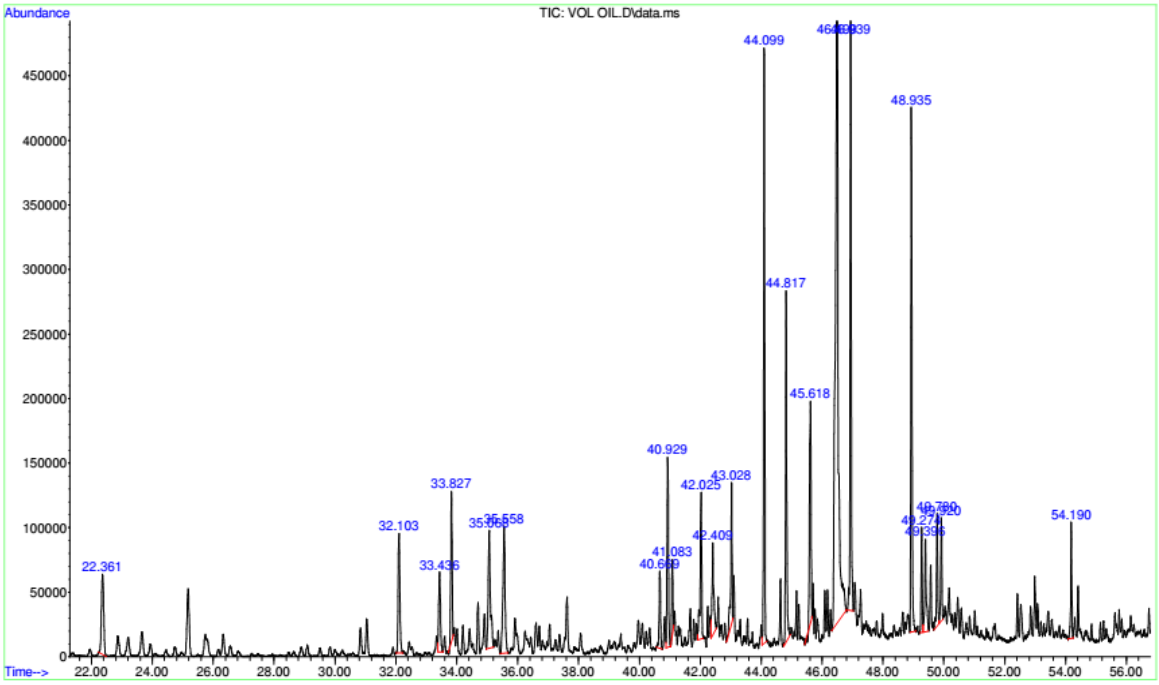


Figure 5.4: GC/MS chromatogram of leaf oil of *P. foetida* L. obtained by hydrodistillation

5.6.1 GC/MS analysis of n-Hexane stem and leaf extract

A total of 35 different phytoconstituents belonging to 9 chemical classes were identified from the n-hexane stem extract. The alkanes/alkenes fraction has the major contribution (65.71%). The other main chemical classes were found to be carboxylic acid and their ester (other than fatty acid and their ester), aromatic carboxylic acid and their ester and fatty acid and their ester. Out of 35 constituents Docosane, Cetene, 2-Methyldecane, 5-Octadecene, Octacosane, n-tricosane, Palmitic Acid, Glycine, 2-Monopalmitin, Hexatriacontane, 2,4-di-tert-butyl phenol, Nonadecane, Triacontane, Ethyl 4-ethoxybenzoate, Eicosane, Heptacosane, Hentriacontane are the major compounds where as n-Tridecan-1-ol, Heptadecane, Tetradecane, Diisobutyl phthalate, n-Pentacosane, Hexadecyl trichloroacetate, 9-Octylhepdecane, Butyric acid, 1-Iodooctadecane, Oxalic acid, Heneicosane, Tetracosane, N-Pentatriacontane, Tritetracontane, 3-Methylindole, Squalene, Octadecane, Pentacosane, Hexadecane are the minor one. The 35 constituents are listed in table 5.4 and the distribution of classes illustrated in figure 5.5.

Table 5.5 showed a total of 34 different chemotypes present in n-hexane leaf extract. This fraction is dominated by palmitic acid (6.64%) whereas Cyclododecane present as minor constituents. The broad chemical classification was shown in figure 5.6.

Table 5.3: Phyto-constituents identified in *P. foetida* L.

Sl No.	Phyto-constituents	% present in n-hexane stem extract	% present in n-hexane leaf extract	% present in hydrodistilled leaf oil
1	Nonadecane	2.62	2.94	0.49
2	N-Tridecan-1-ol	1.42	-	-
3	Triacontane	2.72	1.51	-
4	2,4-ditert-butyl phenol	4.31	3.83	0.72
5	Ethyl 4-ethoxybenzoate	2.51	4.06	2.66
6	Docosane	3.19	1.72	0.75
7	Cetene	2.07	1.82	-
8	n-Hexeadecane	0.80	1.23	1.09
9	Heptadecane	1.15	1.62	1.10
10	2-Methyldecane	3.31	-	-
11	Heptacosane	2.37	2.44	2.50
12	5-Octadecene	2.74	-	-
13	Tetradecane	0.94	-	-
14	Diisobutyl phthalate	0.76	0.90	0.82
15	Octacosane	2.88	1.78	4.04
16	n-tricosane	2.39	-	-
17	Palmitic Acid	3.17	6.64	20.82
18	Hexadecyl trichloroacetate	1.11	-	-
19	9-Octylhepdecane	1.84	-	-
20	Butyric acid	1.55	-	-
21	1-iodooctadecane	1.58	-	-
22	Oxalic acid	0.89	-	-
23	Heneicosane	0.69	2.91	2.01
24	Tetracosane	0.68	-	-
25	Glycine	2.62	-	-
26	2-Monopalmitin	7.91	-	-
27	Tritetracontane	0.98	1.60	-
28	3-Methylindole	1.59	-	-
29	Squalene	0.57	0.78	-
30	Eicosane	2.13	3.06	1.87
31	Hexatriacontane	3.06	3.30	-
32	Octadecane	1.25	1.45	0.78
33	Phytane	-	2.13	1.52
34	Cyclododecane	-	1.23	-
35	n-Dodecane	-	3.31	2.72
36	Pentadecane	-	3.56	-
37	1-Octadecene	-	2.41	-
38	10-Methylnonadecane	-	0.77	-

39	2-Bromotetradecane	-	3.66	-
40	Myristic acid	-	1.09	2.23
41	Methyl linoleate	-	1.69	-
42	Hentriacontane	2.15	0.65	-
43	Sulfurous acid, butyl tetradecyl ester	-	1.32	-
44	Dotriacontane	-	2.64	-
45	Geranylacetone	-	-	2.14
46	Beta Ionone	-	-	1.47
47	Palmitaldehyde	-	-	0.86
48	Ethyl myristate	-	-	2.23
49	2-pentadecanone, 6,10,14-trimethyl	-	-	6.47
50	Cetanol	-	-	4.53
51	Methyl palmitate	-	-	0.56
52	Ethyl palmitate	-	-	7.21
53	Phytol	-	-	6.03
54	Hexacosane	-	0.86	1.15
55	Pentacosane	0.67	0.73	1.19
56	Pentatriacontane	0.52	0.95	0.52
57	Diisooctyl phthalate	-	1.37	-

Among all the characterized constituents *viz.* Nonadecane, Phytane, Heneicosane, Phenol, 2,4-di-tert-butyl, Ethyl 4-ethoxybenzoate, n-Dodecane, Pentadecane, 1-Octadecene, 2-Bromotetradecane, Palmitic acid, Heptacosane, Dotriacontane, Hexatriacontane, Eicosane, were found to be present in major amount where as Octadecane, Squalene, Myristic acid, Methyl linoleate, Tritetracontane, Octacosane, Hentriacontane, Sulfurous acid, butyl tetradecyl ester, Pentatriacontane, diisooctyl phthalate, Triacontane, Diisobutyl phthalate, 10-Methylnonadecane, Docosane , Pentacosane, Cetene , n-Hexadecane, Heptadecane , Hexacosane etc. as minor compounds.

5.6.2 GC/MS analysis of hydro-distilled leaf oil

GC/MS chemometric profiling of distilled leaf oil characterized the presence of a total of 28 chemotypes listed in table 5.6. The alkanes/alkenes fraction has the major contribution (50%). The other main chemical classes were found to be fatty acid and their ester (17.86%) and aldehyde and ketones (14.29%). Among the 28 Compounds, ethyl palmitate, Phytol, Octacosane, Cetanol, 2-pentadecanone,6,10,14-trimethyl,

Table 5.4: GC/MS characterize phytoconstituents present in n-hexane stem extract of *P. foetida* L.

