Mechanism of Action of Multi–Potent Ulcer Blockers in *In Vitro* and *In Vivo* Models

Ph.D. thesis submitted to the

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In

Biotechnology

By

Srikanta B.M., M.Sc.

Under the guidance of

Dr. Shylaja M Dharmesh, Ph.D.

Department of Biochemistry and Nutrition
Central Food Technological Research Institute
Mysore – 570 020, India

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DECLARATION

I hereby declare that the thesis entitled **"Mechanism of action of multi–potent ulcer blockers in *in vitro* and *in vivo* models"** submitted to the University of Mysore, Mysore for the award of the degree of Doctor of Philosophy in Biotechnology, is the result of the research work carried out under the guidance of Dr. Shylaja M Dharmesh, Scientist, Department of Biochemistry and Nutrition, CFTRI, Mysore, during the period 2004 - 2010.

I further certify that this thesis has not been submitted by me for award of any other degree of this or any other University.

Srikanta B.M.

CERTIFICATE

I **Srikanta B.M.,** certify that this thesis is the result of research work done by me under the supervision of **Dr. Shylaja M Dharmesh** at Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore. I am submitting this thesis entitled **"Mechanism of action of multi–potent ulcer blockers in *in vitro* and *in vivo* models"** for the award of Doctor of Philosophy (Ph.D.) degree in Biotechnology of the University of Mysore, Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree of this or any other University.

Signature of Doctoral candidate
Date:

Signature of Guide
Date:

Signature of Head of the Department
Date:

Dedicated to

My Beloved Parents

Teachers & Friends

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Srikanta B.M.

Contents

Abbreviations and units

λ	-	Lambda
°C	-	Degree centigrade
µg	-	Microgram
µL	-	Microliter
µM	-	Micromolar
ηM	-	nanomolar
AB	-	Alcian blue
AC	-	Ammonium carbonate
ALP	-	Alkaline phosphatase
ANOVA	-	Analysis of variance
AOX	-	Antioxidant
ATP	-	Adenosine triphosphate
AU	-	Absorbance units
AUI	-	Antiulcer index
b.w.	-	Body weight
BC	-	Black cumin
Bcl-2	-	prototype for a family of mammalian proteins
BCPP	-	Black cumin pectic polysaccharide
BSA	-	Bovine serum albumin
CagA	-	Cytotoxicity associated gene A
cAMP	-	Cyclic adenosine monophosphate
CAT	-	Catalase
CFU	-	Colony forming units
COX	-	Cyclo oxygenase
Crtl	-	Control
d	-	Days
Da	-	Dalton
DMRT	-	Duncan's multiple range test
DNA	-	Deoxyribonucleic acid
DTNB	-	5, 5'-Dithionitrobenzoic acid
ECM	-	Extracellular matrix
EDTA	-	Ethylenediaminetetraacetic acid
EGF	-	Endothelial growth factor
ELISA	-	Enzyme-linked immunosorbent assay

ERK	-	Extracellular signal-regulated protein kinase
ES	-	Ethanol stress
EtBr	-	Ethidium bromide
EtOH	-	Ethanol
FBS	-	Fetal bovine serum
FTIR	-	Fourier transformer infrared spectrometry
g	-	g force
g	-	Gram
GAE	-	Gallic acid equivalents
GLC	-	Gas liquid chromatography
GPx	-	Glutathione peroxidase
GSH	-	Reduced glutathione
GSSG	-	Oxidized glutathione
GST	-	Glutathione-S-transferase
H & E	-	Hematoxylin and Eosin
h	-	Hour
HSA	-	Human serum albumin
HAU	-	Hemagglutination unit
HCl	-	Hydrochloric acid
HEPES	-	(4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid)
HMBA	-	2-Hydroxy-4-methoxy benzaldehyde
HPLC	-	High performance liquid chromatography
IC_{50}	-	Half maximal inhibitory concentration
Kg	-	Kilogram
M	-	Molar
MAPK	-	Mitogen-activated protein kinase
MDA	-	Malondialdehyde
mg	-	Milligram
MIC	-	Minimum inhibitory concentration
min	-	Minute
mL	-	Milliliter
mM	-	Millimolar
MMP	-	Matrix metalloproteases
MTT	-	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium
N	-	Normality
NBT	-	Nitro blue tetrazolium

NF-kB	-	Nuclear factor Kappa B
nm	-	Nanometer
NMR	-	Nuclear magnetic resonance
NSAIDS	-	Non-steroidal anti-inflammatory drugs
OD	-	Optical density
PB	-	Phosphate buffer
PBS	-	Phosphate buffer saline
PcAb	-	Polyclonal Antibodies
PGE_2	-	Prostaglandin E_2
Pi	-	Inorganic phosphate
PKA	-	Protein Kinase A
PNPP	-	Para nitrophenyl phosphate
RG-I	-	Rhamnogalacturonan type I
RNA	-	Ribonucleic acid
ROS	-	Reactive oxygen species
SD	-	Standard deviation
SEM	-	Scanning electron microscopy
SGOT	-	Serum glutamate oxaloacetate transaminase
SGPT	-	Serum glutamate pyruvate transaminase
SOD	-	Superoxide dismutase
SR	-	Swallow root
SRAE	-	Swallow root aqueous extract
SRPP	-	Swallow root pectic polysaccharide
SS	-	Swim stress
TBA	-	2-Thiobarbituric acid
TBARS	-	Thiobarbituric acid reactive substances
TCA	-	Trichloro acetic acid
TMP	-	1, 1, 3, 3-Tetramethoxy propane
UI	-	Ulcer index
UV	-	Ultraviolet
VacA	-	Vaculating gene A
w/v	-	Weight/Volume
w/w	-	Weight/Weight

Abstract

Thesis Title: **Mechanism of action of multi–potent ulcer blockers in *in vitro* and *in vivo* models.**

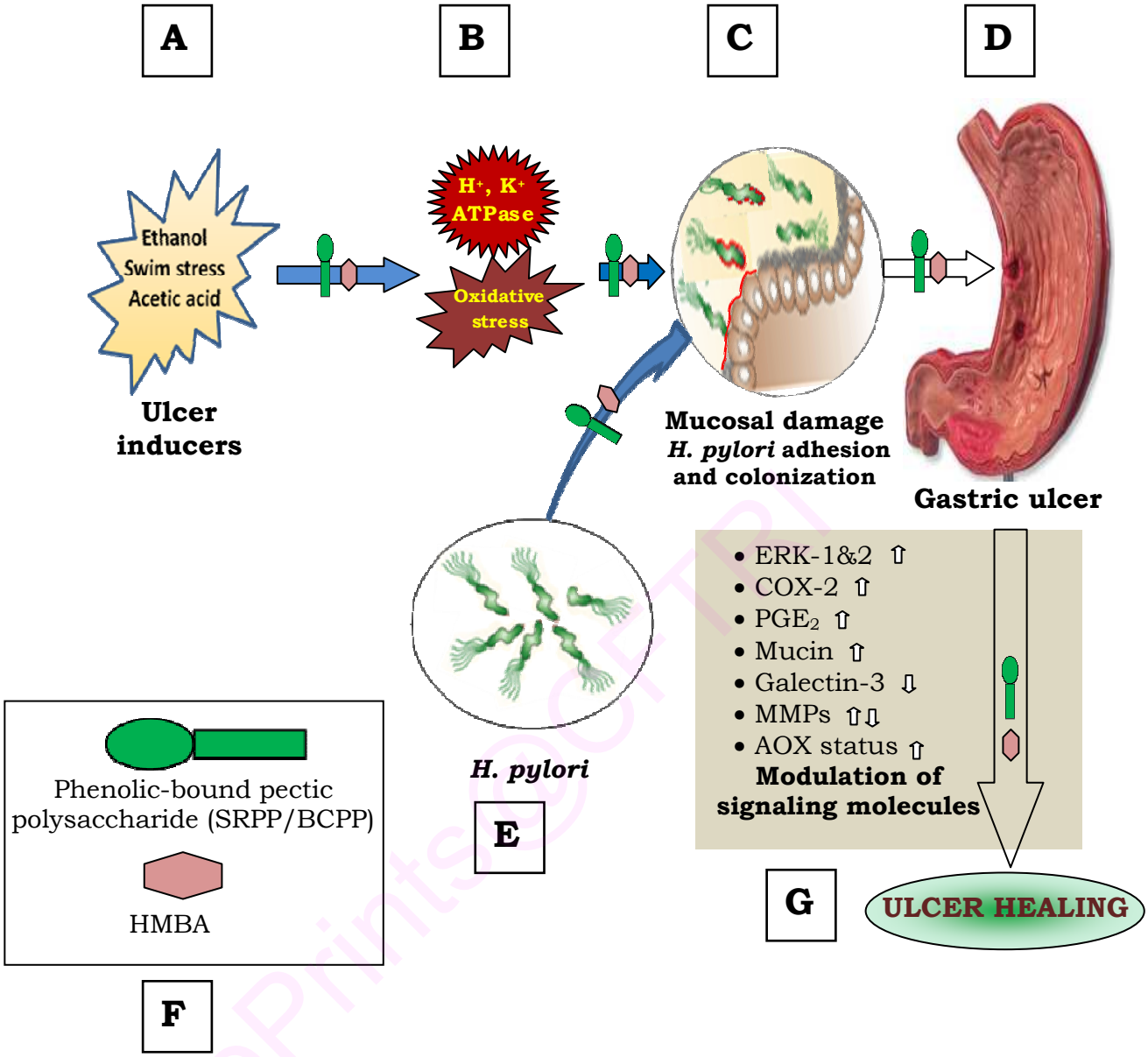
Ulcer is a common global problem with increasing incidence and prevalence. Worldwide 14.5 million people have ulcers with a mortality of 4.08 million. The increasing incidence and prevalence of ulcers have been attributed to several factors encountered during day-to-day life, such as stress, exposure to bacterial infection, use of non-steroidal anti-inflammatory drugs (NSAIDs) etc. Ulcers are resulted from excess secretion of hydrochloric acid from gastric parietal cells via activation of H^+, K^+ -ATPase enzyme, which releases H^+ into the lumen of the stomach leading to acidity. Released acid act on gastric mucosa leading to loss of mucosal damage and hence impaired mucosal protection. Gastric lesions thus develop due to loss of the delicate balance between gastroprotective and aggressive factors. Reduction in gastric mucin, enhanced the secretion of gastric and susceptibility to *Helicobacter pylori* infection adding to the severity of the disease.

Sustainable efforts and constant research in the area lead to the development of several drugs that can act at multi-steps during ulcer pathogenicity such as proton pump blockers (Lansoprazole, Omeprazole), histamine receptor blockers (Ranitidine, Cimitidine, Famotidine) and *H. pylori* inhibitors (Amoxicillin, Erythromycin, Metronidazole). However, majority of them have been documented to pose problems of adverse effects.

In the light of the above, it was pertinent to study natural products from dietary sources as potential antiulcer compounds. In the current thesis therefore dietary components have been explored as a potential effective and safer antiulcer compounds. Series of commonly used dietary sources were examined for inhibition of H^+, K^+ -ATPase, inhibition of *H. pylori* growth and antioxidant activity. The Antiulcer index (AUI) was calculated based on the results. Highest AUI sources – Swallow root *(Decalepis hamiltonii)* and Black cumin *(Nigella Sativa)* were selected for further studies. Pectic polysaccharide

and one of the abundant Swallow root components (2-hydroxy-4-methoxy benzaldehyde) was examined for anti-*H. pylori,* ulcer preventive or ulcer healing properties. Structure - function analysis of pectic polysaccharides of Swallow root (SRPP) and Black cumin (BCPP) revealed that galacturonan; either arabino or rhamnogalacturonan may be responsible for the antiulcer activity. The i*n vivo* efficacy of these indicated that they are potent in inhibiting 73 to 85% of either swim/ethanol stress induced ulcers. Besides, 80 to 90% ulcer healing together with normalization of H^+, K^+ - ATPase, oxidative stress, antioxidant levels were also observed. Further, the selected components were also effective against multi-steps of *H. pylori* induced pathogenicity such as colonization on gastric mucin, invasion and cytotoxicity.

Overall, the current study for the first time provided evidence for the ability of dietary pectic polysaccharides to elicit the signaling cascade of ulcer prevention and healing including the multi-step inhibition of potential ulcer pathogenic steps as indicated in **scheme 1**. Outcome of this study thus may result in the potential use of dietary compounds as better gastroprotective compounds that are safer with no side effects. Further, the ability of these dietary factors in eliciting the signaling cascade of ulcer healing process is quite intriguing and throws insights into the use of such compounds for designing of gastroprotective nutraceutical for gastric ulcer patients.



Scheme 1: Summary of the work envisaged in the thesis

Acetic acid, ethanol and swim stress (**A**) generates oxidative stress and hyper activation of H^+, K^+ ATPase (**B**), which causes loss of gastric mucin layer and hence gastric mucosal damage (**C**) leading to gastric ulcers (**D**). The damaged gastric mucosa is more susceptible to *H. pylori* infection and successful pathogenesis (**E**) and further aggravation of ulcers. The phenolic-bound pectic polysaccharides (SRPP & BCPP) and HMBA (**F**) could act at multi-steps such as inhibition of free radical generation & oxidative stress, inhibition of H^+, K^+ ATPase activity and inhibition of *H. pylori* growth, adhesion, colonization and pathogenesis, thereby preventing gastric ulcerogenesis. On the other hand SRPP and BCPP could also induce rapid healing of already formed gastric ulcers by inhibiting galectin-3 mediated inflammation, regulation of matrix metalloproteinases (MMPs) and increasing gastric mucin synthesis via ERK - COX-2 - PGE_2 pathway in addition to strengthening antioxidant defence of gastric mucosa (**G**).

Synopsis of the thesis submitted for the award of Ph.D. degree in Biotechnology of the University of Mysore, Mysore, India.

Title: **Mechanism of action of multi–potent ulcer blockers in *in vitro* and *in vivo* models.**

Candidate: Srikanta B.M.

Gastric ulcer is a major disorder of gastrointestinal system. It is a common global problem, highly prevalent all over the world with increasing incidences every year and has been attributed to several factors encountered during day-to-day life, such as stress, exposure to bacterial infection, continuous use of non-steroidal anti-inflammatory drugs (NSAIDs) etc. Gastric ulcer is a deep necrotic lesion characterized by disruption of mucosal integrity leading to local defect or excavation due to active inflammation. It develops due to loss of the delicate balance between gastro-protective and aggressive factors. Reduction in gastroprotective factors, such as mucus, bicarbonate secretion, gastric mucosal blood flow & prostaglandins and; enhancement of aggressive factors, such as increase of acid/pepsin secretion, *Helicobacter pylori* infection and NSAIDs consumption results in gastric ulceration. Mucosal damage, an initial step in ulcer development, has been known to be due to oxidative stress by reactive oxygen species, hypersecretion of hydrochloric acid through H^+, K^+ -ATPase action, the blockade of the cyclooxygenase enzyme system by NSAIDs in addition to *H. pylori* mediated cytotoxicity. Many experimental studies have been carried out to understand the aetiology of ulcers; however ulcer healing being an active and complicated process of reconstruction of mucosal architecture with the involvement of inflammation, cell proliferation, re-epithelialization, formation of granulation tissue, interaction between various cells, matrix and tissue remodeling etc; understanding of healing or gastroprotective process by any source appear to be difficult.

Reconstruction or rejuvenation of mucosal architecture, etc., needed for ulcer healing is dependent on elicitation of signaling molecules that can modulate the complete healing process including modulation of specific markers. A modest approach to control ulceration, therefore, is via stimulation of gastric mucin synthesis, enhancement of antioxidant levels in

the stomach, scavenging of reactive oxygen species, inhibition of H^+, K^+-ATPase and *H. pylori* growth. Although the antisecretory drugs, such as H^+, K^+-ATPase pump inhibitors, histamine H_2-receptor blockers and antibiotics are being used to control acid secretion and acid related disorders; they are not the drugs of choice since they produce potential adverse effects on human health. Mere blockade of induction of damage can offer effective prevention. However, unless the molecule has inherent capacity to activate defence or deactivate ulcer pathogenicity routes and stimulation of cells to undergo appropriate mechanisms to selectively activate the cascade of ulcer healing process, the compound may not be considered as effective ulcer healing agent.

In light of the above, it is pertinent to study natural products from food as potential antiulcer compounds. Due to the lack of side effects compared to synthetic drugs, approximately 60% of the world's population relies entirely on such natural medications. In Indian traditional medicine, several plants have been employed to treat gastrointestinal disorders, including gastric ulcers.

Decalepis hamiltonii (Swallow root) roots and *Nigella sativa* (Black cumin) seeds were selected as potential sources, after screening series of dietary sources for antioxidant activities and inhibition of H^+, K^+ -ATPase and *H. pylori* growth, since they exhibited better activities in all the three assays employed during the screening for antiulcer sources. Selected sources were further employed for understanding the mechanism of action at multi-steps during prevention and healing of gastric ulcers and inhibition of *H. pylori* mediated cytotoxicity. Hence the following objectives have been proposed:

Objectives:

1. **Screening of dietary sources for multi-step ulcer blockers**
2. **Isolation and characterization of active antiulcer compounds from selected sources**
3. **Determination of antiulcer potency of identified compounds *in vivo* in comparison with known antiulcer drugs; mechanism of action**
4. **Establishment of mechanism of action of effective antiulcer compounds employing cell models**

The aim of the present study was to identify multi-potent compounds from selected dietary sources, which acts against multi-steps of gastric ulcer pathogenesis. Identified compounds implicated in bioactivity envisage a higher impact for designing gastroprotective nutraceutical for gastric ulcer patients. Development of such gastroprotective compounds are warranted, since most of the available antiulcer drugs are either fail to show multi-potency or cause side effects. Outcome of this study resulted in the identification of potent dietary sources for their effective uses against gastric ulcer without causing side effects and with multi-mechanistic action. Results of the study also highlight the mechanism of action in comparison with that of the known antiulcer drugs and helps in highlighting the importance of nutraceutical as a potential alternative for health benefits. The research work carried out towards achieving these objectives makes the subject matter of the thesis. The thesis is divided into 4 chapters with appropriate general introduction with review of literature, and the contents provided in each chapter is highlighted as follows:

General introduction:

General introduction deals with general account on gastric ulcer. The statistical data on gastric ulcer incidence, prevalence and mortality and the past and current status of gastric ulcer have been described. Since gastric ulcer is a multi-step and multiple factors mediated disease, the process of pathogenesis and alteration of molecular components involved in gastric ulcer pathogenesis have been depicted. This section describes also the available drugs and their adverse effects; in addition to providing an overview of current literature on use of natural products from dietary sources including polysaccharides for treating gastric ulcers. The general account on selected dietary sources – *D. hamiltonii* (Swallow root) and *N. sativa* (Black cumin), their uses as food and medicine, composition of their bioactive compounds also have been depicted.

Further, predicted antiulcer molecules being polysaccharide and phenolic compounds, overview on structure and function of polysaccharides and phenolics have been depicted. At the end of this section, the health benefits of dietary antiulcer compounds have been highlighted, including the aims and scopes of the current study.

Subsequent chapters - chapters 1 to 4 deals with objectives 1 to 4 respectively. These chapters have a uniform format depicting hypothesis underlying the specific objective, work concept adapted, a brief introduction pertaining to the respective objectives, materials and methods, results, discussion and a relevant summary and conclusions highlighting the important outcome of the chapter. List of references for literatures cited and methodologies used are provided at the end of the thesis.

Chapter 1: Screening and selection of dietary sources for multi-step ulcer blockers

This chapter deals with collection of different dietary sources that have been shown to possess health beneficial properties as per the traditional knowledge. Since aqueous extracts of these plants are effective in traditional medicine; aqueous extracts which are rich in polysaccharides and phenolic compounds were prepared and tested for their efficacy against gastric ulcers. Array of *in vitro* assays such as H^+, K^+ -ATPase inhibition, *H. pylori* growth inhibition and antioxidant assays such as free radical scavenging and reducing power assays were employed to arrive at the potential sources to treat or prevent gastric ulcers, since gastric ulcer has been known to be initiated by the action of free radicals/reactive oxygen species that triggers parietal cell H^+, K^+ -ATPase, leading to increased acid secretion, mucosal damage, successful invasion of *H. pylori* to mucosal layer and progress of ulcer pathogenicity. Therefore attention has been paid during the screening for selection of potential antiulcer dietary/plant sources. Results of this chapter enable us to identify the very good, good, moderate and poorer sources with varied activity. Chapter 1 thus deals with the criteria for selection of potential dietary sources – *Decalepis hamiltonii* (Swallow root) and *Nigella sativa* (Black cumin) to employ potentially against ulcer pathogenicity.

Chapter 2: Isolation and characterization of active antiulcer compounds from Swallow root *(Decalepis hamiltonii)* and Black cumin *(Nigella sativa)*

Results of the chapter 1 showed that aqueous extracts of Swallow root and Black cumin are potent gastroprotective sources. Previous studies from our laboratory and also from others had shown that plant phenolics and

polysaccharides are potential components for treating gastric ulcers. In this chapter, attempt has been made to isolate different fractions of polysaccharides and also phenolics and examined their efficacy employing *in vitro* antiulcer and antioxidant assays as described in chapter 1. The pectic polysaccharide fractions of both Swallow root and Black cumin showing potent activity when compared to other fractions were isolated employing classical protocol and purified using specific chromatographic methods. The structure was delineated adapting analytical techniques such as FTIR and NMR analysis and structure-function relationship has been depicted based on both *in vitro* and *in vivo* studies. Further since Swallow root has been shown to be the best source against gastric ulcer; one of its major compound – 2-hydroxy-4-methoxy benzaldehyde has been isolated, confirmed its identity and tested its ability to inhibit potential ulcer pathogenic factors *in vitro*.

Chapter 3: Determination of *in vivo* antiulcer potency and mechanism of action of active antiulcer compounds

In this chapter *in vivo* efficacy in comparison with known antiulcer drugs and mechanism of action of selected antiulcer compounds such as pectic polysaccharides and 2-hydroxy-4-methoxy benzaldehyde has been determined. Chapter 4 constituting 'Part 4A' and 'Part 4B' enumerate ulcer preventive and ulcer healing potentials respectively. Swim/ethanol stress induced gastric ulcer models were employed to test ulcer preventive potential and acetic acid induced gastric ulcer model was used to test the ulcer healing ability. Modulation of key molecules/enzymes and specific events involved in gastric ulcer pathogenesis were analyzed to determine the mechanism of action of selected antiulcer compounds either during ulcer prevention or healing. Results of the study reveal the efficacy of phenol-bound pectic polysaccharides by virtue of their antioxidant mechanism along with inhibition of H^+, K^+ -ATPase and protection to gastric mucin during swim stress and ethanol induced gastric ulcer prevention. During ulcer healing, pectic polysaccharides triggered prostaglandin E_2 and gastric mucin synthesis in addition to participate in the intervention of signaling cascades such as MAP kinase (ERK-1 & 2), matrix metalloproteinases, galectin-3, cyclooxygenase-2 and strengthening of antioxidant defence mechanism. The

study for the first time revealed the efficacy of phenolics in combination with polysaccharide enhancing gastroprotection via modulation of signaling cascades also.

Chapter 4: Establishment of mechanism of action of anti-*Helicobacter pylori* compounds.

This chapter deals with the mechanism of action of antiulcer compounds in *in vitro* cell models. This has been employed particularly to understand the role of selected dietary compounds against *H. pylori* – a potential ulcerogen. The chapter emphasizes on the mechanism of selected compounds against *H. pylori* colonization, growth and *H. pylori* induced cytotoxic effects in *in vitro* cell model studies. Polyclonal antibody was produced against *H. pylori* in rabbit and utilized the same to determine the mode of action of anti-*H. pylori* compounds. Different *in vitro* assays such as agar diffusion, colony count inhibition and scanning electron microscopic studies were performed to determine the bactericidal effect of the selected antiulcer compounds. The results of this chapter showed 2-hydroxy-4-methoxy benzaldehyde and Swallow root pectic polysaccharide as potent bactericidal compounds against *H. pylori* and pectic polysaccharides of both Swallow root and Black cumin as potent anti-adhesive compounds.

Study could highlight the detailed molecular mechanism of action of selected dietary antiulcer compounds in suppressing aggressive factors and amplifying mucosal defence, both of which are key steps for gastroprotection.

Overall research work of the thesis highlights the mechanism of action of pectic polysaccharides and phenolics as multi-potent ulcer blockers that act at multiple steps of ulcer pathogenesis in stress, ethanol and acid induced gastric ulcer conditions and also provide insights into the role of phenolics bound to polysaccharides, polysaccharide alone and phenolics during gastroprotection.

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GENERAL

INTRODUCTION

Introduction

The first gastric ulcer described in human history probably belongs to a Chinese man who died 2000 years ago in the Western Han Dynasty from perforated gastric ulcer and whose well-preserved body was recently discovered in Jinzhou, China (Tien et al., 1982). Before the 19th century, ulceration was uncommon, be it in East or West (Lam, 2000) and up to the late period of 19th century, the stomach was often not clearly recognized as a source of symptoms (Brzozowski, 2003).