S. No.	Peak RT (min)	Compound detected	Peak area (%)	Mol. Formula	Mol. Wt.	CAS No.
1	22.35	Nonadecane	2.62	$C_{19}H_{40}$	268.5209	629-92-5
2	29.090	n-Tridecan-1-ol	1.42	$C_{13}H_{28}O$	200.36082	112-70-9
3	33.82	Triacontane	2.72	$C_{34}H_{70}$	478.9196	638-68-6
4	34.69	2,4-di-tert-butyl phenol	4.31	$C_{14}H_{22}O$	206.32388	96-76-4
5	35.07	Ethyl 4-ethoxybenzoate	2.51	$C_{11}H_{14}O_3$	194.22706	23676-09-7
6	35.55	Docosane	3.19	$C_{22}H_{46}$	310.60064	629-97-0
7	37.37	Cetene	2.07	$C_{16}H_{32}$	224.42528	629-73-2
8	37.62	n-Hexeadecane	0.80	$C_{16}H_{34}$	226.44116	544-76-3
9	40.66	Heptadecane	1.15	$C_{17}H_{36}$	240.46774	629-78-7
10	40.93	2-Methyldecane	3.31	$C_{11}H_{24}$	156.30826	6975-98-0
11	42.02	Heptacosane	2.37	$C_{27}H_{56}$	380.73354	593-49-7
12	42.94	5-Octadecene (e)	2.74	$C_{18}H_{36}$	252.47844	7206-21-5
13	43.09	Tetradecane	0.94	$C_{14}H_{30}$	198.39	629-59-4
14	44.63	Diisobutyl phthalate	0.76	$C_{16}H_{22}O_4$	278.34348	84-69-5
15	45.16	n-Pentacosane	0.67	$C_{25}H_{52}$	352.68038	629-99-2
16	45.62	Octacosane	2.88	$C_{28}H_{58}$	394.76012	630-02-4
17	46.40	n-tricosane	2.39	$C_{23}H_{48}$	324.62722	638-67-5
18	46.44	Palmitic Acid	3.17	$C_{16}H_{32}O_2$	256.42408	57-10-3
19	46.88	Hexadecyl trichloroacetate	1.11	$C_{18}H_{33}Cl_3O_2$	387.81242	74339-54-1
20	49.27	9-Octylhepdecane	1.84	$C_{25}H_{52}$	352.68038	7225-64-1
21	49.57	Butyric acid	1.55	$C_4H_8O_2$	88.11	107-92-6
22	49.92	1-iodooctadecane	1.58	$C_{18}H_{37}I$	380.39	629-93-86
23	50.13	Oxalic acid	0.89	$C_2H_2O_4$	90.03	144-62-7
24	52.41	Heneicosane	0.69	$C_{21}H_{44}$	296.57	629-94-7
25	53.08	Tetracosane	0.68	$C_{24}H_{50}$	3 38.65	646-31-1
26	54.19	Glycine	2.62	$C_2H_5NO_2$	75.07	56-40-6
27	54.63	2-monopalmitin	7.91	$C_{19}H_{38}O_4$	330.5	23470-00-0
28	55.62	N-pentatriacontane	0.52	$C_{35}H_{72}$	492.95	630-07-9
29	55.68	Tritetracontane	0.98	$C_{43}H_{88}$	605.158813	7098-21-7
30	56.74	3-methylindole	1.59	C_9H_9N	131.17	83-34-1
31	56.90	Hentriacontane	2.15	$C_{31}H_{64}$	436.84	630-04-6
32	58.50	Squalene	0.57	$C_{30}H_{50}$	410.72	7683-64-9
33	60.32	Eicosane	2.13	$C_{20}H_{42}$	282.55	112-95-8
34	61.39	Hexatriacontane	3.06	$C_{36}H_{74}$	506.97	630-06-8
35	62.42	Octadecane	1.25	$C_{18}H_{38}$	254.49432	593-45-3

Table 5.5: GC/MS characterize phytoconstituents present in n-hexane leaf extract of *P. foetida* L.

S. No.	Peak RT (min)	Compound detected	Peak area (%)	Mol. Formula	Mol. Wt	CAS No
1	22.36	Nonadecane	2.94	$C_{19}H_{40}$	268.52	629-92-5
2	25.17	Phytane	2.13	$C_{20}H_{42}$	282.55	638-36-8
3	29.09	Cyclododecane	1.23	$C_{12}H_{24}$	168.32	294-62-2
4	33.82	Heneicosane	2.91	$C_{21}H_{44}$	296.57	629-94-7
5	34.69	2,4-di-tert-butyl Phenol	3.83	$C1_4H_{22}O$	206.32	96-76-4
6	35.06	Ethyl 4-ethoxybenzoate	4.06	$C_{11}H_{14}O_3$	194.23	23676-09-7
7	35.55	n-Dodecane	3.31	$C_{12}H_{26}$	170.33	112-40-3
8	37.37	Cetene	1.82	$C_{16}H_{32}$	224.42	629-73-2
9	37.62	n-Hexeadecane	1.23	$C_{16}H_{34}$	226.44	544-76-3
10	40.66	Heptadecane	1.62	$C_{17}H_{36}$	240.47	629-78-7
11	40.83	Hexacosane	0.86	$C_{26}H_{54}$	366.71	630-01-3
12	40.93	Pentadecane	3.56	$C_{15}H_{32}$	212.41	629-62-9
13	42.02	Docosane	1.72	$C_{22}H_{46}$	310.6	629-97-0
14	42.94	1-Octadecene	2.41	$C_{18}H_{36}$	252.48	112-88-9
15	43.09	Pentacosane	1.16	$C_{25}H_{52}$	352.680	629-99-2
16	44.63	Diisobutyl phthalate	0.90	$C_{16}H_{22}O_4$	278.34	84-69-5
17	45.16	10-Methylnonadecane	0.77	$C_{20}H_{42}$	282.55	56862-62-5
18	45.61	2-Bromotetradecane	3.66	$C_{14}H_{29}Br$	277.28	74036-95-6
19	46.43	Palmitic acid	6.64	$C_{16}H_{32}O_2$	256.42	57-10-3
20	46.88	Myristic acid	1.09	$C_{14}H_{28}O_2$	228.37	544-63-8
21	48.64	Methyl linoleate	1.69	$C_{19}H_{34}O_2$	294.47	112-63-0
22	49.78	Tritetracontane	1.60	$C_{43}H_{88}$	605.15	7098-21-7
23	49.92	Octacosane	1.78	$C_{28}H_{58}$	394.76	630-02-4
24	52.41	Hentriacontane	0.65	$C_{31}H_{64}$	436.84	630-04-6
25	52.98	Sulfurous acid, butyl tetradecyl ester	1.32	$C_{18}H_{38}O_3S$	334.25	999541-21-2
26	53.08	Pentatriacontane	0.95	$C_{35}H_{72}$	492.95	630-07-9
27	55.14	Diisooctyl phthalate	1.37	$C_{24}H_{38}O_4$	390.56	27554-26-3
28	55.68	Triacontane	1.51	$C_{34}H_{70}$	478.91	638-68-6
29	56.90	Heptacosane	2.44	$C_{27}H_{56}$	380.73	593-49-7
30	58.08	Dotriacontane	2.64	$C_{32}H_{66}$	450.87	544-85-4
31	58.50	Squalene	0.78	$C_{30}H_{50}$	410.72	7683-64-9
32	59.22	Hexatriacontane	3.30	$C_{36}H_{74}$	506.97	630-06-8
33	61.39	Eicosane	3.06	$C_{20}H_{42}$	282.55	112-95-8
34	62.42	Octadecane	1.45	$C_{18}H_{38}$	254.49	593-45-3

Table 5.6: GC/MS characterize phytoconstituents present in leaf oil of *P. foetida* L. obtained by hydrodistilation

S. No.	Peak RT (min)	Compound detected	Peak area (%)	Mol. Formula	Mol. Wt	CAS No
1	22.36	Eicosane	1.87	$C_{20}H_{42}$	282.55	112-95-8
2	25.17	Phytane	1.52	$C_{20}H_{42}$	282.55	638-36-8
3	32.10	Geranylacetone	2.14	$C_{13}H_{22}O$	194.31	3796-70-1
4	33.43	Beta Ionone	1.47	$C_{13}H_{20}O$	192.3	79-77-6
5	33.82	Heneicosane	2.01	$C_{21}H_{44}$	296.57	629-94-7
6	34.69	2,4-di-tert-butyl Phenol	0.72	$C_{14}H_{22}O$	206.32	96-76-4
7	35.07	Ethyl 4-ethoxybenzoate	2.66	$C_{11}H_{14}O_3$	194.23	23676-09-7
8	35.55	Dodecane	2.72	$C_{12}H_{26}$	282.55	112-40-3
9	37.62	Hexeadecane	1.09	$C_{16}H_{34}$	226.44	544-76-3
10	40.66	Heptadecane	1.10	$C_{17}H_{36}$	240.47	629-78-7
11	40.92	Heptacosane	2.50	$C_{27}H_{56}$	380.73	593-49-7
12	41.08	Palmitaldehyde	0.86	$C_{16}H_{32}O$	240.42	629-80-1
13	42.41	Myristic acid	2.23	$C_{14}H_{28}O_2$	228.37	544-63-8
14	43.02	Ethyl myristate	2.61	$C_{16}H_{32}O_2$	256.42	124-06-1
15	43.09	Octadecane	0.78	$C_{18}H_{38}$	254.49	593-45-3
16	44.10	2-pentadecanone, 6,10,14-trimethyl	6.47	$C_{18}H_{36}O$	268.48	502-69-2
17	44.63	Diisobutyl phthalate	0.82	$C_{16}H_{22}O_4$	278.34	84-69-5
18	44.81	Cetanol	4.53	$C_{16}H_{34}O$	242.44	36653-82-4
19	45.16	n-Nonadecane	0.49	$C_{19}H_{40}$	268.52	629-92-5
20	45.61	Octacosane	4.04	$C_{28}H_{58}$	394.76	630-02-4
21	45.69	Methyl palmitate	0.56	$C_{17}H_{34}O_2$	270.45	112-39-0
22	46.49	Palmitic acid	20.82	$C_{16}H_{32}O_2$	256.42	57-10-3
23	46.94	Ethyl palmitate	7.21	$C_{18}H_{36}O_2$	284.48	628-97-7
24	48.93	Phytol	6.03	$C_{20}H_{40}O$	296.53	150-86-7
25	49.27	Hexacosane	1.15	$C_{26}H_{54}$	366.71	630-01-3
26	49.92	Pentacosane	1.19	$C_{25}H_{52}$	352.68	629-99-2
27	52.99	Docosane	0.72	$C_{22}H_{46}$	310.6	629-97-0
28	54.41	Pentatriacontane	0.52	$C_{35}H_{72}$	492.95	630-07-9

Myristic acid, Ethyl myristate, Heptacosane, Ethyl 4-ethoxybenzoate, Heneicosane, Geranylacetone, Dodecane, Palmitic acid are categorized as major compounds and Hexacosane, Pentacosane, Docosane, Pentatriacontane, methyl palmitate, n-Nonadecane, Diisobutyl phthalate, phytane, Octadecane, Palmitaldehyde, Hexadecane, Heptadecane, Beta Ionone, 2,4-di-tert-butyl Phenol, Eicosane categorized as minor compounds. The distribution of chemical classes is shown in figure 5.7. From figure 5.8 we can conclude that stem

produced more volatile principles (61.40%) as compared to the leaf and oil. 26.31% of compounds are similar in all the samples in spite of their different extraction methodology, 38.59% compounds are common in leaf and stem, 33.33% common in leaf and leaf oil where as 26.31% common in stem and leaf oil.

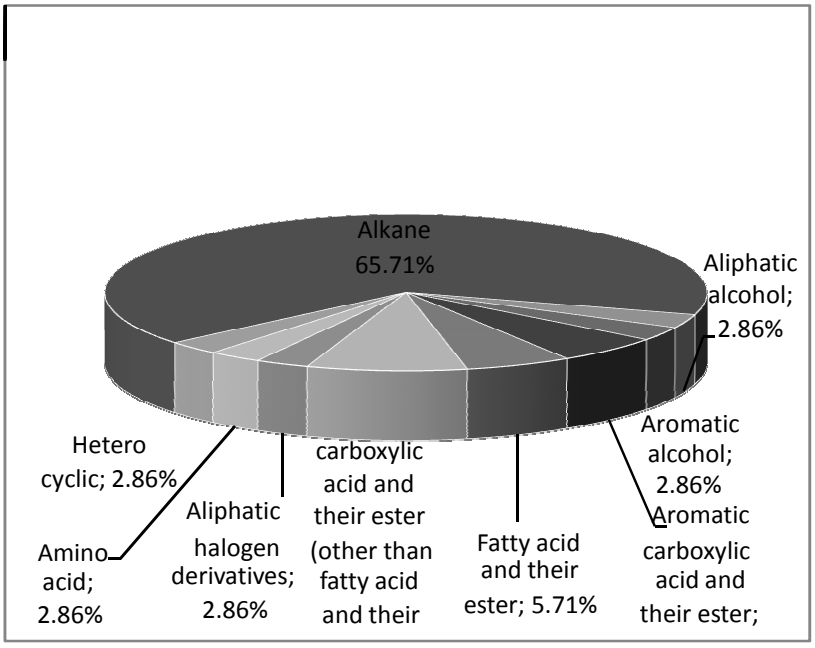


Figure 5.5: Group distributions of phytochemicals in n- Hexane stem extract of *P. foetida* L.

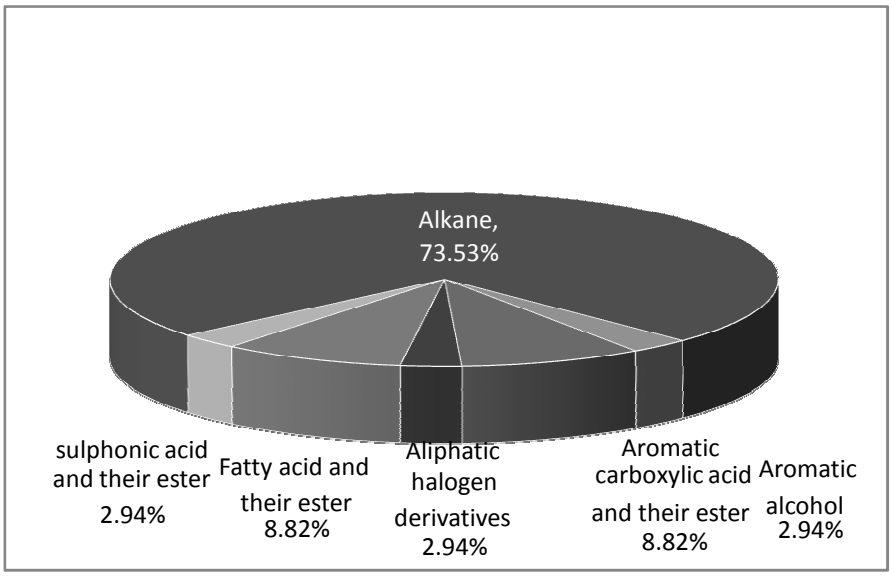


Figure 5.6: Group distributions of phytochemicals in n- Hexane leaf extract *P. foetida* L.

In order to get a clear outlook on the loss of volatile principles while heating, extraction of leaf was done by two methods i.e. aids of heat and without aid of heat. Considering the same starting extract material, in the current study solvent extraction is presuming most productive technique as compared to the distillation technique (aid of heat). By solvent extraction, we obtained more than 10% of volatile principles as compared to distillation technique. In the other way water distillation technique was able to produce 50% of oxygenated compounds (Figure 5.9) as compared to 28.57% and 23.53% with solvent extraction of stem and leaf respectively, indicating the quality of extract/oil. As presence of more oxygenated components recognize as high quality of oil. Structures of all the identified compounds are captured in figure no. 5.10.

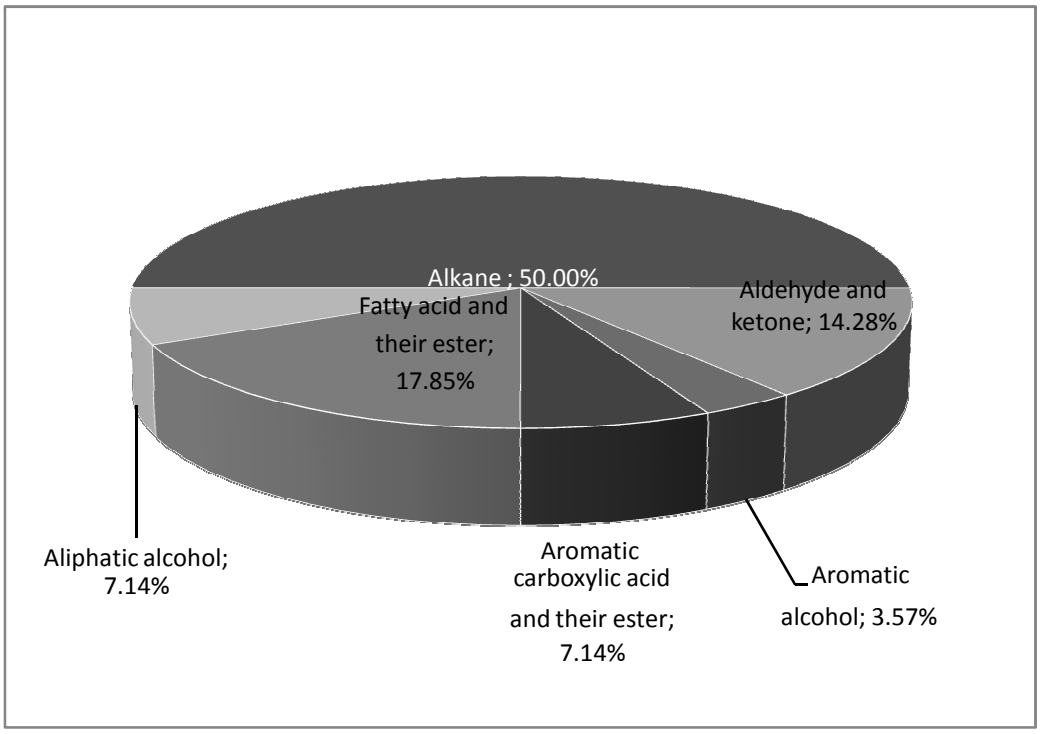


Figure 5.7: Group distributions of phytochemicals in hydrodistilled leaf oil of *P. foetida* L.

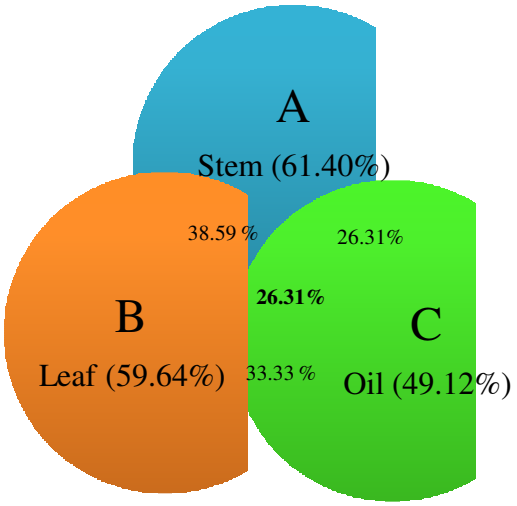


Figure 5.8: Distribution pattern (%) of volatile constituents in stem, leaf and oil (A ∩ B: 38.59%; B ∩ C: 33.33%; A ∩ C: 26.31; A∩B∩ C: 26.31%) of *P. foetida* L.

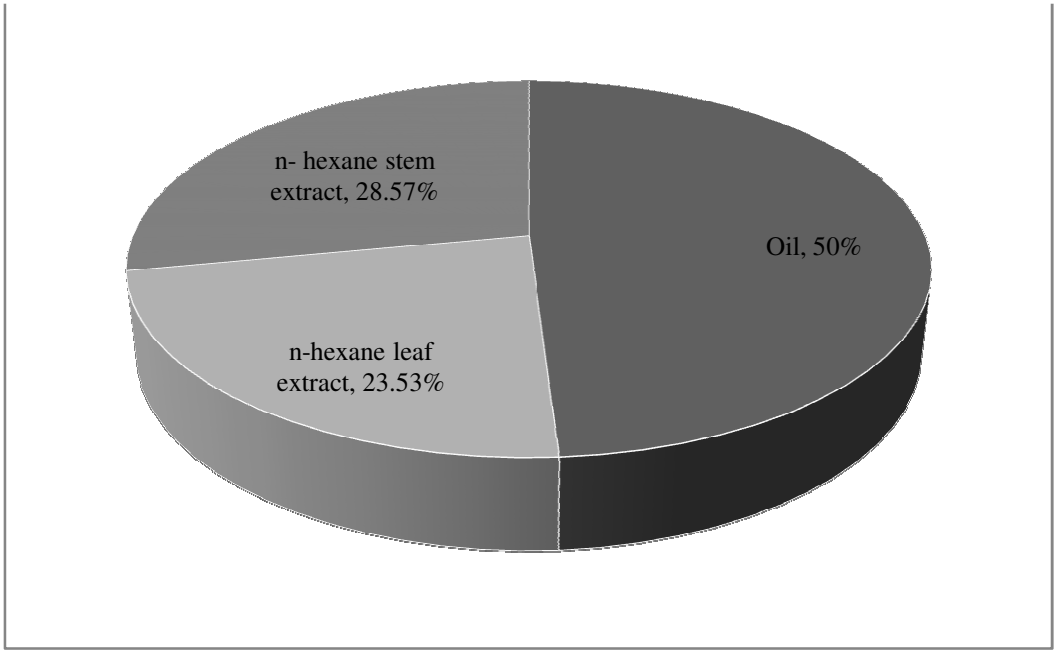
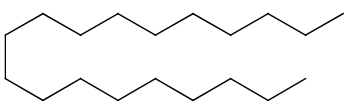
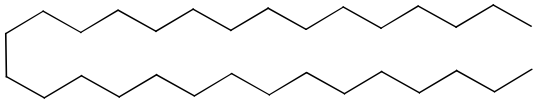
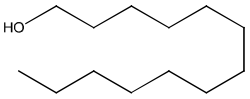


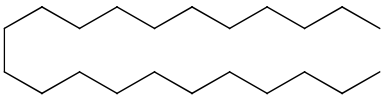
Figure 5.9: Percentage of oxygenated compounds of n-hexane stem, leaf extract and leaf oil of *P. foetida* L.