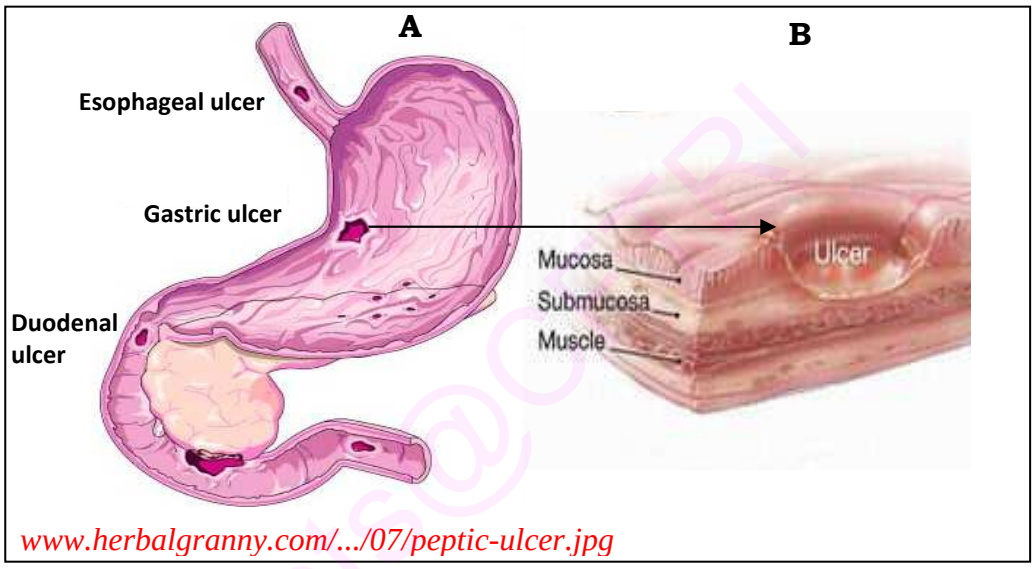


Fig 1. Picture showing esophageal, gastric and duodenal ulcers (A), penetrating deep into the mucosa and submucosal layer (B).

Although there were various descriptions of gastric and intestinal diseases throughout the 18th century, neither specific symptomatic descriptions nor the remedy for the same were defined. The pathology of gastric ulcer was first described in 1835 by Jean Cruveilhier, one of the foremost authorities on stomach ulcers in France and the condition was earlier therefore referred as “Cruveilhier's disease” (Cruveilhier, 1835). Since then the elucidation of the ulcer disease of the stomach and duodenum had accelerated dramatically within 25 years due to the identification of several techniques, which have facilitated the study of the gastric mucosa. As depicted in Fig 1 gastric mucosa has been known to contain three layers which includes innermost mucosal layer towards lumen, followed by submucosal layer and muscle layer. Generally gastric ulcers have been noticed in the gastric

mucosal layer, apart from ulcerations in esophagus called as esophageal ulcers (Fig 1A) and in the duodenum called as duodenal ulcer. The gastric ulcers may penetrate deep into the mucosa and submucosal layer (Fig 1B) or it may perforate through entire stomach wall.

History and definition of ulcer

The word ulcer is first attested from ca. 1400 CE, deriving from Old French *ulcere,* which came from Latin *ulcus* "ulcer". Further the word "ulcer" traveled across the English Channel from the French "ulcere" meaning sore, sore spot or painful spot. Generally, ulcer is an area of erosion, disintegration and necrosis of epithelial tissue of the skin or mucous membrane. Ulcer is defined as a deep necrotic lesion characterized by disruption of mucosal integrity leading to local defect or excavation, penetrating through entire mucosal thickness and the muscularis mucosae due to active inflammation in the lining of the esophagus or stomach or duodenum. The peptic ulcer is classified into esophageal ulcer (esophagus), gastric ulcer (stomach) and duodenal ulcer (duodenum) based on the anatomical location. Although general information is available on total of peptic ulcers, principles of pathogenesis and healing mechanisms apply to the other gastrointestinal tract ulcers, the main focus of the thesis work is on gastric ulcers which is statistically blowing due to several factors.

Epidemiology and statistics of ulcer

Epidemiology is the study of the distribution and determinants of health-related states or events (including disease), and the application of this study to the control of diseases and other health problems. The time trends in the epidemiology of ulcer reflect complex, multifactorial aetiologies. Epidemiological data for this disease and its complications have shown striking geographical variations in incidence and prevalence (Fig 2). Before the 19th century, gastric ulcers were occasionally seen and duodenal ulcers were rare. At the turn of the century, ulcer, in particular duodenal ulcer rose to become one of the most common medical conditions in Western countries, affecting 10% of men in their lifetime (Grossman, 1980; Kurata & Haile, 1984; Sonnenberg & Baron, 2010). In the East, its occurrence is equally common, and its prevalence has also been documented to be 10-11%. Ulcer disease had been shown to have a tremendous effect on morbidity and

mortality until the last decades of the 20th century. Midway through the 20th century, however, the incidence of ulcer started to fall in Western countries, while that in Asian countries continued to rise. However, in the past decade, although there has been no documented formal report in Asian countries, it is generally noted that the incidence of ulcer has also been falling (Lam, 2000). The recent study also confirmed that the prevalence of ulcer in Asia is substantially higher (17.2%) than in Western populations (4.1%) (Li et al., 2010). Two important developments are associated with the decrease in rates of ulcer disease: the discovery of effective and potent acid suppressants, and of *Helicobacter pylori* (Malfertheiner et al., 2009). Despite substantial advances, this disease remains an important clinical problem, largely because of the increasingly widespread use of non-steroidal anti-inflammatory drugs (NSAIDs) and low-dose aspirin. At the same time in the West and especially in USA, the increase in non-*H. pylori*, non-NSAID peptic ulcer were documented, which is about 30% of all ulcers seen (Maher et al., 1997) and such ulcers appear to be much less common in Asia and represents only 5% of the ulcers (Lam et al., 2000). As noted above, it is now evident that the epidemiology of ulcer largely reflects environmental factors, primarily *H. pylori* infection, NSAID use, cigarette smoking, environmental stress and dietary factors (Lam, 2000; Graham, 2003).

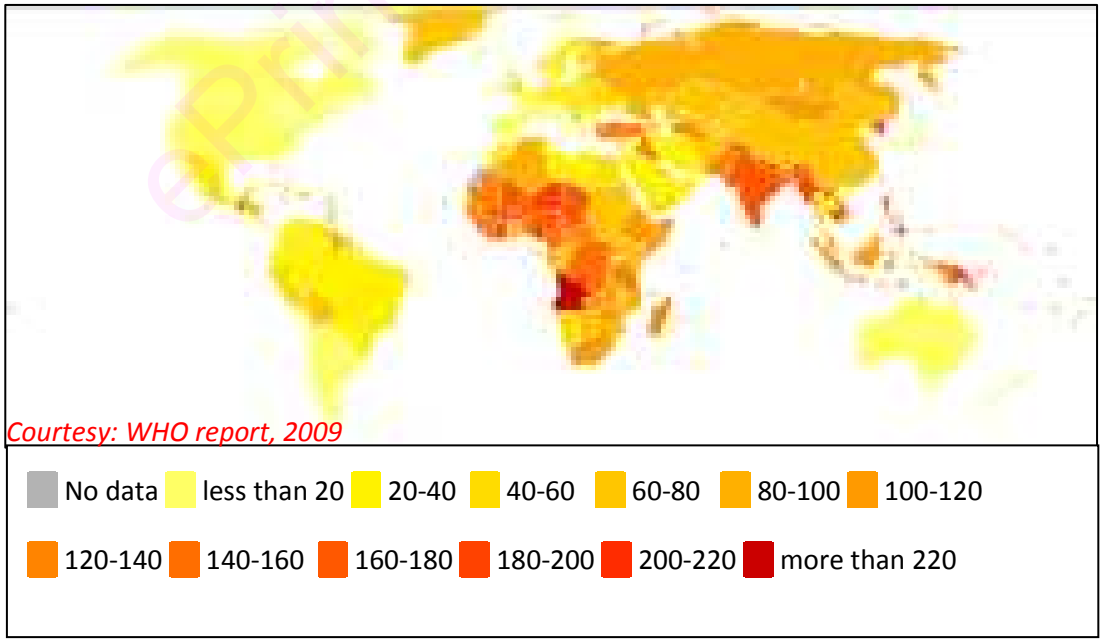


Fig 2. Prevalence of ulcer in terms of "Disability-adjusted life year" per 100,000 inhabitants in 2004.

It is estimated that, 14.5 million people have ulcers with a mortality of 4.08 million worldwide (http://digestive.nidk.nih.gov/statistics.htm/ pepticulcer prevalence). There are about 2% of the adult population in the United States has active peptic ulcers, and that about 10% will develop ulcers at some point in their lives. There are about 500,000 new cases of peptic ulcer in the United States every year, with as many as 4 million recurrences. The male/female ratio for ulcers of the digestive tract is 3:1. The most common forms of ulcer are duodenal and gastric ulcers. About 80% of all ulcers in the digestive tract are duodenal ulcers. This type of ulcer may strike people in any age group but is most common in males between the ages of 20 and 45. The incidence of duodenal ulcers has dropped over the past 30 years. Gastric ulcers account for about 16% of peptic ulcers. They are most common in males between the ages of 55 and 70 (Kurata & Haile, 1984).

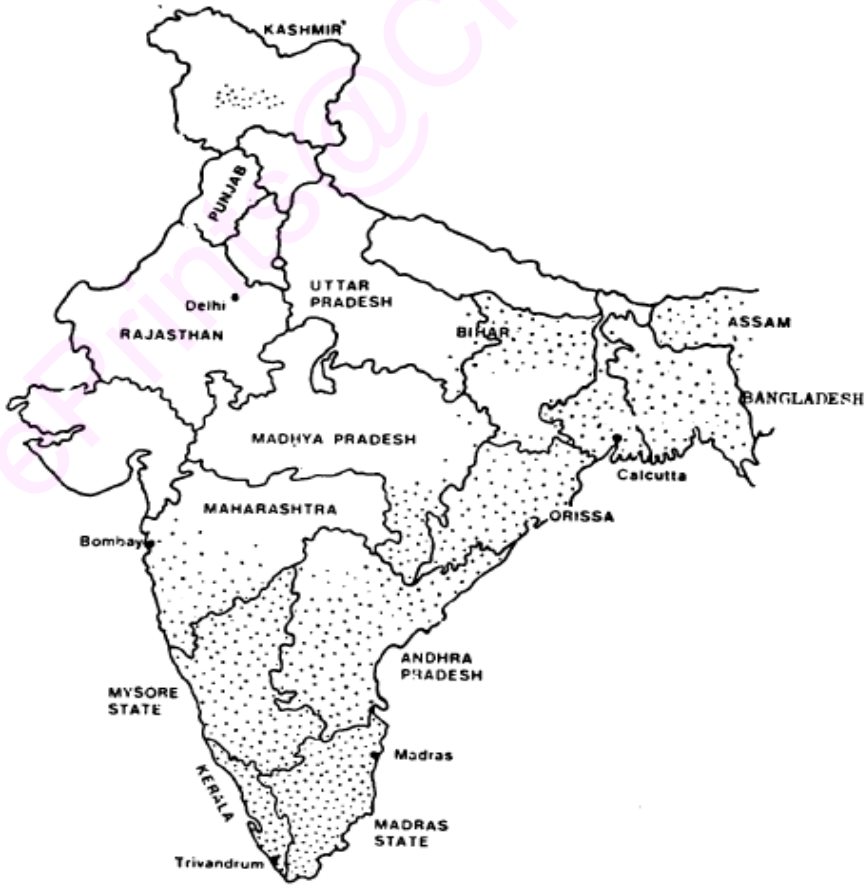


Fig 3. Distribution of ulcer in India. Composite map based on information from all available literature. The small dots indicate the prevalence of ulcers. (*Ref: Tovey, Gut 1979; 20: 329-347)*

However, contradictions to decreased incidences of duodenal ulcer, gastric ulcer incidences are on raise. Threatening factors for gastric ulcers are gastritis, *H. pylori* infection, NSAID use, alcohol, dietary factors etc. Although there is poor statistical data on ulcers in India, a report by Tovey (1979) showed a marked regional differences in India, ulcer being more prevalent in southern than in northern India as shown in Fig 3. High-prevalence areas stretch from the south up the west coast to Bombay, all the way up the east coast, and into the plains of Assam and Kashmir. A study by Khuroo et al., (1989) showed 11% lifetime prevalence of peptic ulcer in Kashmir region alone. A recent study in Chandigarh region showed the peptic ulcer lifetime prevalence of 8.8% with the point prevalence of active peptic ulcer of 3.4% (Virendra et al., 2002).

Epidemiological studies revealed a very strong association between *H. pylori* infection and duodenal/gastric ulcers. Factors determining whether the infection will produce disease are the pattern of histological gastritis induced; changes in homeostasis of gastric hormones and acid secretion; gastric metaplasia in the duodenum; interaction of *H. pylori* with the mucosal barrier and immunopathogenesis; ulcerogenic/virulent strains; and genetic factors (Malfertheiner et al., 2009) is yet to be understood.