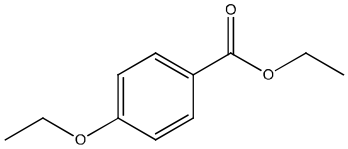

Nonadecane


Triacontane

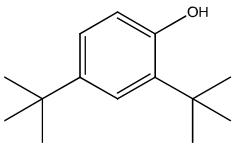


n-Tridecan-1-ol

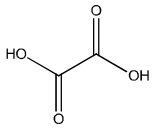

Docosane



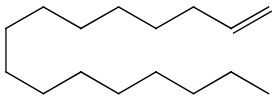
ethyl 4-ethoxybenzoate

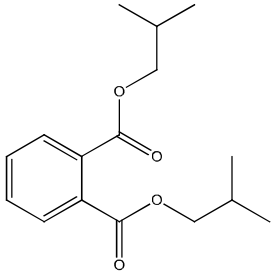


2,4-di-tert-butyl phenol

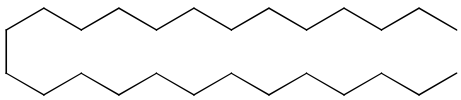


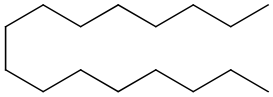
Oxalic acid

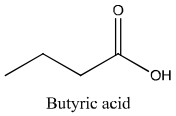

Cetene



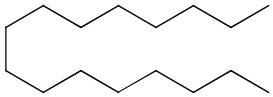
Diisobutyl phthalate

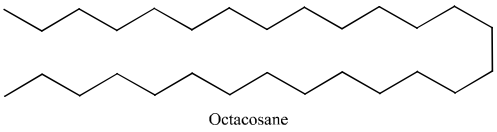

Heptacosane

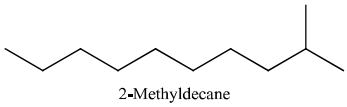

n-Hexadecane

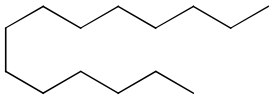


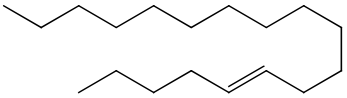
Butyric acid

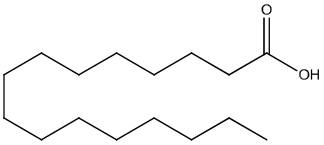

Heptadecane


Octacosane

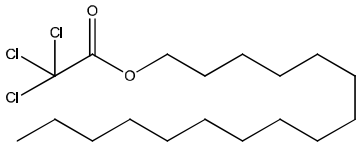

2-Methyldecane


Tetradecane

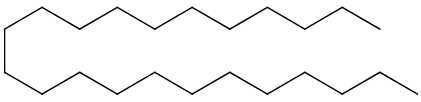

5-Octadecene

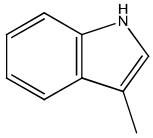


Palmitic Acid

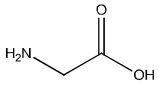


Hexadecyl trichloroacetate

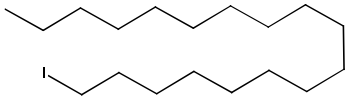

n-tricosane



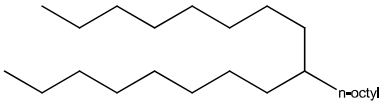
3-methylindole



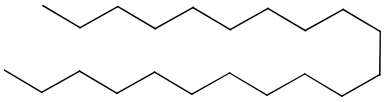
Glycine

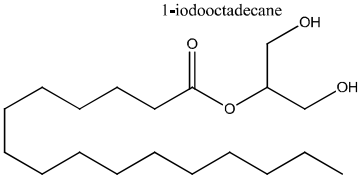


1-iodooctadecane

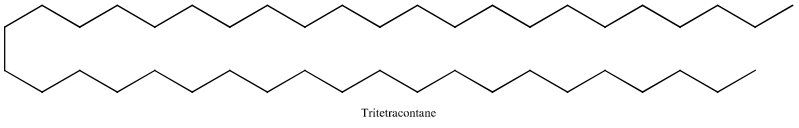


9-octylheptdecane


Heneicosane



2-monopalmitin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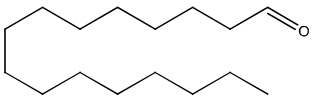
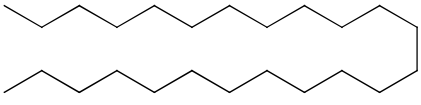
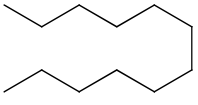
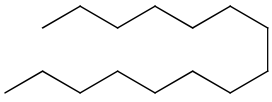
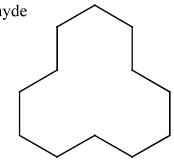


Palmitaldehyde


Tetracosane


n-Dodecane


Pentadecane


Cyclododecane

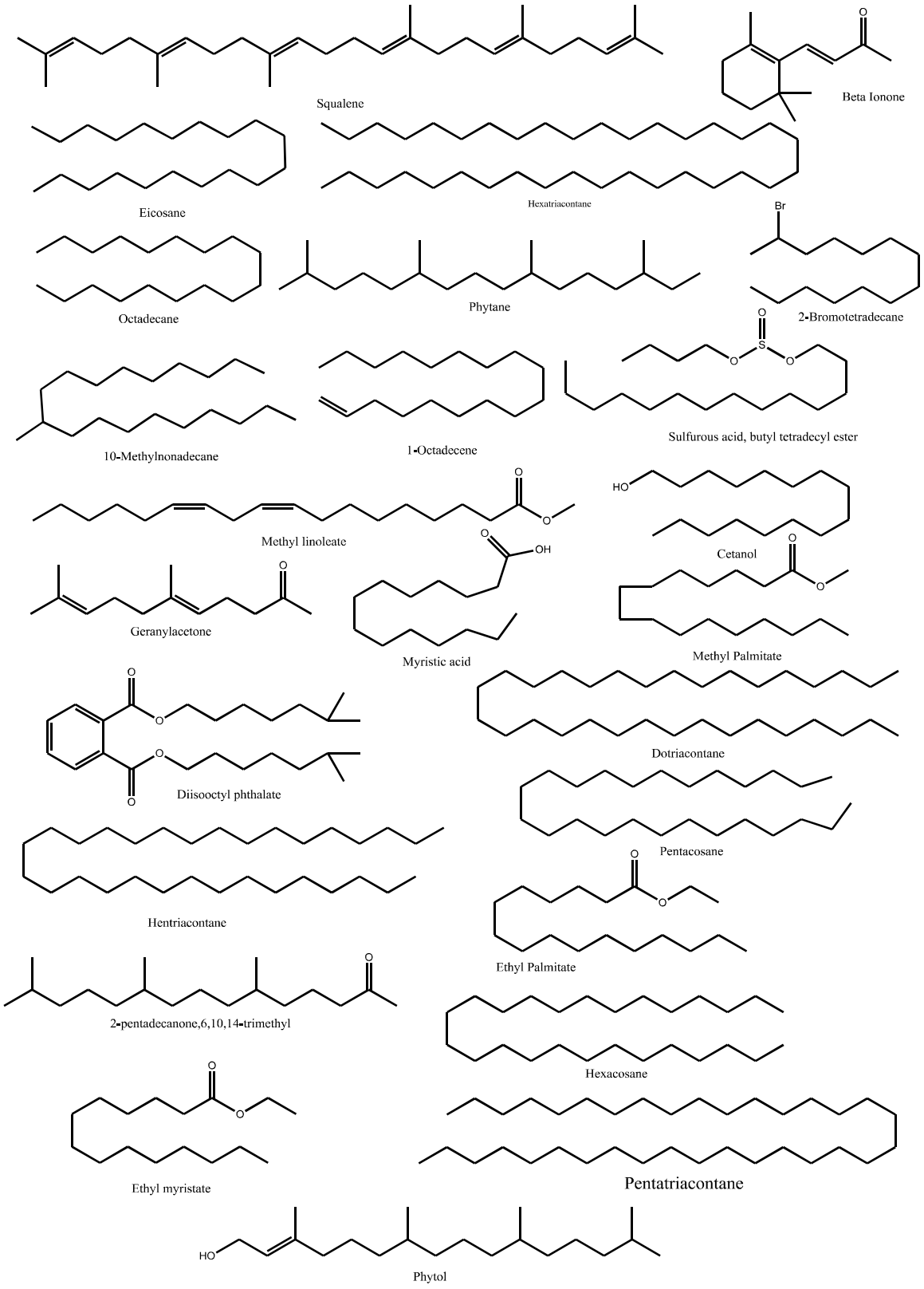


Figure 5.10: Identified phytoconstituents from *P. foetida* L. by GC-MS technique

5.7 Standardization of *P. foetida* extract

5.7.1 Optimization of Thin Layer chromatography solvent system

As per the chemical nature of the extract and literature survey, several solvent systems were used for the development of TLC solvent system. Maximum band and better separation was obtained with the solvent system tolune : ethyl acetate at a ratio of 8:2 (v/v) and toluene: ethyl acetate: formic acid (8:2:0.1 v/v/v) but the better movement of marker compounds and appearance of standards in the extract was achieved by using the solvent system methanol: chloroform and formic acid in a ratio of 8:2.5:0.1(v/v/v). The chromatograms of different solvent system are shown in figure 5.11 (a-g).

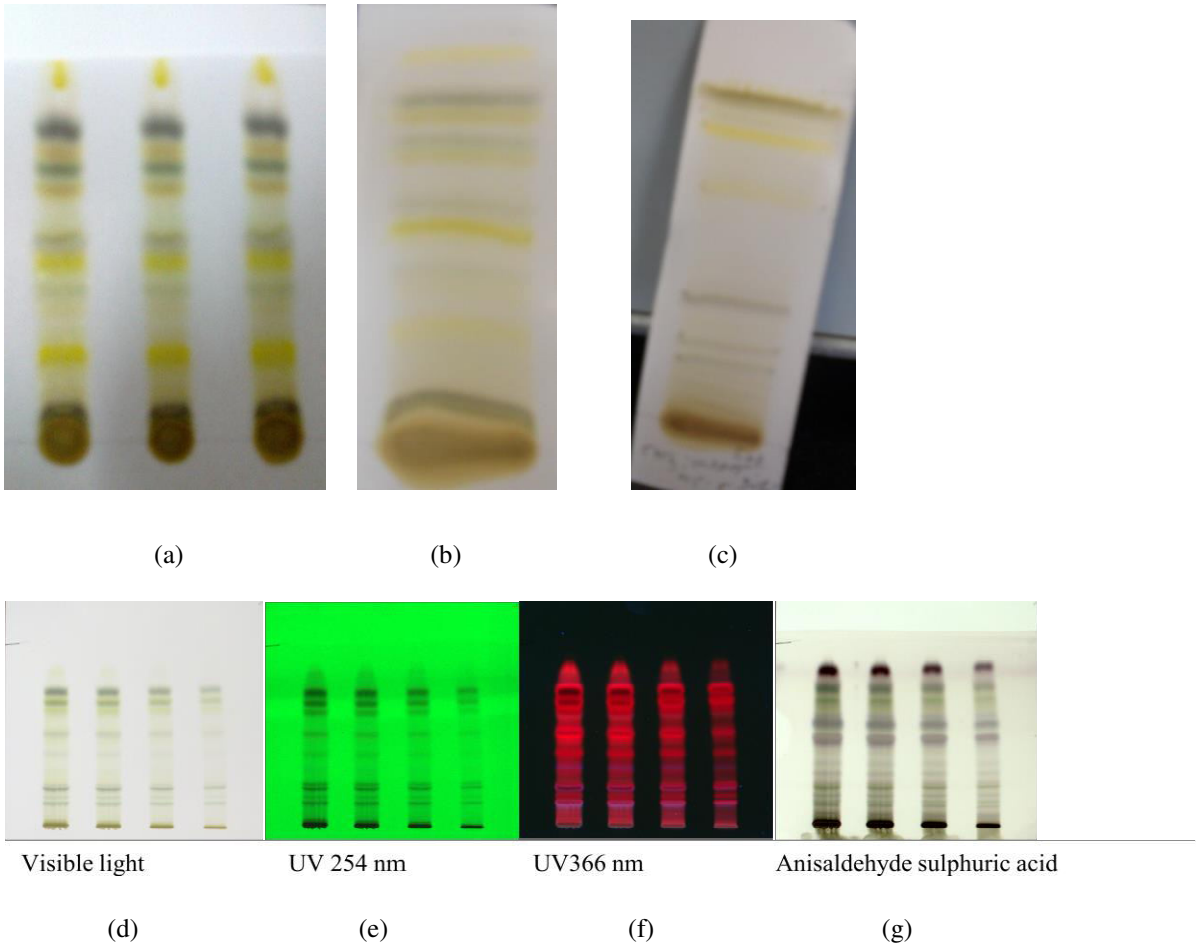


Figure :5.11: TLC of *P. foetida* L. methanol extract, Solvent system used **(a)** Toluene :ethyl acetate (8:2), visible light; **(b)**: Chloroform: methanol (95:5) visible light; **(c)** ethyl acetate: methanol:acetic acid(18:5:0.2), visible light **(d-g)**: TLC of *P. foetida* L. methanol extract, Solvent system used Toluene: ethyl acetate: formic acid (8:2:0.1)

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

Better resolution with compact spot and sharp and well-defined-resolved symmetric band of asperuloside and paederoside were obtained by using the mobile phase chloroform: methanol : formic acid (8:2.5:0.1v/v/v). The HPTLC densitogram of the whole extract was given in figure no. 5.12. The R_f value asperuloside and paederoside was obtained 0.56 and 0.65 respectively (Figure 5.13).

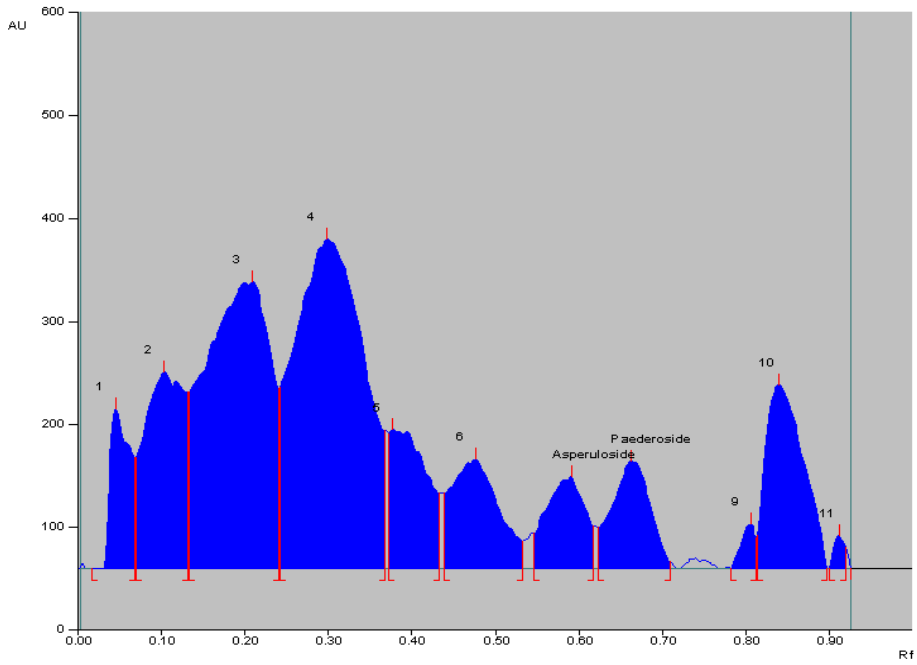


Figure 5.12: HPTLC densitogram of methanol extract of leaf of *P. foetida* L. at 245 nm

The developed HPTLC method was validated in terms of accuracy, precision, repeatability, robustness and other validation parameter. The ICH guidelines were followed throughout the method. The relationship between the concentration of standard solutions and the peak responses were linear (Table 5.7) and within the concentration range of 400-3000ng/spot with a correlation coefficient of 0.998 for asperuloside and 0.994 for paederoside. Figure 5.14 and 5.15 represent the linearity curve of asperuloside and paederoside respectively. Table 5.8 showed good recoveries of the substances i.e. 98 to 99% for both the standards. Accuracy was obtained when spiked with standard at four different concentration levels. The % RSD values with theoretical content are tabulated in table 5.8.

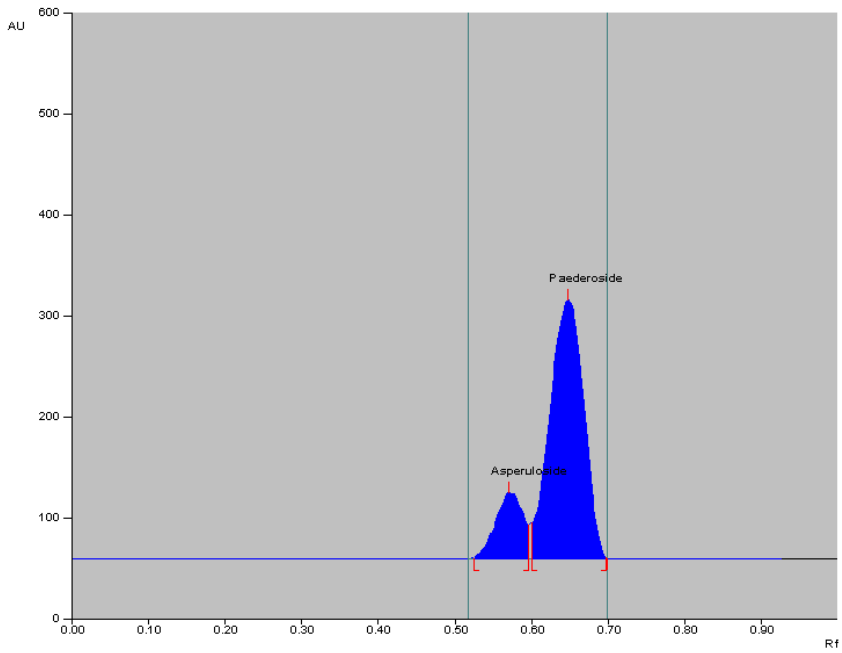


Figure 5.13: HPTLC densitogram of standard asperuloside and paederoside at 245 nm

Table 5.7: Linear regression data for the calibration plot (n=3) of standard Asperuloside and Paederoside

	Asperuloside	Paederoside
Linearity range	400-3000 ng/spot	400-3000 ng/spot
Regression Equation	y = 1.801x - 369.1	y = 6.440x - 174.3
Correlation coefficient	0.998	0.994
Slope	1.801	6.44
Intercept	-369.1	-174.3

The HPTLC method was found to give repeatable results at three different concentrations (600, 800, 1000 ng/spot) showing the same R_f values and thereby the method is found to be reliable. The % RSD tabulated in Table 5.9. Table 5.10 summarizes the intra-inter-day and inter analyst precision. There were insignificant variations in the peak areas (Table 5.10). The LOD and LOQ were found to be 121.23 and 363.69 ng/spot, for asperuloside and 94.11 and 228.33 ng/spot for paederoside. During robustness studies, the %RSD was <2% in each case. Table 5.11 showed the low values of %RSD which represents the robustness of the method. The specificity of the analytes was checked by checking the peak purity or by checking the resolution when mixed with the other related compounds. The spots of asperuloside and paederoside were confirmed by comparing the R_f value and spectra of the

sample with respect to standards. 0.12% w/w asperuloside and 0.097% w/w of paederoside present in the extract was determined from the regression equation.

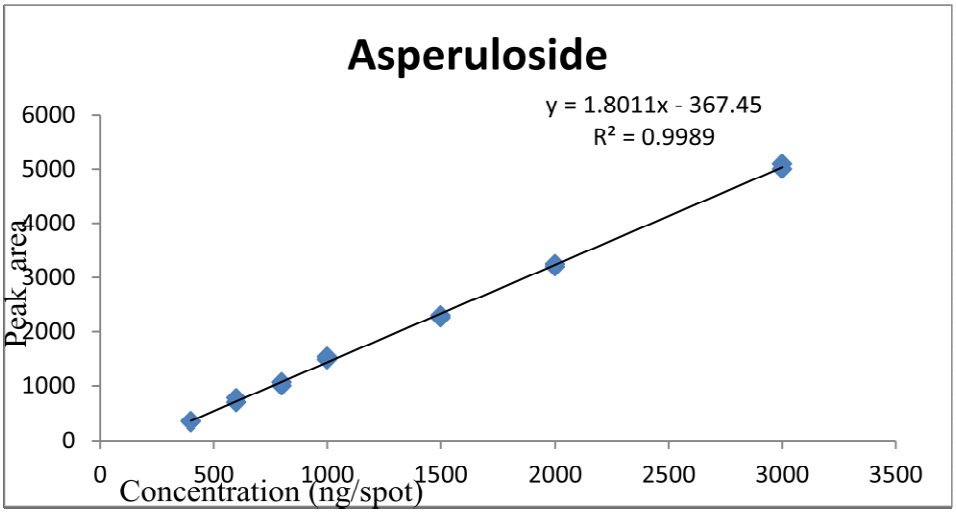


Figure 5.14: Calibration curve for Asperuloside at concentration 400-3000ng/spot scanned at 245nm