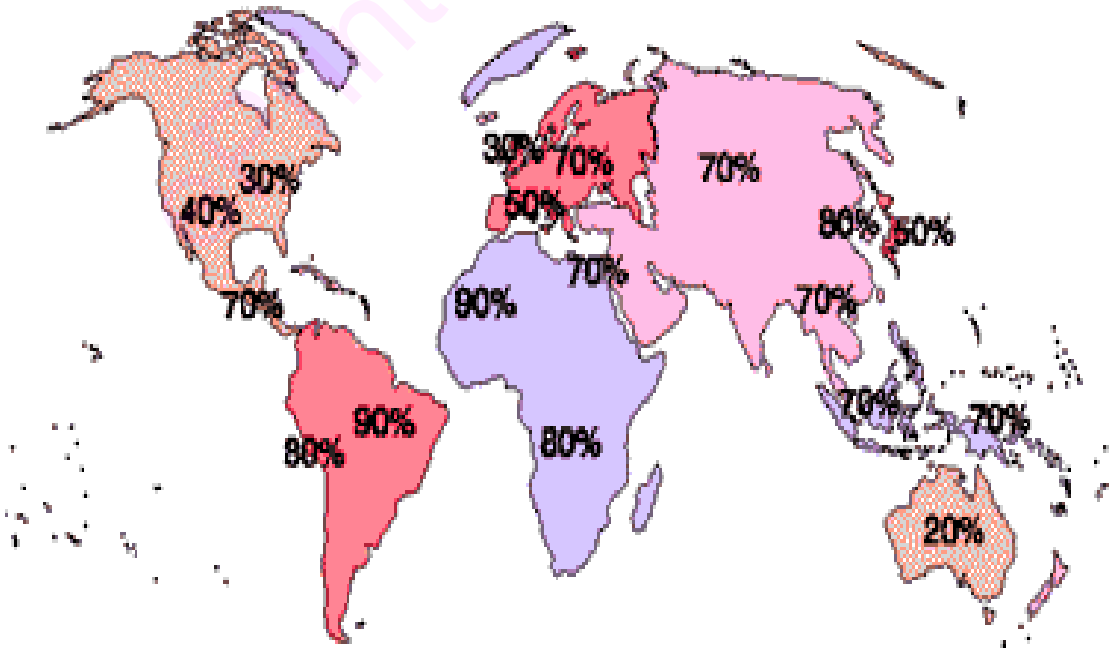


Fig 4. Prevalence rate of *H. pylori*

More than 50% of the world's population has a chronic *H. pylori* infection of the gastric mucosa, yet only 5–10% of those infected develop ulcers. This again necessitates the understanding on whether it directly relates to incidence of gastritis or linked to other factors such as strain, host resistance, immunotolerence etc., and is very much warranted. The prevalence of *H. pylori* varies throughout the world as shown in Fig 4 and depends to a great extent on the overall standard of living in the region. In developing parts of the world, 80% of the population may be infected by the age of 20. In contrast, in the United States, this organism is rare in childhood. The overall prevalence of *H. pylori* in the United States is ~30%, whereas in Asia it is about 70%.

Causative factors of ulcer pathogenesis

Ulcer is a multi-factor induced disease. The complex and multi-factorial pathogenesis of ulcer has been studied over several decades. Ulcers are produced from an imbalance of aggressive gastric luminal factors acid and pepsin, combined with superimposed injury from environmental or immunologic agents and defensive mucosal barrier function (Malfertheiner et al., 2009).

Several environmental and host factors contribute to ulcer formation by increasing gastric acid secretion or weakening the mucosal barrier (Kato et al., 1992). Apart from **genetic factor**, at least 5 other factors which are contributing to ulcer pathogenesis such as **stress, use of NSAIDs, *H. pylori* infection, smoking** and **alcohol consumption,** as described below.

Among environmental factors, smoking, excessive alcohol use, and drug use are most often quoted but none of them, apart from NSAID use, were identified as an individual ulcerogenic agent (Kato et al., 1992). The discovery of *H. pylori* switched the notion from an acid-driven disease to an infectious disease, opening a huge area for intensive research that resulted in the reconciliation of previously suggested mechanisms of pathogenesis. Based on these aetiological factors ulcers were classified as shown in Table 1 (Malfertheiner et al., 2009).

Aetiological classification of peptic ulcers

- Positive for *Helicobacter pylori* infection
- Drug induced (ie, NSAIDs)
- *H. pylori* and NSAIDs positive
- *H. pylori* and NSAIDs negative*
- Acid hypersecretory state (ie, Zollinger-Ellison syndrome)
- Anastomosis ulcer after subtotal gastric resection
- Tumours (ie, cancer, lymphoma)
- Rare specific causes
  - Crohn's disease of the stomach or duodenum
  - Eosinophilic gastroduodenitis
  - Systemic mastocytosis
  - Radiation damage
  - Viral infections (eg, cytomegalovirus or herpes simplex infection, in particular in immunocompromised patients)
  - Colonisation of stomach with *H. heilmanii*
  - Severe systemic disease
- Cameron ulcer (gastric ulcer where a hiatus hernia passes through the diaphragmatic hiatus)
- True idiopathic ulcer

*Requires search for other specific causes.

Ref: Malfertheiner et al., Lancet 2009; 374: 1449–61.

Table 1. Aetiological classification of ulcers.

Stress

Emotional stress and psychosocial factors are frequently identified as important contributors to ulcer pathogenesis (Peters & Richardson, 1983). Although stress cannot be neglected as a contributing factor, convincing evidence for it being the sole cause of duodenal ulcer is scarce. A good example of stress as a contributory factor was the rise in bleeding gastric ulcers in elderly people after a severe earthquake in Japan (Aoyama et al., 1998). The generations of free radicals are mainly implicated as a causative mechanism in stress induced ulcers (Das et al., 1997).

NSAIDs

Long-term use of NSAIDs is the second most common cause of ulcers and the rate of NSAID-caused ulcers is increasing. About 20 million people take prescription of NSAIDs regularly, and over 25 billion tablets of over-the-

counter brands are sold each year in America. The detailed mechanism of NSAID induced ulceration is described below.

***H. pylori* infection**

H. pylori infection is strongly associated with gastroduodenal ulcers as evidenced by epidemiological studies. More than 50% of the world's population has a chronic *H. pylori* infection of the gastric mucosa. The detailed mechanism of *H. pylori* induced ulceration is explained later in following sections.

Alcohol

Although alcohol has been shown to induce damage to the gastric mucosa in animals, it seems to be related to the absolute ethanol administered. Pure ethanol solubalises lipids and hence results in frank, acute mucosal layer damage. Because most humans do not drink absolute ethanol, it is unlikely that there is a mucosal injury at ethanol concentrations of less than 10%. Ethanol at low concentrations (5%) may modestly stimulate gastric acid secretions. The knowledge available in the literature indicate that alcohol intake is affecting gastric mucosal damage (Chou 1994) and acute gastritis or hyperacidity with increased acid secretion affects gastric mucosal layer justifies the alcohol induced gastric ulcer episodes which are quite fatal.

Smoking

The literature reveals a strong positive correlation between cigarette smoking and the incidence of ulcer disease, mortality, complications, recurrences and delay in healing rates. Smokers are about two times more likely to develop ulcer disease than nonsmokers. Cigarette smoking and *H. pylori* were found to act as co-factors for the formation of ulcer disease. A strong association between *H. pylori* infection and cigarette smoking in patients with and without ulcers has been shown to be common. Cigarette smoking may increase susceptibility, diminish the gastric mucosal defensive factors, or may provide a more favorable milieu for *H. pylori* infection.

Food and beverages

Some types of food and beverages are reported to cause dyspepsia. Myers et al., (1987) have reported deleterious effect of red pepper and black pepper on the stomach. Both have induced a significant enhancement in parietal

secretion, pepsin secretion, and potassium loss, as well as a dose dependent gastric cell exfoliation and mucosal microbleeding, which are comparable to those induced by aspirin. Epidemiologic studies have failed to reveal a correlation between caffeinated, decaffeinated, or cola-type beverages, beer, or milk with an increased risk of ulcer disease. Dietary alteration, other than avoidance of pain-causing foods, is unnecessary in ulcer patients.

Genetic factors

Genetic factors play a role in the pathogenesis of ulcer disease. The lifetime prevalence of developing ulcer in first-degree relatives of ulcer patients is about three times greater than the general population. Approximately 20–50% of duodenal ulcer patients report a positive family history; gastric ulcer patients also report clusters of family members who are likewise affected.

Ulcer pathogenesis

The well defined mechanism of ulcer pathogenesis was established only in stress, NSAIDs and *H. pylori* induced ulcers and these are the major causative factors of gastric ulcer. Therefore the detailed mechanism of stress, NSAIDs and *H. pylori* induced ulcer pathogenesis is described as follows.

Stress induced ulcer pathogenesis

Numerous studies have revealed conflicting conclusions regarding the role of psychological factors in the pathogenesis and natural history of peptic ulcer disease. The role of psychological factors is far from established. Acute stress results in increases in pulse rate, blood pressure and anxiety, but only in those patients with duodenal ulcers did acute stress actually result in significant increases in basal acid secretion. Ulcer patients typically exhibit the same psychological makeup as the general population, but they appear to perceive greater degrees of stress. The results of most studies indicate that 75% to 100% of patients in the ICU have abnormalities of the gastric mucosa within hours after admission (Spirt, 2004).

The association between severe physiologic stress and gastrointestinal (GI) ulceration is well established. The pathogenesis of stress-related mucosal disease (SRMD) has not been described completely, but there is a strong evidence that hypoperfusion of the upper GI tract is the major cause.

Aggressive management of the underlying disease is the most important factor in the prevention of stress ulceration (Spirt, 2004). In a rat model, Itoh and Guth (1985) found that oxygen derived free radicals, particularly O_2^-, appear to play an important role in the formation of gastric lesions produced by ischemia plus hydrochloric acid during stress condition. In another study in rats, they have also found that even without intragastric hydrochloric acid, sys-temic ischemia followed by retransfusion of shed blood caused histologic mucosal injury in the corpus and antrum. These investigators reported that a longer period of ischemia caused more lesions and that reperfusion (retransfusion) was a critical factor in lesion development. The term stress-related mucosal disease (SRMD) represents a continuum of conditions ranging from stress-related injury (superficial mucosal damage) to stress ulcers (focal deep mucosal damage) as shown in Fig 5.

Stress is known to induce oxidative stress mediated by generation of free radicals resulting in a) up regulation of H^+, K^+- ATPase; b) increase in acidity; c) disruption of mucosal epithelium and; d) susceptibility for *H. pylori* infection ultimately leading to e) gastric ulceration as depicted in Fig 6 & 7. Ulcer therefore is caused due to imbalance between aggressive and defensive (gastroprotective) factors during stress condition.

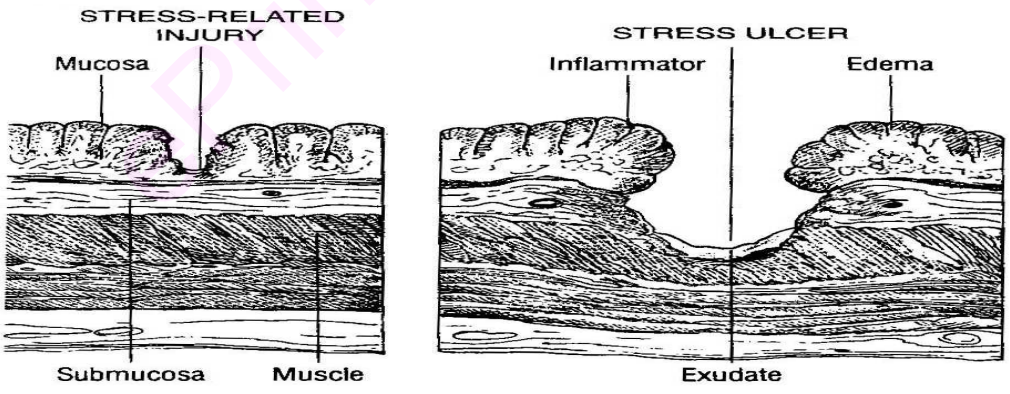


Fig 5. Depth of tissue injury in stress-related injury (A) and stress ulcer (B).

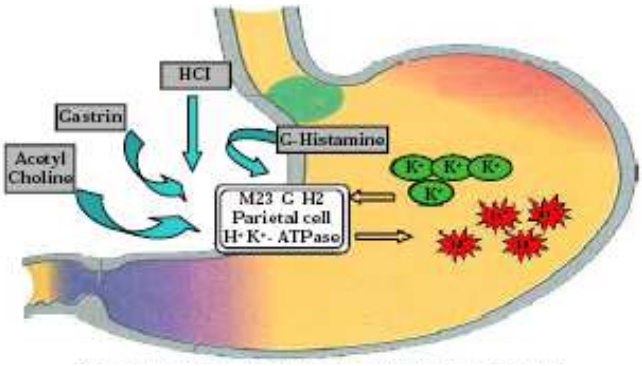


Fig 6. Disregulation of parietal cell activity results in hyperacidity during stress

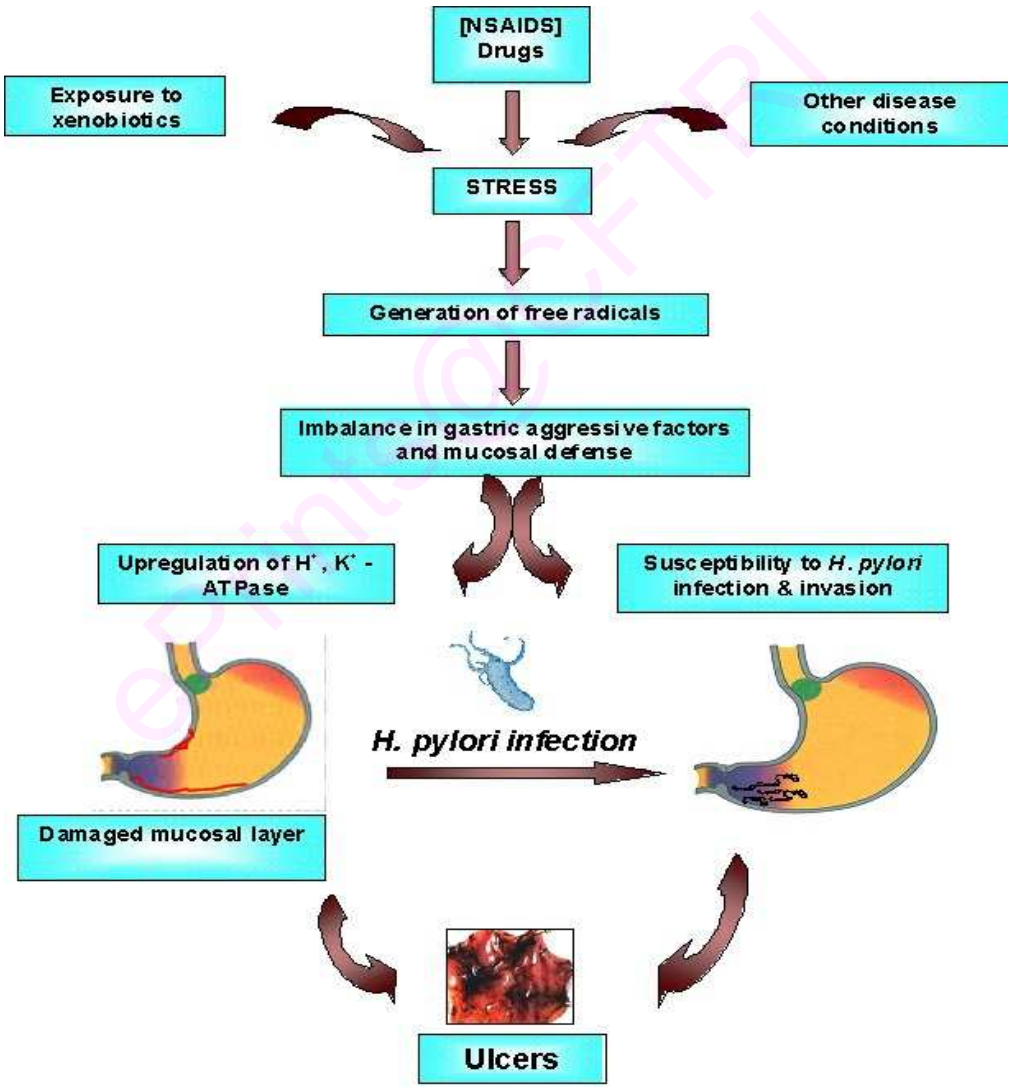
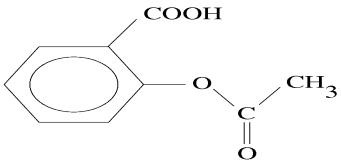


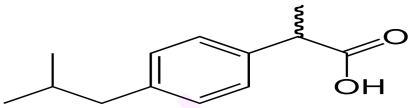
Fig 7. Multi-steps involved in stress induced ulcer pathogenicity.
Ref: Dharmesh & Srikanta, 2009.

NSAIDs induced ulcer pathogenesis

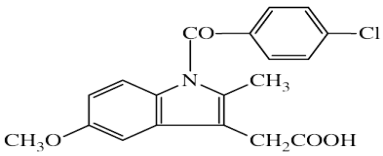
The most common NSAIDs are aspirin, ibuprofen, and naproxen, although many others are available (Fig 8). The concept of gastroduodenal mucosal injury has evolved from the notion of topical injury to concepts that involve multiple mechanisms of mucosal defense. Topical injury by ion trapping (Davenport, 1969) and reduction of mucus gel hydrophobicity (Lichtenberger et al., 1995) was once thought to be an important mechanism of NSAID-induced gastric damage.



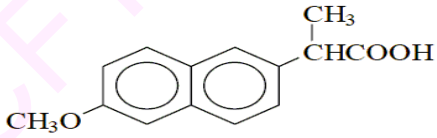
Aspirin
2-acetoxybenzoic acid



Ibuprofen
(*RS*)-2-(4-(2-methylpropyl)phenyl) propanoic acid



Naproxen
(+)-(*S*)-2-(6-methoxynaphthalen-2-yl) propanoic acid



Indomethacin
2-{1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl}acetic acid

Fig 8. Structures of most commonly used NSAIDs

Since Vane's discovery in 1971 (Vane, 1971) that, NSAIDs damage the stomach mainly by suppression of gastric prostaglandin synthesis, there is substantial evidence that the ulcerogenic effect of an NSAID correlates well with its ability to suppress prostaglandin synthesis (Wallace, 2008). The study by Wallace and colleagues (Wallace et al., 2001) showed that selective inhibition of either COX-1 or COX-2 is not associated with gastrointestinal damage. Rather, it was suggested to be the dual inhibition of COX-1 and COX-2 that is important. Selective inhibition of COX-2 delays healing of experimental ulcers, suggesting that COX-2 is important in restoring gastric mucosal integrity (Mizuno et al., 1997). Neutrophil adherence damages the mucosa by liberating oxygen free radicals, releasing proteases, and obstructing capillary blood flow in NSAID user as shown in Fig 9. Inhibition

of neutrophil adherence alleviates NSAID induced damage in animal models. Therefore attention is focused on the role of nitric oxide (NO) and hydrogen sulphide (H_2S), in maintenance of gastric mucosal integrity. NO and H_2S increase mucosal blood flow, stimulate mucus secretion, and inhibit neutrophil adherence (Wallace et al., 1990). Unlike animal ulcer models, however, NSAID gastropathy in man is characterised by an absence of inflammatory cells unless *H. pylori* infection is present. Whether neutrophils initiate NSAID injury in man is unknown.

On the other hand, there are number of studies showed the necessity of prostaglandins, particularly PGE_2 in mucoprotection (Konturek et al., 1981). Prostaglandins stimulate several factors felt to be important in maintaining normal mucosal integrity, such as mucus synthesis and secretion, mucosal bicarbonate secretion, mucosal blood flow, and cellular repair (Wilson, 1991; Kokoska et al., 1998). Thus systemic effects of NSAIDs appear to play a predominant role through the decreased synthesis of mucosal prostaglandins by inhibition of COX enzyme (Fig 9).

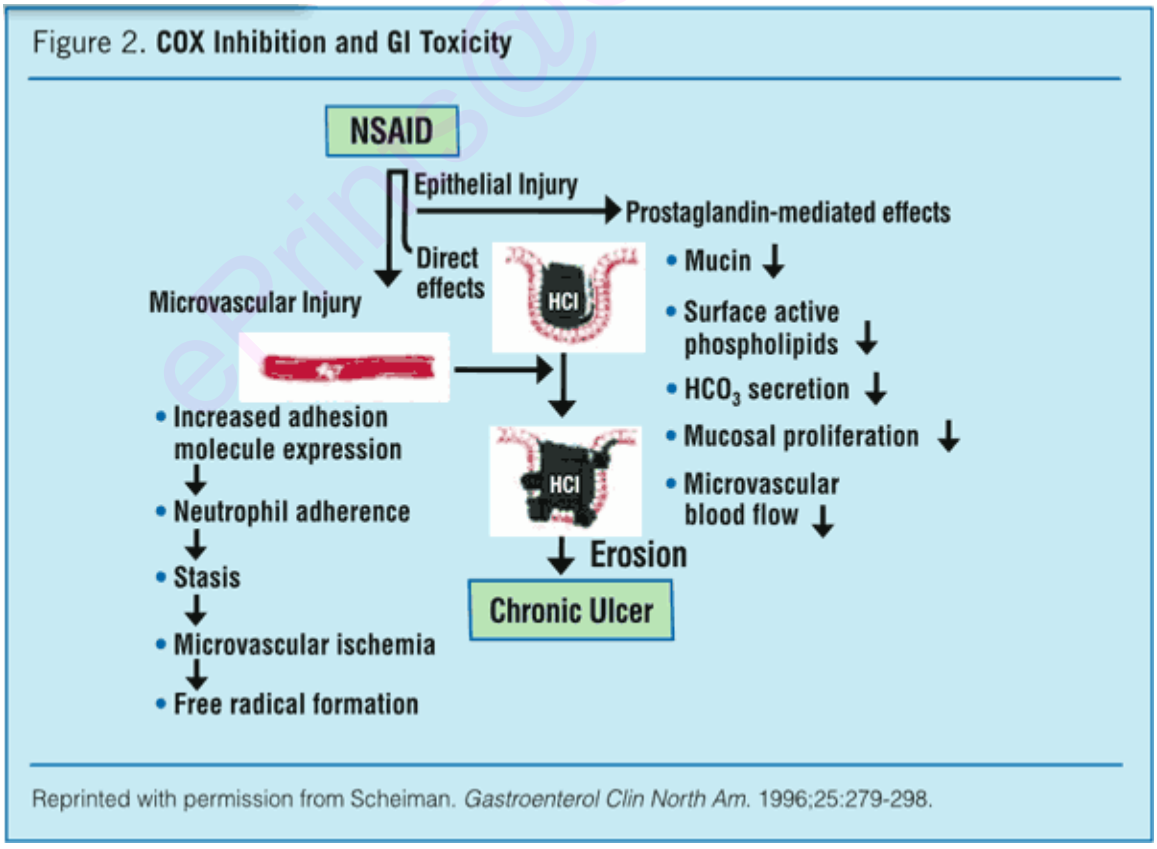


Fig 9. Mechanism of gastric mucosal damage induced by NSAIDs.

In addition, two systematic reviews have shown that *H. pylori* infection substantially increases risk of peptic ulcer and ulcer bleeding in chronic NSAID users due to combinational effects as shown in Fig 10 (Huang et al., 2002). Therefore, although mucin protection and acid suppressions are the mainstay of management of NSAID-associated ulcer disease, *H. pylori* eradication is needed in case of *H. pylori* positive ulcers.

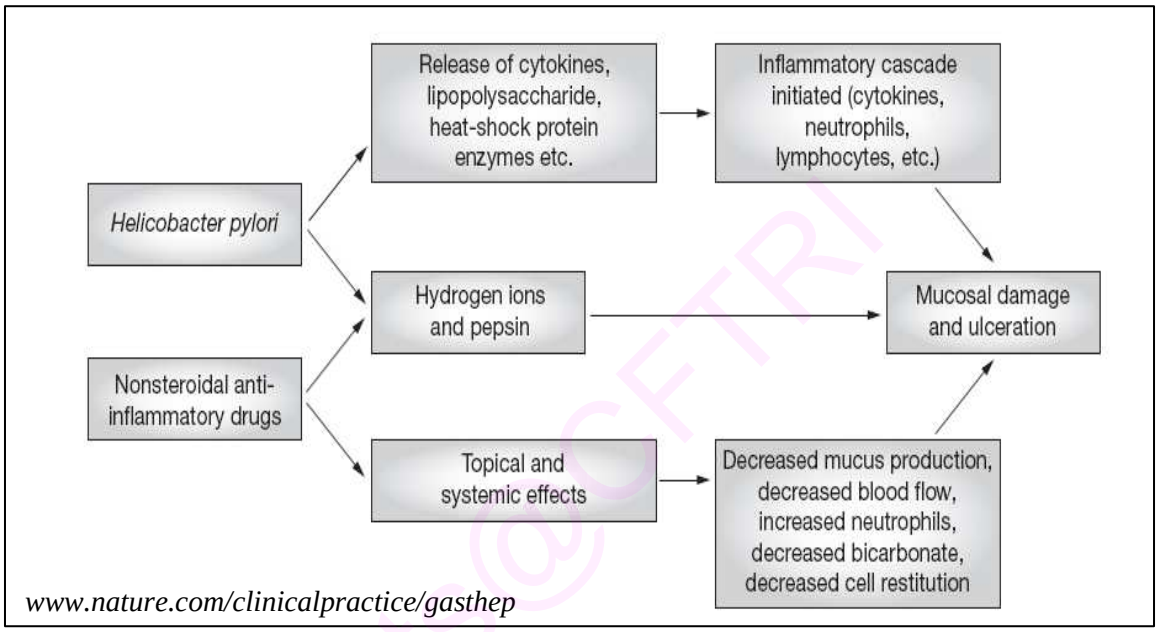


Fig 10. NSAIDs and *H. pylori* act synergistically through inflammatory pathways to develop gastric ulcers

H. pylori induced ulcer pathogenesis

Marshall and Warren's (**Fig 11A)** seminal discovery that a humble bacterium, *H. pylori* (**Fig 11B)**, causes gastric ulcers, and in some cases, gastric cancer, merits the 2005 Nobel Prize in Physiology or Medicine for its remarkable impact on public health and for opening up new avenues of research. Among the more than 20,000 articles about *H. pylori* published since 1983, many have been devoted to the study of the bacterium and understanding its secrets (Mégraud, 2005).