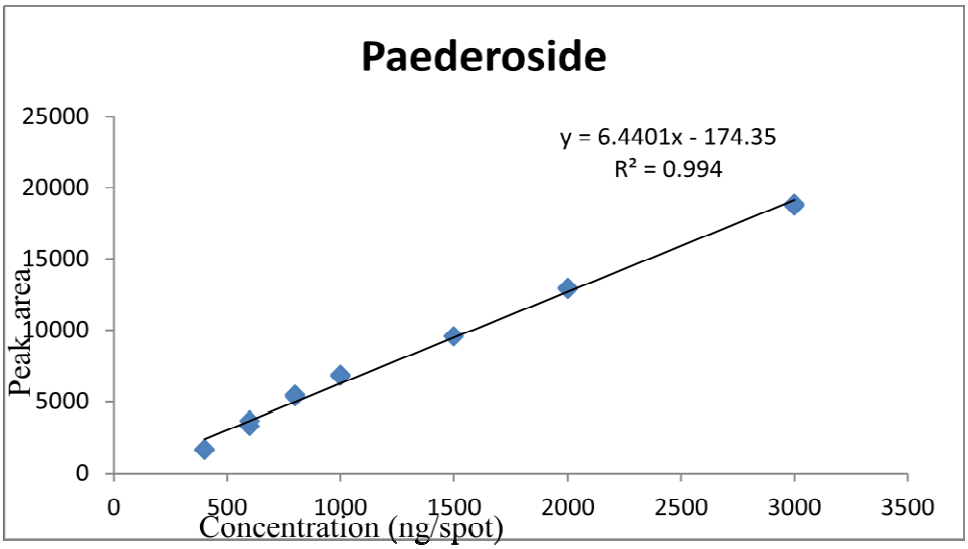


Figure 5.15: Calibration curve for Paederoside at concentration 400-3000ng/spot scanned at 245nm

Table 5.8: Recovery studies for determination of accuracy (n=3; n=No. of times repetition of the experiment) in pre-analyzed samples determined by HPTLC

Substance	Amount (%) of standard spiked in sample	Theoretical content (ng/spot)	Amount of substance recovered (ng/spot)	SD	% of substance recovered	RSD (%)
Asperuloside	0	455	452.89	3.65	99.54	0.81
	50	682.5	678.77	5.02	99.45	0.74
	100	910	903.86	6.35	99.33	0.70
	150	1137.5	1122.59	13.97	98.69	1.24
Paederoside	0	390	385.92	4.01	98.95	1.04
	50	585	577.34	6.99	98.69	1.21
	100	780	774.22	5.23	99.26	0.68
	150	975	970.21	10.89	99.51	1.12

Table 5.9: Repeatability of the HPTLC method (n=6; n=No. of times repetition of experiment)

Standard	Concentration (ng/spot)	Peak area	SD	%RSD	Rf	SD	% RSD
Asperuloside	600	726.21	9.43	1.30	0.56	0.01	0.92
	800	1063.07	15.38	1.45	0.56	0.00	0.73
	1000	1430.12	19.29	1.35	0.56	0.01	0.92
Paederoside	600	3644.99	33.72	0.93	0.65	0.00	0.63
	800	4939.41	30.91	0.63	0.65	0.01	1.16
	1000	6262.32	31.54	0.50	0.65	0.01	1.38

Table 5.10: Precision of the method (n= 3) determined by HPTLC

Standard	Concentration (ng/spot)	Peak area	SD	%RSD
INTER DAY				
Asperuloside	600	721.97	14.27	1.98
	800	1072.89	10.13	0.94
	1000	1453.15	19.22	1.32
Paederoside	600	3668.28	40.18	1.10
	800	4960.64	35.04	0.71
	1000	6234.38	28.82	0.46
INTRA DAY				
Asperuloside	600	718.62	11.96	1.66
	800	1061.25	7.82	0.74
	1000	1448.75	18.95	1.31
Paederoside	600	3648.85	44.95	1.23
	800	4960.41	27.27	0.55
	1000	6229.47	30.50	0.49
INTER ANALYST				
Asperuloside	600	716.85	13.69	1.91
	800	1069.73	11.57	1.08
	1000	1453.09	20.39	1.40
Paederoside	600	3669.92	14.83	0.40
	800	4909.57	64.69	1.32
	1000	6256.60	25.05	0.40

Table 5.11: Robustness of HPTLC method (n= 3)

Standard	Parameter	Used levels	Peak Area	SD	%RSD
Asperuloside	Chloroform : methanol : formic acid (v/v/v)	7.9:2.6:0.1	1414.04	8.21	0.58
		8:2.5:0.1	1426.60	12.14	0.85
		8.1:2.4:0.1	1427.33	8.49	0.59
Paederoside	Chloroform : methanol : formic acid (v/v/v)	7.9:2.6:0.1	6265.47	35.35	0.56
		8:2.5:0.1	6278.10	27.93	0.44
		8.1:2.4:0.1	6281.97	24.87	0.40
Asperuloside	Scanning wavelength (nm)	243	1439.88	12.50	0.87
		245	1432.57	13.53	0.94
		247	1448.97	19.27	1.33
Paederoside	Scanning wavelength (nm)	243	6268.03	44.32	0.71
		245	6321.03	33.43	0.53
		247	6316.10	23.00	0.36

5.8 Evaluation of *in-vitro* antioxidant activity

In vitro anti-oxidant activity of the methanol extract of fresh and dried leaf of *P. foetida* was determined by evaluating DPPH* radical scavenging activity. The experiment was done in triplicate for both the sample. Table 5.12 presents the data of the antioxidant activity of methanol extract of fresh sample, whereas table 5.13 showing the antioxidant activity of methanol extract of dried sample. Rutin was used as reference standard (Table 5.14).
Calculation of percentage of inhibition was done by using the following formula:
% inhibition= $[(A_0-A_1/A_0)]$ X 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 mean percentage inhibition by each concentration was plotted against the log concentration. The 50% inhibitory dose (IC_{50} value) was found by interpolation by using graph pad (prism 6 software) and compared with standard [207]. The IC_{50} value of Methanol extract of fresh and dried leaf of *P. foetida* L. was found to be 40 µg/ml and 43µg/ml respectively.

Table 5.12: Antioxidant activity of methanol extract of fresh leaves of *P. foetida*

Concentration(µg/ml)	OD 1	OD 2	OD 3	% inhibition (OD1)	% inhibition (OD2)	% inhibition (OD3)
10	0.7195	0.7123	0.7898	24.33	22.66	18.82
20	0.5821	0.5902	0.5992	38.78	35.92	38.82
40	0.4502	0.4721	0.4869	52.65	48.74	50.33
80	0.2539	0.3123	0.3211	73.30	66.99	67.25
100	0.1723	0.1998	0.1964	81.88	78.31	79.98
120	0.1634	0.1936	0.1903	82.81	78.98	80.59
Blank	0.9508	0.9210	0.9804	-	-	-

Table 5.13: Antioxidant activity of methanol extract of dried leaves of *P. foetida*

Concentration (µg/ml)	OD 1	OD 2	OD 3	% inhibition (OD1)	% inhibition (OD2)	% inhibition (OD3)
10	0.7629	0.7416	0.8012	19.76	19.48	18.28
20	0.6300	0.6020	0.5969	33.74	34.63	39.12
40	0.5022	0.4912	0.5523	47.18	46.66	43.67
80	0.4294	0.3021	0.3569	54.83	67.19	63.59
100	0.3297	0.2005	0.2417	65.32	78.23	75.35
120	0.2122	0.1998	0.2019	77.68	78.30	79.40
Blank	0.9508	0.9210	0.9804	-	-	-

Table 5.14: Antioxidant activity of standard reference drug (Rutin)

Concentration (µg/ml)	OD 1	OD 2	OD 3	% inhibition (OD1)	% inhibition (OD2)	% inhibition (OD3)
10	0.6704	0.6917	0.7123	24.29	24.89	27.35
20	0.6078	0.6393	0.6065	36.7	30.59	38.13
40	0.4923	0.5123	0.5265	48.22	44.38	46.30
80	0.2729	0.3423	0.3362	70.64	62.83	65.70
100	0.1319	0.1329	0.1371	86.02	85.57	86.02
120	0.1314	0.1316	0.1217	86.12	85.71	87.69
Blank	0.9508	0.9210	0.9804	-	-	-

The antioxidant activity was higher in the fresh sample as compared to the dried one. This indicates the loss of active constituents during the drying or else extraction procedure, which might lead to the reduction of antioxidant activity. The *in vitro* antioxidant activity may be attributed to the presence of β-sitosterol.

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

As *H. pylori* is one of the major risk factor for the development of peptic ulcer disease so it is becoming very necessary to evaluate the anti *H. pylori* activity of any extract before going to do study on animal. Moreover the development of antibiotic resistance by the *H. pylori* is becoming a crucial issue in the success of treatment. Hence it is an urgent need to find out new entities in the treatment of PUD. The methanol extract of *P. foetida* showed promising results against eight different clinical strains of *H. pylori.* Out of the eight strains one *H. pylori* strains were metronidazole (MIC>8µg/ml) resistant. Our result suggested that the methanol extract of Paederia leaf acts through mechanisms distinctly different from the mode of action of these antibiotics for inhibition of *H. pylori* growth. Overall, this study provides novel insights into the therapeutic potential of Paederia extract against *H. pylori* infections, although further studies are required to extrapolate its effect on humans. Table 5.15 shows the zone of inhibition of different strains at different concentrations.

5.10 Evaluation of *in-vivo* anti-ulcer activity

Albino Wistar rats weighing 150 to 180 g of both sexes were used in the current study. There are several methods for the evaluation of gastric and duodenal antiulcer agents like pylorus ligation, stress ulcers, histamine, acetic acid, NSAIDs induced ulcers, reserpine induced solitary chronic gastric ulcers, serotonin-induced gastric mucosal lesions,

cysteamine, dimaprit, endotoxin, dulcerozine, indomethacin plus histamine, MPTP (1-methyl-4phenyl-1,2,3,6 tetrahydro pyridine) induced duodenal ulcers etc. Here we used four different models to evaluate the basic mechanism of action involved, i.e. H2 antagonism or anti-secretory or proton pump hydrogen potassium ATPase inhibitors (PPIs) or cytoprotection etc. A total of four different models used to evaluate the anti-ulcer activity of the plant extract.