The genus Helicobacter belongs to the subdivision of the Proteobacteria, order Campylobacterales, family Helicobacteraceae. This family also includes the genera Wolinella, Flexispira, Sulfurimonas, Thiomicrospira, and Thiovulum. To date, the genus Helicobacter consists of over 20 recognized

species, with many species awaiting formal recognition (Fox, 2002). Members of the genus Helicobacter are all microaerophilic organisms and in most cases are catalase and oxidase positive, and many but not all species are also urease positive.

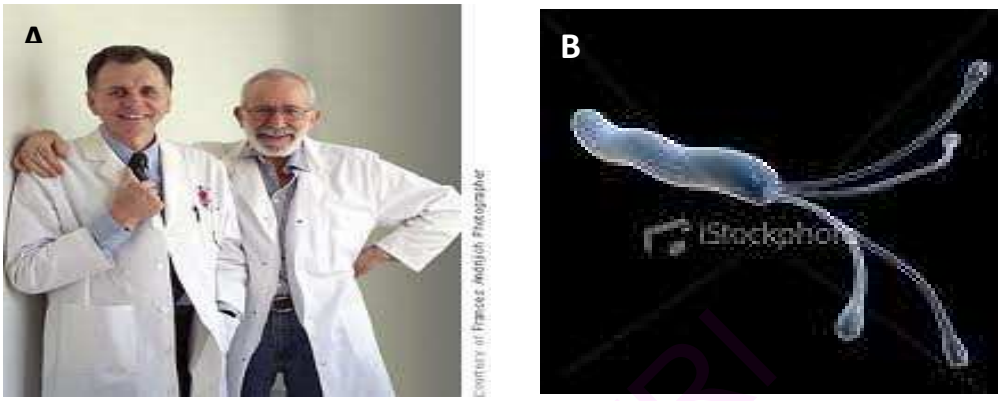


Fig 11. Barry Marshall (left) and Robin Warren (right) in Fig A and image of *Helicobacter pylori* (B); winners of the 2005 Nobel Prize for their discovery of "the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease". **Fig B** is a image of *H. pylori*.

A key feature of *H. pylori* is its urease activity and microaerophilicity, with optimal growth at O_2 levels of 2 to 5% and the additional need of 5 to 10% CO_2 and high humidity. Growth occurs at 34 to 40°C, with an optimum of 37°C. Although its natural habitat is the acidic gastric mucosa, *H. pylori* is considered to be a neutralophile. The bacterium will survive brief exposure to pH of <4, but growth occurs only at the relatively narrow pH range of 5.5 to 8.0, with optimal growth at neutral pH. *H. pylori* is a fastidious microorganism and requires complex growth media. Often these media are supplemented with blood or serum (Kusters et al., 2006).

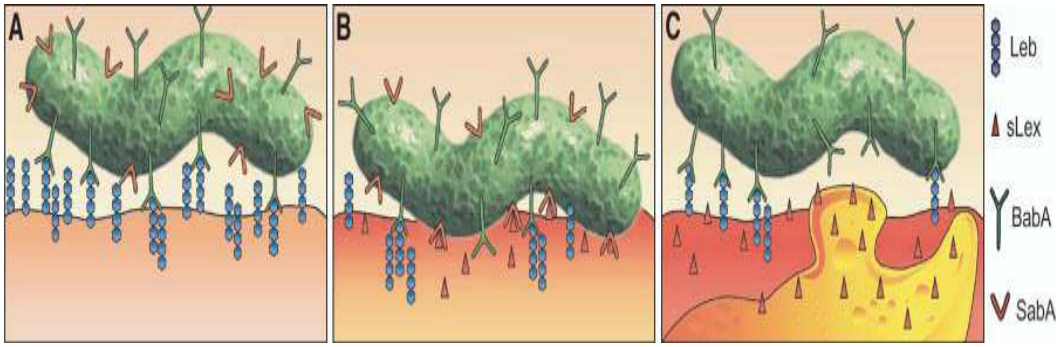
H. pylori (Fig 11B) is a gram-negative bacterium, measuring 2 to 4 μm in length and 0.5 to 1 μm in width. Although usually spiral-shaped, the bacterium can appear as a rod, while coccoid shapes appear after prolonged *in vitro* culture or antibiotic treatment. The organism has 2 to 6 unipolar, sheathed flagella of approximately 3 μm in length, which often carries a distinctive bulb at the end. The flagella confer motility and allow rapid movement in viscous solutions such as the mucus layer overlying the gastric epithelial cells. In contrast to many other pathogens of the gastrointestinal tract, it lacks fimbrial adhesions, which are commonly associated with the majority of bacterial adhesins (Kusters et al., 2006).

Colonization of *H. pylori*

The process of colonization consists of four steps: (i) transmission to a new host; (ii) bacterial adherence to a specific niche within the host; (iii) avoidance, subversion, or exploitation of host defense mechanisms; and (iv) acquisition of nutrients resulting in successful replication (Traci et al., 2001). Transmission of *H. pylori* occurs from person to person, following an oral-oral or fecal-oral route. Both microbial and host factors for attachment of *H. pylori* are thought responsible for the variety of pathogenic outcomes of infection. The genetic diversity not only of humans, but of *H. pylori* may provide various combinations of adhesins and receptors as shown in Table 2. Although there are several host molecules which can act as receptors for *H. pylori* adhesins (Table 2), gastric mucin has been known to attract very well and hence *H. pylori* can induce ulcers. The figure shown below (Fig 12) illustrates the proficiency of *H. pylori* for adaptive multistep mediated adhesion to host cells and adhesion induced changes in expression of host cell surface antigens.

***H. pylori* adhesins**	**Host cell receptors**
AlpA, AlpB	Unknown
BabA	Lewis b
HopZ	Unknown
HpaA, Nap; 64 kDa, 62 kDa, 56 kDa, 20 kDa	*N*-acetylneuraminyllactose (sialic acid)
Hsp60, Hsp70	Lactosylceramide sulfate, galactosylceramide sulfate (sulfatides)
LPS	97-kDa mucin receptor
LPS core	Laminin
LPS O antigen (Lewis X); Nap	Lewis X
Nap	Gastric mucin
19.6 kDa (ferritin)	Laminin
25 kDa	Laminin
61-kDa protein	H type 2 (O antigen), Lewis b, Lewis a
63 kDa (Exoenzyme S-like adhesin)-catalase; Nap	Heparan sulfate and other sulfated polysaccharides; phosphatidylethanolamine, gangliotriaosylceramide (GM3), gangliotetraosylceramide (GM2), gangliotriaosylceramide; gangliotetraosylceramide
Unknown	Class II MHC
Unknown	β1 integrins
AlpA, AlpB	Unknown
BabA	Lewis b

Table 2. List of known *H. pylori* adhesions and host cell receptors.
(*Ref: Traci et al., 2001, ASM Press*)



Source: Mahdavi et al., 2002

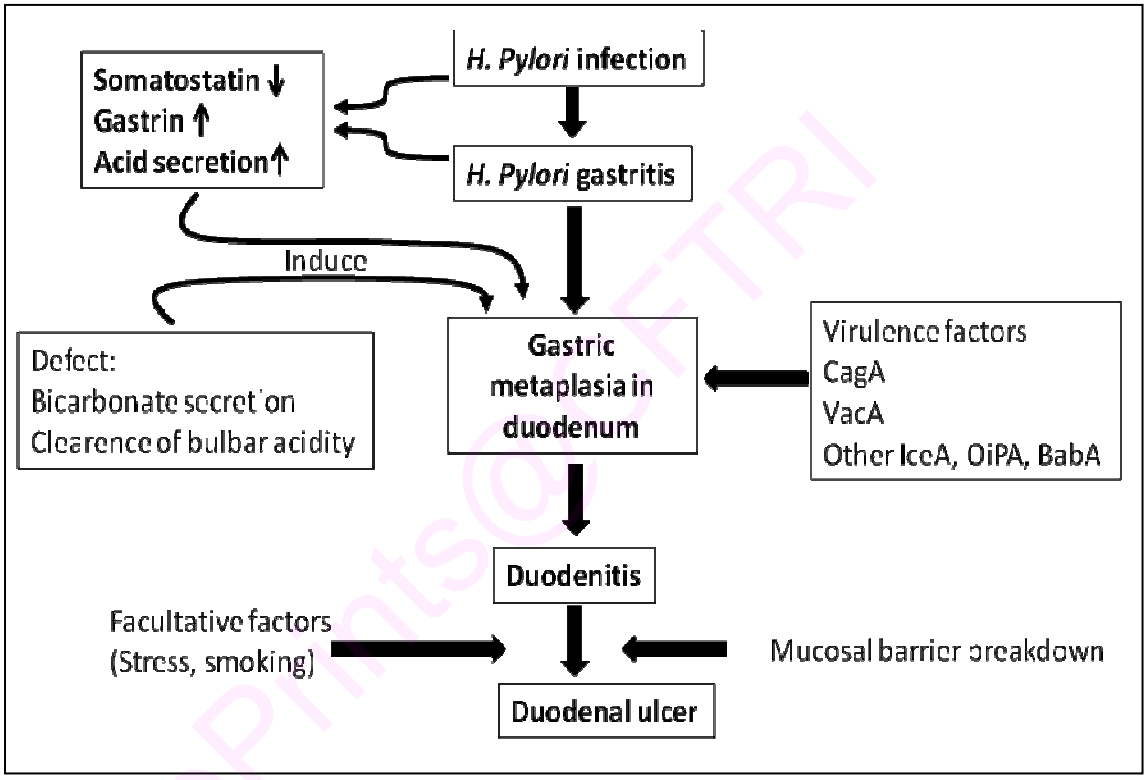
Fig 12. *H. pylori* adherence induced changes in host cells.

This figure illustrates the proficiency of *H. pylori* for adaptive multistep mediated attachment. (**A**) *H. pylori* (in green) adherence is mediated by the Leb blood group antigen expressed in glycoproteins (blue chains) in the gastric surface epithelium (the lower surface). *H. pylori* uses BabA adhesins (green Y's) for strong and specific recognition of the Leb antigen. Most of the sLex binding isolates also bind the Leb antigen (SabA, in red Y's). (**B**) During persistent infection and chronic inflammation (gastritis), *H. pylori* triggers the host tissue to retailor the gastric mucosal glycosylation patterns to up-regulate the inflammation-associated sLex antigens (red host, triangles). Then, SabA (red Y structures) performs Selectin-mimicry by binding the sialyl-(di)-Lewis x/a glycosphingolipids, for membrane close attachment and apposition. (**C**) At sites of vigorous local inflammatory response, as illustrated by the recruited activated white blood cell (orange "bleb"), those *H. pylori* subclones that have lost sLex binding capacity due to ON/OFF frameshift mutation might have gained local advantage in the prepared escaping of intimate contact with (sialylated) lymphocytes or other defensive cells. Such adaptation of bacterial adherence properties and subsequent inflammation pressure could be major contributors to the extraordinary chronicity of *H. pylori* infection in human gastric mucosa.

H. pylori infection impairs negative feedback regulation of gastrin release and thus acid secretion.

H. pylori colonizes the entire gastric epithelium, from the prepyloric antrum to the cardia (Gillen et al., 1998). In gastric ulcer, inflammation affects the body and antral mucosa to a similar degree, although it varies dependent on

ulcer location (Schultze et al., 1998). *H. pylori* infection impairs acid secretion by upregulation of gastrin and downregulation of somatostatin secretion leading to increased acid secretion in the duodenum leading to duodenal metaplasia (Fig 13). Metaplasia is a prerequisite for *H. pylori* colonization of duodenal epithelium necessary for *H. pylori* colonization in the duodenum leading to duodenal ulcer (Khulusi et al., 1996). Unlike in duodenal ulcer, acid secretion in gastric ulcer can be decreased because of the more severe damage to acid-secreting body mucosa by *H. pylori*.



Ref: Malfertheiner et al., Lancet 2009; 374: 1449–61.

Fig 13. Changes in gastrin and acid secretion due to *H. pylori* infection.

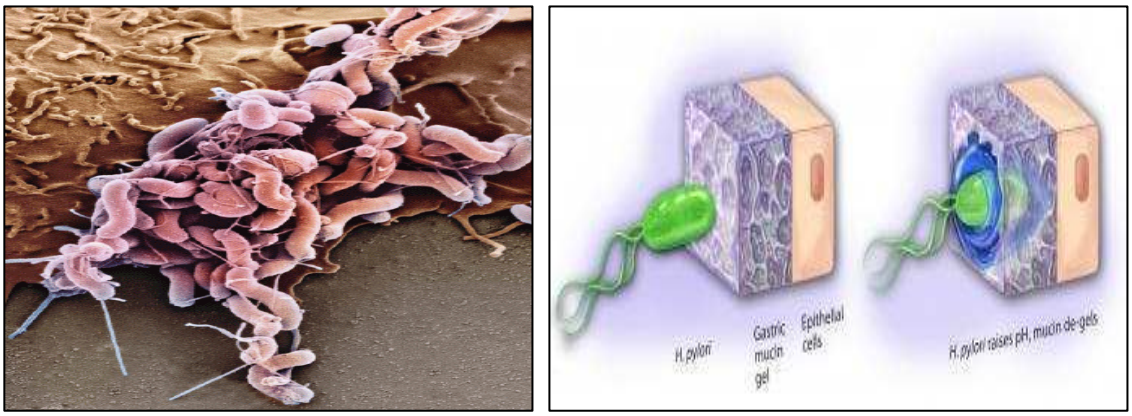
Virulent factors of *H. pylori*

H pylori isolated from patients with ulcer disease carry a high virulence. Features of increased virulence include a strong adhesive property and increased production of enzymes with toxic potential. Strains from ulcer patients might produce higher amounts of urease than do those from people without ulcers. Urease catalyses production of ammonia, which in high concentrations is followed by formation of toxic complexes such as NH_4Cl. Urease activity and motility using flagella are essential factors for

colonization of *H. pylori*. Urease of *H. pylori* exists both on the surface and in the cytoplasm, and is involved in neutralizing gastric acid by hydrolyzing urea to produce ammonia and helps in chemotactic motility. *H. pylori* senses the concentration gradients of urea in the gastric mucus layer, then moves toward the epithelial surface by chemotactic movement. The energy source for the flagella movement is the proton motive force. The hydrolysis of urea by the cytoplasmic urease possibly generates additional energy for the flagellar rotation in the mucus gel layer (Yoshiyama & Nakazawa, 2000). A team of researchers from Boston University, Harvard Medical School and Massachusetts Institute of Technology recently made a discovery that changes a long held paradigm about how bacteria move through soft gels. They showed that, contact with stomach acid keeps the mucin lining the epithelial cell layer in a spongy gel-like state at acidic pH. This consistency is impermeable to *H. pylori*. However, the bacterium releases urease which neutralizes the stomach acid. This causes the mucin to liquefy, and the bacterium can swim right through watery fluids using their tails to propel them as shown in the Fig 14 (Cellia et al., 2009).

Bacterial phospholipases A and C impair the phospholipid-rich layer in the mucosa that maintains mucosal hydrophobicity and integrity of the gastric epithelial barrier (Fig 15) (Smoot et al., 1990). Specific *H. pylori* genotypes are associated with severe morbidity. The most prevalent *H pylori* genotypes in patients with peptic ulcerations are vacA-positive and cagA-positive (Van Doorn et al., 1998). *H pylori*-derived vacuolating cytotoxin (VacA), an ~97 kDa protein, causes vacuolar degeneration. The cytotoxin-associated gene A (*cagA*), restricted to cytotoxin-producing strains of *H pylori*, is within an island of 31 genes defined as CagA pathogenicity island.

CagA encodes a ~120–160 kDa immunodominant protein that is a marker of increased virulence and enhances the local inflammatory response as shown in Fig 15 (Van Doorn et al., 1998). A series of other virulence genes with a higher prevalence in ulcer disease, such as the adhesion protein BabA and the outer membrane inflammatory protein OipA, are likely to play a rather modest part in pathogenesis (Fig 15). The body's immune system responds to the presence of *H. pylori* and sends infection-fighting cells to the area.



Ref: www.jaapa.com/.../article/173479/ *Ref: ScienceDaily (Aug. 19, 2009)*

Fig 14. *H. pylori* colonizing on human gastric epithelial cells (A), and diagrammatic representation of *H. pylori* crossing mucus layer of stomach (B).

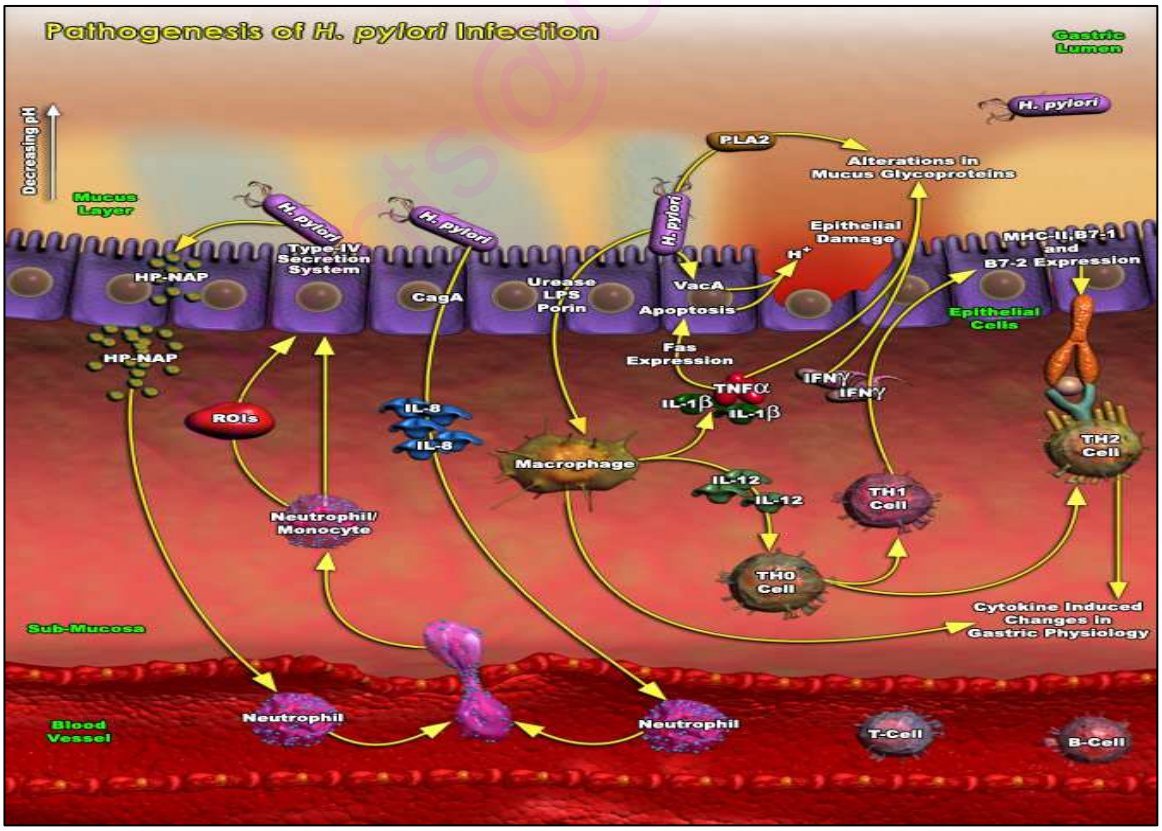


Fig 15. Molecular interplay during *H. pylori* induced ulcer pathogenesis.

H. pylori causes an inflammatory response in gastric mucosa, with induction of epithelium-derived cytokines, predominantly interleukin 8 and interleukin 1β (Dixon, 2000). However, the neutrophils cannot reach the *H. pylori* infection because they cannot easily get through the stomach lining. Inflammation in the stomach tissue occurs as the neutrophils die and release superoxide radicals on the stomach wall, damaging tissue. The immune system sends in more nutrients to help the neutrophils, and the *H. pylori* can feed on these nutrients. It may not be the *H. pylori* itself that causes a stomach ulcer, but inflammation in the stomach lining as part of the immune response as shown in Fig 15 (Wilson and Crabtree, 2007).

Strategies of *H. pylori* to avoid host immune response

Alongside the capability of breaching the cellular and physical barriers, *H. pylori* has additionally developed ways of avoiding both the innate and the adaptive immune response of the host organism. Many potential mechanisms for the failure of the host response have been postulated, and these include apoptosis of epithelial cells and macrophages, inadequate effector functions of macrophages and dendritic cells, vacA inhibition of T-cell function, and suppressive effects of regulatory T cells (Wilson and Crabtree, 2007) as follows.

To date, several bacterial strategies have been described (some of them are illustrated in Fig 16 and Table 3). For example, *H. pylori* flagellar proteins have developed in a way that masks them from the Toll-like receptor 5 (an innate immune receptor that recognizes various other bacterial flagellins). LPS from *H. pylori* is 1000 times less pyrogenic and 500-folds less toxic than that of gram-negative enteric bacteria due to modifications that mimic human glycans and therefore induces less inflammation. *H. pylori* is also capable of obtaining cholesterol directly from epithelial cell membranes and incorporating it into its own lipid layer. To escape macrophage phagocytosis it modifies obtained cholesterol by adding a glucosyl group (a reaction catalysed by cholesterol-α glucosyltransferase). Additionally, *H. pylori* reduces generation of bactericidal nitric oxide by the macrophage. Its enzyme arginase competes with macrophages inducible nitric oxide synthase for substrate L-arginine that is used by the bacteria for the synthesis of urea (a substrate for urease).

A two-way interaction might exist between *H pylori* and gastric acid that determines pattern of gastritis and hence clinical outcome. Environmental factors and host factors are associated with low acid secretion status. These factors favour the colonization of *H pylori* in the corpus with intense inflammation and hence further reduction in acid secretion. The resultant hypochlorhydria promotes *H pylori* colonization, more intense corpus inflammation with subsequent development of gastric atrophy, gastric ulcer, and cancer (Chan & Leung, 2002). The Fig 17 summarizes the *H. pylori* infection, colonization and ulcer pathogenesis.

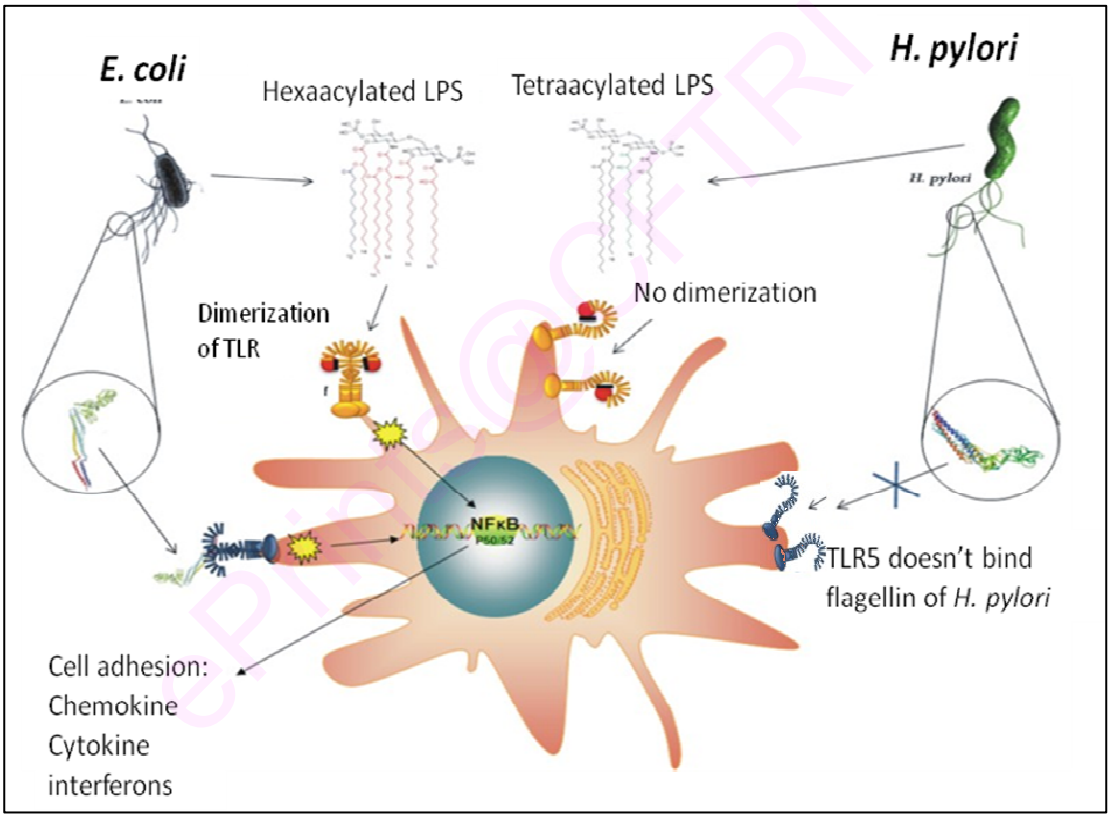


Fig 16. Mechanism of avoiding host immune response by *H. pylori*:

MECHANISM	EXAMPLE
Bypassing the TLR system	• Flagellin fails to activate TLR5
	• Tetraacylated LPS poorly activates TLR4 and may actually work as an antagonist
	• DNA is rich in A/T nucleotides and frequently methylated, making TLR9 response more unprobable
Minimization of innate immunity	• Modification of LPS (long 3-hydroxy fatty acids of lipid A, unusual phosphorylation pattern…)
Mimicry of host antigens	• Lewis expression (O antigen region of H. pylori LPS)
Antigenic variation	• CagY
Host gene expression modulation	• Upregulation of specific inflammatory and immune mediators including β-defensin, protease inhibitor, chemokine receptor, interleukin-1β, tumor necrosis factor-α-inducible protein
Downregulation of immune effectors	• Blocking the proliferation of T cell through VacA and B cell proliferation through CagA-induced supression- Interference with phagocytosis
Avoidance of attack by ROIs and RNIs	• Enzymes involved in ROI scavenging, such as catalase and superoxide dismutase
	• Arginase regulates NO synthesis
Ability to colonize gastric environment	• Neutralizing pH around the organism with urease enzyme
	• Interaction between adhesins and local cell receptors
	• Expressing mucolytic molecules
	• Relative absence of immune cells in gastric mucosa and rare competition with commensal bacteria
High mutational and recombination frequency, high diversity	• Rapid development in the bacterial population of high-level resistance to commonly used antibiotics
	• High competence for uptake of DNA from other *H. pylori* strains

Table 3. Strategies of *H. pylori* to avoid host immune system.