Table 5.15: Anti *H. pylori* activity of methanol extract of leaf of *P. foetida* L. at different concentration

Strain No.	**Concentration (µg/ml)**											
	25	**50**	**100**	**200**	**400**	**500**	**800**	**1000**	**1600**	**1800**	**2000**	**3000**
340 A	-	-	0	0	7mm	-	9mm	-	11mm	-	11mm	-
347A	-	0	6mm	6mm	-	7mm	-	8mm	-	12mm	12mm	-
339A	-	0	6mm	6mm	-	7mm	-	9mm	-	13mm	13mm	-
354A	-	-	0mm	-	-	0	-	6mm	-	11mm	11mm	-
137A	-	0	6mm	-		6mm	-	7mm	-	-	8mm	11mm
122A	-	-	0mm	0	-	6mm	-	9mm		-	10mm	11mm
383A	0	6	11mm	-	-	11mm	-	11mm	-	-	-	-
399A	0	4	11mm	-	-	11mm	-	11mm	-	-	-	-

Number indicating *H. pylori clinical strains*; Strain 399A Metronidazole resistant strain; Except 399A all Metronidazole sensitive strains; (-) indicate not done; 0 indicate no zone of inhibition.

5.10.1 Indomethacin-pylorus ligation model

In this model administration of methanol extract at two different doses i.e.100 and 200 mg/kg were used for the evaluation of anti ulcer activity. The parameters evaluated for anti ulcer activity were volume, pH, acid output, ulcer index, ulcer protection etc. (Table 5.16). The extract in both the doses inhibited the increase level of gastric secretion volume, acid output whereas there is an increase in pH. The effect was more significant with higher dose. It showed ulcer protection, 72% and 78% at the dose of 100 and 200mg/kg, respectively, where ranitidine used as reference drug showed 82% of ulcer protection. Indomethacin inhibits activity of cyclo-oxygenase and decreases the formation of prostaglandins (PGs) [221-225]. It causes drastic changes in arachidonic acid metabolism, particularly during the

first hour of its treatment. Indomethacin does not inhibit the lipoxygenase pathway of arachidonic acid while it strongly inhibits the cyclo-oxygenase pathway. The increased level of LTs in gastric mucosa has potent action on the mucosal vasculature [225]. Lipid mediators play important role in causing inflammation and pain. Indomethacin potentiates the secretary response elicited by histamine due to the inhibition of PGE_2 synthesis.

Therefore, induction of gastric ulcers and erosions is accompanied by progressive decrease in the level of PGE_2 [226,227]. In indomethacin-pylorus induced ulcer model methanol extract significantly decreased the gastric secretion volume, acid out-put and ulcer index while gastric pH was increased significantly in treated rats.

Table 5.16: Effect of methanol extract on gastric secretion volume, pH, acid-output and ulcer index against indomethacin-pylorus ligation induced ulcer in rats

Treatment	**Gastric secretion (Mean ± SEM)** Volume (ml)	pH	acid output (Eq/100g/4hr)	**Ulcer index (mm) Mean ± SEM**	**% of ulcer protection**
Group 1 (5ml distilled water/kg)	2.114±0.13	2.80±0.01	2.80±0.01	2.850±0.07	-
Group 2 Indomethacin (25mg/kg)	9.280±0.09 $^{\alpha}$	1.07±0.01 $^{\alpha}$	244.7±2.28 $^{\alpha}$	39.12±0.81 $^{\alpha}$	-
Group3 MeOH PF (100mg/kg)	4.296±0.11*	2.51±0.02*	10.99±0.11*	10.57±0.15*	72.98
Group 4 MeOH PF (200mg/kg)	2.670±0.21*	2.71±0.71*	7.62±0.14*	8.38±0.05*	78.58
Group5 Ranitidine (10mg/kg)	2.426±0.52*	2.89±0.03*	5.30±0.31*	6.69±0.06*	82.89

MeOH PF= methanolic extract of *Paederia foetida*; n=5; Data = Mean±SEM; $^{\alpha}$P<0.001 (G1 Vs G2); *P<0.001, (Group 2 Vs Group 3, 4 & 5)

This model indicates its anti secretary mechanism and revealed that methanol extract of *P. foetida* must contain active anti-ulcer constituents as they effectively antagonized the gastric ulcerogenic effects.

5.10.2 Alcohol induced gastric ulcer model

Oral administration of ethanol induces ulcer. In this model ulcer score was studied. Pretreatment of such rats with different doses of the extracts showed highly significant protection to ulceration. 59 % and 72% ulcer protection was seen in the dose of 100 and 200mg/kg, respectively, whereas standard drug sucralfate showed 81% of ulcer protection (Table 5.17). Alcohol causes erosion and necrosis of superficial epithelial cells on gastric mucosa. Gastric damage in rats may be due to the production of leukotrienes production and also involvement of 5-lipooxygenase.

Table 5.17: Effect of methanol extract of *P. foetida* on alcohol-induced gastric ulcer in rats

Treatment	Ulcer score	% of ulcer protection
Group 1 (5ml distilled water/kg, p.o.)	2.0 ± 0.12	-
Group 2 (5ml distilled water + 70% ethanol, 10ml/kg, p.o.)	3.70 ± 0.49 $^{\alpha}$	-
Group 3 MeOH PF (100mg/kg)	1.40 ± 0.29*	59.45
Group 4 MeOH PF (200mg/kg)	1.0 ± 0.31*	72.97
Group 5 Sucralfate (100mg/kg, p.o.)	0.70 ± 0.12*	81.08

MeOH PF= methanolic extract of *Paederia foetida*; n=5; Data = Mean±SEM; $^{\alpha}$P<0.05 (G1 Vs G2); *P<0.05, (Group 2 Vs Group 3, 4 & 5)

Prostaglandin also plays role in alcohol induced ulcer [228,229]. When compared with positive control, decrease in mucosal resistance is considered to be a most important etiological reason in alcohol induced gastric ulcers. Even, there are reports that alcohol increases the secretion of protein into the gastric juice. In addition, alcohol also causes ulcer by producing toxic oxygen derived free radicals.

5.10.3 Water immersion stress induced ulcer (WISIU) model

Anti-ulcer effect of *P. foetida* L. is further confirmed by WISIU model, in this case the lesion form due to both emotional and physiological stress. Lansoprazole is used to evaluate its proton pump inhibition mechanism. Ulcer score was studied to evaluate the anti ulcer activity. The methanol extract (100 and 200mg/kg) dose dependently showed highly significant ($p<0.001$) ulcer protective (62 and 67%) to stomach mucosa of rats against WISIU model where as lansoprazole showed 70% protection (Table 5.18).

Table 5.18: Effect of methanol extract of *P. foetida* on water immersion stress induced ulcer in rats

Treatment	Ulcer score	% of ulcer protection
Group 1 (5ml distilled water/kg, p.o.)	2.0 ± 0.12	-
Group 2 (ulcer control)	4.00±0.2236	-
Group 3 (MeOH PF 100mg/kg, p.o.)	1.50±0.2739*	62.50
Group 4 (MeOH PF 200mg/kg, p.o.)	1.30±0.1225*	67.50
Group 5 (Lansoprozole 8mg/kg, p.o.)	1.20±0.2550*	70.00

MeOH PF= methanolic extract of *P. foetida*; n=5; Data = Mean±SEM; *P<0.001, (Group 2 Vs Group 3, 4 & 5)

5.10.4 Western blot analysis of stomach tissue from pylorus ligatured rats

Table 5.19 and figure 5.16 (a-d) showed the effects of the methanol extract of leaf of *P. foetida* L. at a dose of 150 mg/kg, p.o. and cimetidine (8mg/kg, p.o.) on pylorus ligatured rats. The model revealed the prevention of the development of acute gastric ulcer. Oral administration of the extract and cimetidine decreased ulcer index to 1.114 ± 0.4569 and 1.000 ± 0.2263 in comparison to ulcer control 7.00 ± 0.6325 ($p<0.001$), but acute gastric ulcer was developed significantly ($p<0.001$) in ulcer control when compared to the ulcer index of normal control animals 0.333 ± 0.1054. Compared to the ulcer control rats, methanol extract of *P. foetida* L. and cimetidine pretreatment rats were shown a significant reduction in gastric volume, free acidity and total acidity ($p<0.01$, $P<0.05$, $P<0.001$).

In current working animal models, ulcer developed in different mechanism and according to that there are several standards we were using. For e.g. in case of indomethacin induced animal model ulcer caused due to the high release of hydrochloric acid, hence we need an anti secretory agent not any gastro-protective or PPIs. Due to this reason, in model 1, ranitidine was chosen as standard, which act as anti secretory agent. In the same way, ulcer developed by alcohol due to the necrosis of the epithelial tissue. So we need a protective in place of any kind anti-secretory agent. So in the alcohol induced ulcer model we used sucralfate as standard drug which is acting as a protective.

Table 5.19: Effect of methanol extract of *P. foetida* on Pylorus-ligatured rats

Treatment	**Mean ulcer Index (SEM)**	**% Protection**	**Volume (ml) of gastric secretion**	**Free Acidity (Eq/l/100gm)**	**Total Acidity (Eq/l/100gm)**	**pH**
Group 1 Normal Control (Distl. Water, 5ml/kg, p.o.)	0.333 ± 0.1054	-	1.5233 ± 0.4991	71.966 ± 1.035	83.229 ± 0.6836	3.65 ± 0.1118
Group 2 Ulcer Control (Distl. Water, 5ml/kg, p.o.)	7.00 ± 0.6325 ***c	-	7.400 ± 0.6110 ***c	88.167 ± 2.136 ***c	130.17 ± 6.405 ***c	2.383 ± 0.1887 ***c
Group 3 Methanol extract (150 mg/kg, p.o.)	1.114 ± 0.4569 ***	84	2.200 ± 0.3502 ***	50.167 ± 5.115 ***	60.333 ± 3.204 ***	5.417 ± 5.500 ***
Group 4 Cimetidine (8mg/kg, p. o.)	1.000 ± 0.2263 ***	85	1.883 ± 0.2167 ***	36.00 ± 5.292 ***	44.00 ± 1.125 ***	7.417 ± 0.1493 ***

Data were expressed as mean ± Standard Error Mean (SEM), where n=6 in each group. Differences were considered significant at ***P<0.001, or **P < 0.01 or * P<0.05 when compared control group vs Ulcer control group (c) and Test groups vs Ulcer control (*) group. For numerical results, one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparison post tests were performed using GraphPad InStat Version 3 (GraphPad Software)

5.10.4.1 Nrf2 Expression

Nrf2 is a redox sensitive leucine zipper transcriptor factor. When Nrf2 express there is a expression of other several antioxidant enzymes for e.g. catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), heme oxygenase 1 (HO-1) etc. Nrf2 is concealed in the cytoplasm via physical attachment to keap-1, causing inhibition of Nrf2 activity due to proteosomal degradation. During oxidative stress Nrf2 dissociate from keap-1 and translocate into the nucleus and dimerizes with a variety of different transcriptor factor (JUN, Maf family). This hetero dimer then binds to ARE (anti oxidant response element) and initiate the transcription of antioxidative gene. Figure 5.17 explains the mechanism of Nrf2. In the normal control animals were allowed for free access of regular diet (wet grams and soya bori) which has anti oxidants properties. Therefore, the expression of Nrf2 was observed in normal control. On the other hand, treatment groups, either the extract or cimetidine showed Nrf2 expression may be due to the antioxidant property of *P. foetida* and cimetidine [230]. But in case of ulcer control group, the animals were subjected to inducible stress. With such a high degree of stress and unavailability of diet, Nrf2 was not able to express. Nrf2 mediated antioxidant response represents a critically important cellular defense mechanism that serves to maintain intracellular redox homeostasis and limit oxidative damage [231]. Thus figure 5.18 explained that there was no expression of Nrf2 in ulcer control (- control) group, whereas in the normal control (+ control) group, methanol extract of *P. foetida* L. group and cimetidine treated group there was an expression of Nrf2.

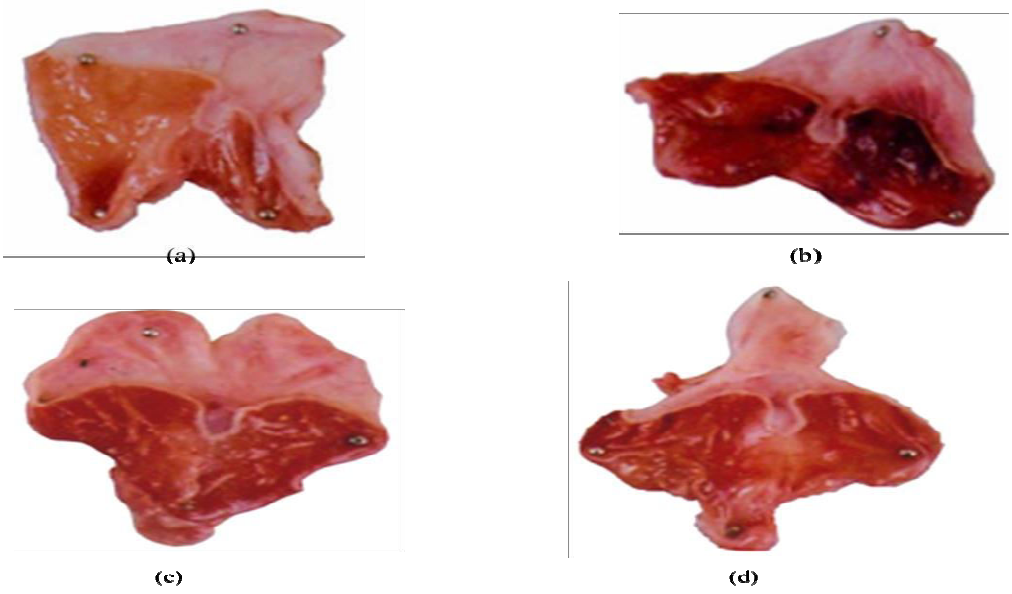


Figure 5.16: Isolated stomach **(a)**: Normal control UI =0.333; **(b)**: Ulcer control UI= 7.0; **(c)**: *P. foetida* treated UI= 1.114; **(d)**: Cimetidine treated UI= 1.0

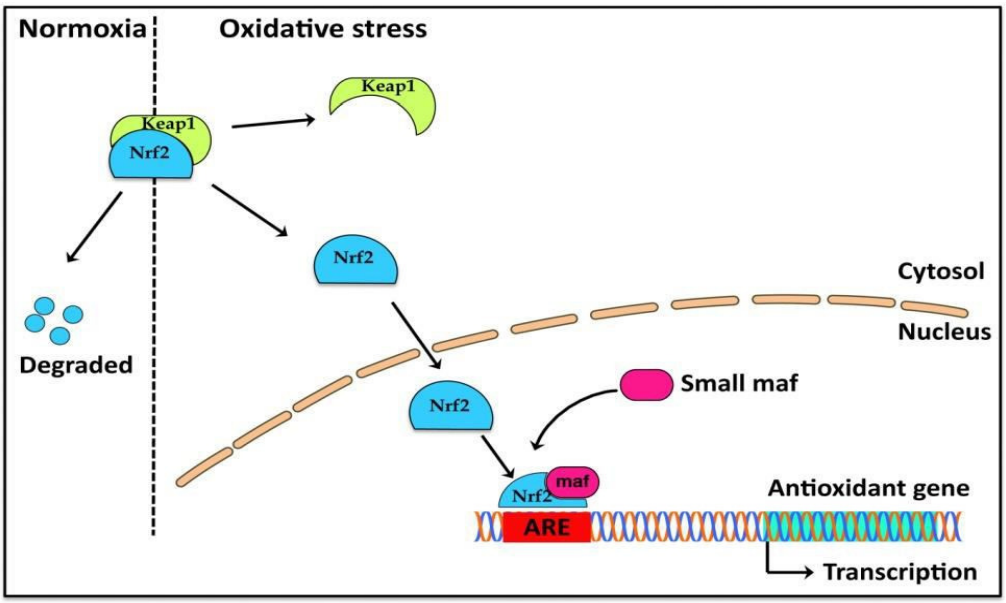


Figure 5.17: Nrf2 pathway of oxidative stress [232]

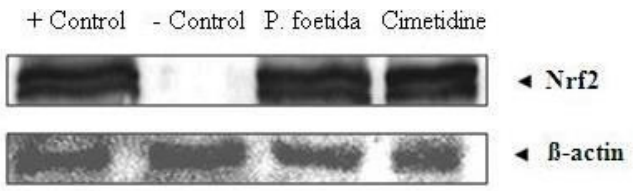


Figure 5.18: Nrf2 expression in rat stomach demonstrated by western blot analysis of pylorus ligatured rat

CHAPTER 6

CONCLUSIONS

The thrust in the area of development of new benefits and alternative treatment for PUD is day by day increasing due to the several existing unavoidable side effects and limitations of currently available drugs. It can only be fulfilled by accepting the herbal approach. Nature always gave us a safe and better solution. Taking into consideration of this fact, an ethnobatanical survey was done among the tribal families in and around Agartala.

After through the study of filled format of the questionnaires and group discussion with the tribal people and medicine men, we came to know about a total of six such plants which were used since long by the Northeast tribes as food and as medicine. We mainly targeted those plants which are having therapeutic effect as gastroprotective or as anti ulcer. The plant *Paederia foetida* L. was chosen for the current study to evaluate the antiulcer potential on the basis of the outcomes of the ethnobatanical survey report. After the selection of the plant, the aerial part of the plant was collected from the tribal market of Agarlatala.

After the collection, authentication and extraction of leaf material by methanol, an effort was made to investigate the phytochemical profile of the extract. Qualitative chemical reaction revealed the presence of glycoside, alkaloid, steroids, volatile oils, tannins and terpenoids. The presence of the different metabolites supports its wide therapeutic use. Qualitative HPTLC analysis revealed the presence of β-sitosterol as one the constituent. GC-MS analysis followed by library search of non polar extract of the leaf and stem and hydrodistilled oil of the leaf revealed the presence of 57 compounds. Out of 57 compounds, 54 were identified for the first time and reported as volatile compounds of *P. foetida* by our study.

Qualitative standardization of the extract was carried out with respect to the two iridoids *viz.* asperuloside and paederoside. HPTLC technique for simultaneous estimation of these two iridoids (asperuloside and paederoside) was reported for the first time. The R_f value of asperuloside and paederoside

was found to be 0.56 and 0.65 respectively. It was obtained as 0.12%w/w asperuloside and 0.097%w/w of paederoside in the extract. The developed method was validated as per the guidelines of ICH, and it was found that the developed method is accurate, precise, linear, rugged, specific, simple and rapid. The method could be used for routine quality control of any polyherbal formulations that contain paederia as one of the ingredients. The method is very simple, easy and cost effective as compared to GC and HPLC.

Our standardized extract was further subjected to evaluate the anti ulcer potential. The anti ulcer activity of the plant extract was evaluated by *in vitro* and *in vivo* assay. The antioxidant potential of the fresh and dried leaf extract was evaluated by DPPH* radical scavenging activity. The IC_{50} value of methanol extract of fresh and dried leaf sample was found to be 40 µ g/ml and 43µ g/ml

respectively. The extract also gave promising results for *in vitro H. pylori* activity against metronidazole sensitive and resistant strains.

The extract was screened for *in vivo* anti ulcer activity by using four different animal models. In all the models the extract showed significant (***$p<0.001$) protective effect. At last but not least the mechanism involved in ulcer protective action was confirmed by Western blotting of pylorus ligatures stomach tissue on the basis of *in vitro* antioxidant activity. Western blot analysis revealed that the activity obtained due to the expression of antioxidant gene.

The result of anti ulcer activity was found promising and first time report on leaf which was found potential as compared to earlier report on stem. The study also provided scientific validation of traditional concepts of tribal of North East.

CHAPTER 7

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PUBLICATIONS

Published paper

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Communicated paper

4. Gastroprotective Mechanism of *Paederia foetida* Linn. (Rubiaceae) - A popular edible tribal plant of North-East India
5. Targeted metabolite analysis of iridoids of *Paederia foetida* : a popular tribal edible plant of northeast, India
6. Analysis and comparison of different extracted metabolites of *Paederia foetida*: An edible tribal plant of Northeast India by hyphenated GC-MS technique.

Book chapter published

7. S. Chanda, B. De and R.K. Tiwari. Traditional and ethnobotanical investigation of some edible plants among the tribes of Tripura, India. In *Status and conservation of bio-diversity in North East India*, Choudhury MD, Sharma GD, Choudhury S, Talukdar AD. Swastik publications: Delhi. (ISBN 978-93-81084-10-6).

Oral/poster presentation

1. Oral presentation: S. Chanda, RK Tiwari & B. De, Title-Traditional and Ethno botanical investigation of some edible plants among the tribes of Tripura, India" in National Conference on Recent Trends in Biodiversity and researches organized by Assam University (Central University), March 2010.
2. Poster presentation: S. Chanda, RK Tiwari, K. Singh & B. De, Title-Traditional and ethnomedicinal investigation of *Paderia foetida* (Rubiaceae) -An edible Trible plant of northeast region in 12th international congress of Ethnopharmacology" organized by school of natural product studies Jadavpur University, Kolkata, 17-19 Feb, 2012.