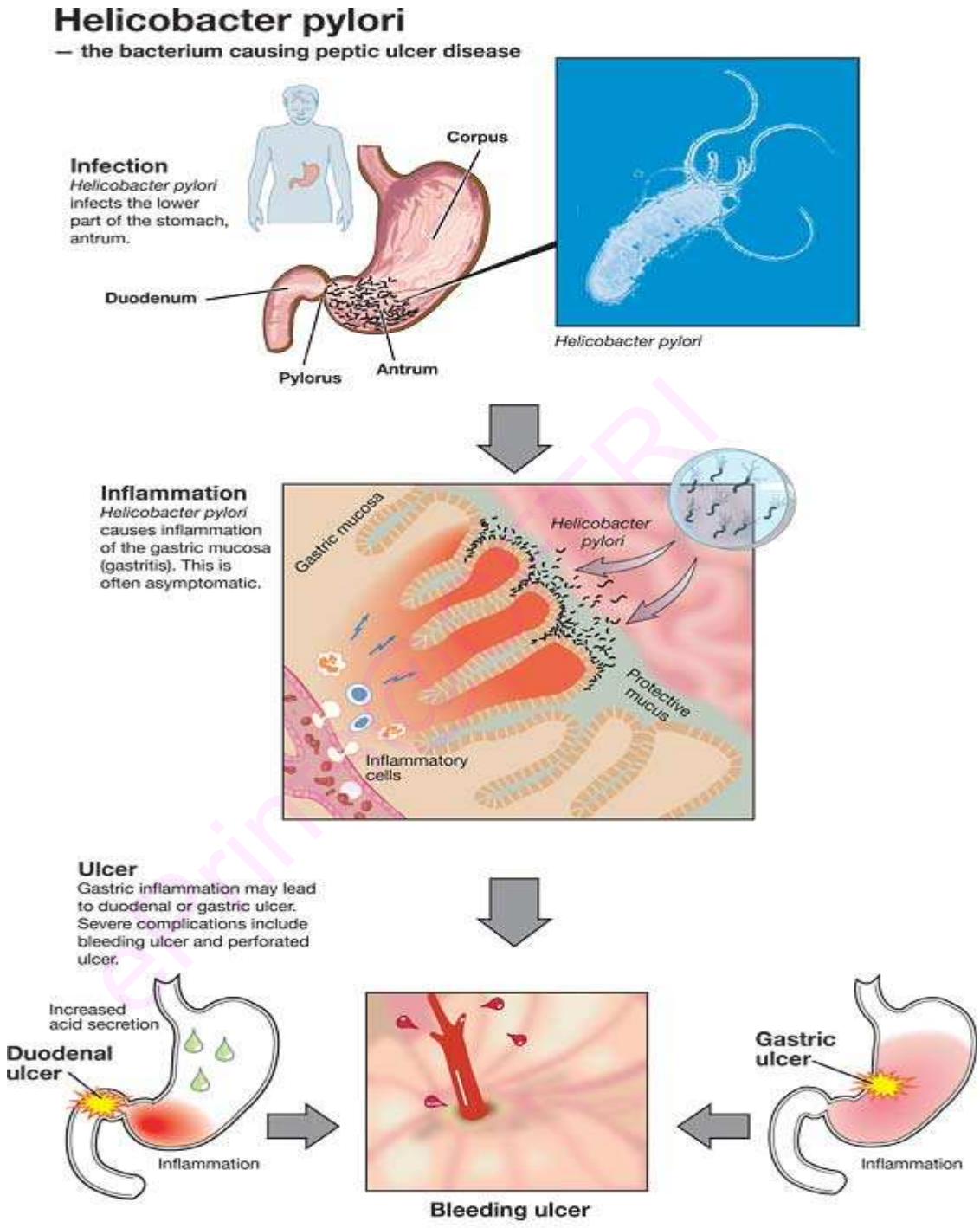


Fig 17. Summary of *H. pylori* infection, colonization and ulcer pathogenesis.

Role of gastric parietal cells and gastric acid in ulcer pathogenesis

Gastric acid is a secretion produced in the stomach. It is one of the main solutions secreted, together with several enzymes and intrinsic factors. Chemically it is an acid solution with a pH of 1 to 3 in the stomach lumen, consisting mainly of hydrochloric acid (HCl) (around 0.5%, or 5000 parts per million), and large quantities of potassium chloride (KCl) and sodium chloride (NaCl). The resulting highly acidic environment in the stomach lumen denatures proteins of the food. This also stimulates chief cells of the stomach to secrete enzymes for protein breakdown (inactive pepsinogen and renin). Gastric acid activates pepsinogen into pepsin–this enzyme then helps digestion by proteolysis. In addition, gastric acid inhibits the growth of many microorganisms and prevents infection.

The disturbance in normal acid secretion leads to gastrointestinal complications. In hypochlorhydria and achlorhydria, there is low or no gastric acid in the stomach, potentially leading to the loss of disinfectant properties of the gastric lumen and in such conditions, there is greater risk of infections of the digestive tract (*H. pylori* infection). In Zollinger-Ellison syndrome and hypercalcemia, there are increased gastrin levels, leading to excess gastric acid production, which can cause mucosal damage leading to gastric ulcers. Gastric acid is produced by parietal cells (also called oxyntic cells) found in the gastric glands of the stomach as shown in Fig 18A. Its secretion is a complex and relatively energetically expensive process. Parietal cells (Fig 18B) are part of the epithelial fundic glands in the gastric mucosa. Recent studies showed ultrastructural and functional changes of gastric parietal cells during stress conditions (Li et al., 2006).

The acidity in the stomach is maintained by the proton pump H^+, K^+ -ATPase enzyme. This enzyme uses the chemical energy of ATP to transfer H^+ ions from parietal cell cytoplasm to the secretory canaliculi in exchange for K^+. The H^+, K^+ -ATPase is located within the secretory canaliculus and in nonsecretory cytoplasmic tubulovesicles of parietal cell. The tubulovesicles are impermeable to K^+, which leads to an inactive pump in this location. The distribution of pumps between the nonsecretory vesicles and the secretory canaliculus varies according to parietal cell activity (Fig 19). Under resting conditions, only 5% of pumps are within the secretory canaliculus, whereas

upon parietal cell stimulation, tubulovesicles are immediately transferred to the secretory canalicular membrane, where 60 to 70% of the pumps are activated. Proton pumps are recycled back to the inactive state in cytoplasmic vesicles once parietal cell activation ceases.

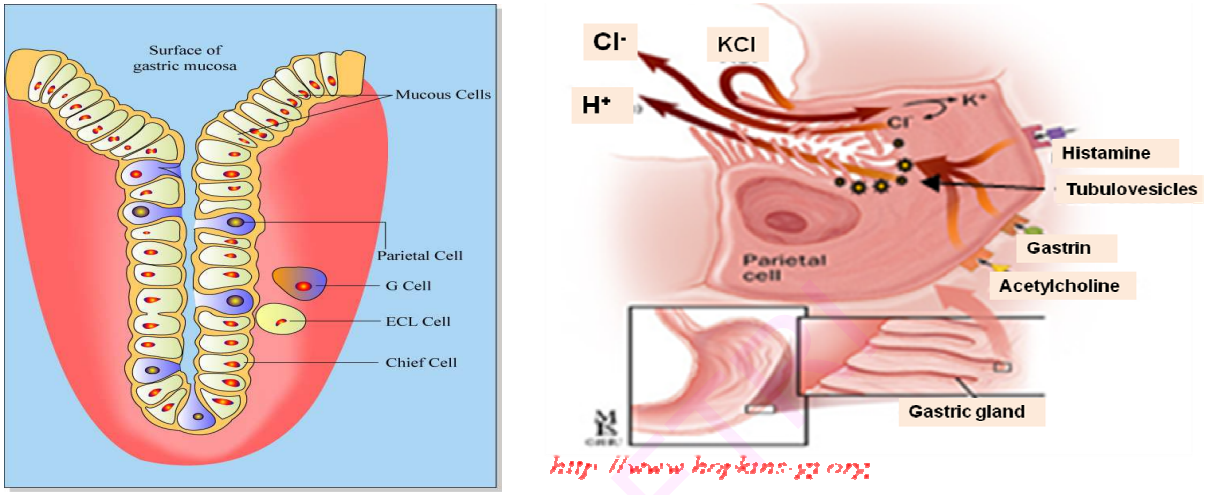


Fig 18. Gastric gland showing different cell types (A) and enlarged view of parietal cell (B).

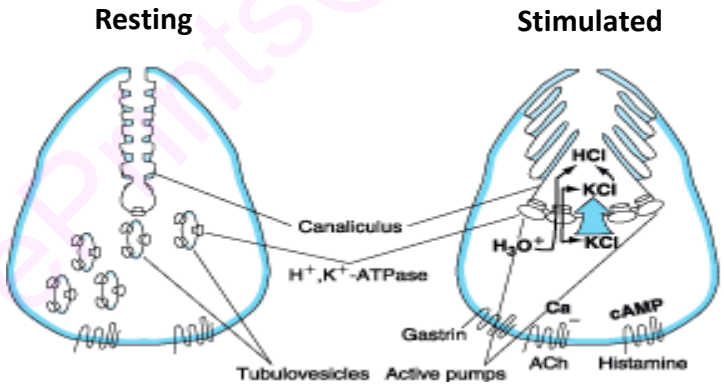


Fig 19. Structural differences of parietal cell between resting and stimulated conditions.

Mechanism of gastric acid secretion

The hydrogen ion concentration in parietal cell secretions is roughly 3 million fold higher than in blood, and chloride is secreted against both a concentration and electric gradient. Thus, the ability of the parietal cell to secrete acid is dependent on active transport. The key player in acid

secretion is a H^+, K^+ -ATPase or "proton pump" located in the cannalicular membrane. The current model for explaining acid secretion is as follows (Fig 20):

- Hydrogen ions are generated within the parietal cell from dissociation of water. The hydroxyl ions formed in this process rapidly combine with carbon dioxide to form bicarbonate ion, a reaction catayzed by carbonic anhydrase.
- Bicarbonate is transported out of the basolateral membrane in exchange for chloride. The outflow of bicarbonate into blood results in a slight elevation of blood pH known as the "alkaline tide". This process serves to maintain intracellular pH in the parietal cell.
- Chloride and potassium ions are transported into the lumen of the cannaliculus by conductance channels, and such is necessary for secretion of acid.
- Hydrogen ion is pumped out of the cell, into the lumen, in exchange for potassium through the action of the proton pump; potassium is thus effectively recycled.
- Accumulation of osmotically-active hydrogen ion in the cannaliculus generates an osmotic gradient across the membrane that results in outward diffusion of water - the resulting gastric juice is 155 mM HCl and 15 mM KCl with a small amount of NaCl.

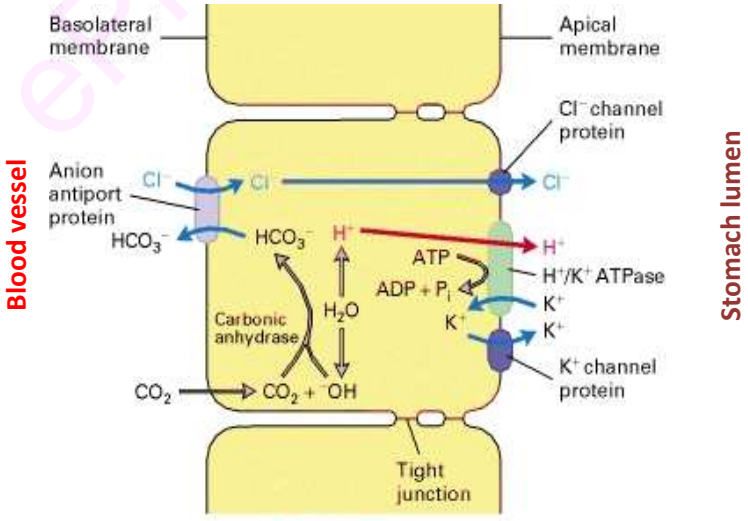


Fig 20. Ion transport in the parietal cell containing H^+, K^+ -ATPase and other ion channels

Regulation of gastric acid secretion

The parietal cell is considered to have at least three types of activating receptors on its basolateral membrane, i.e., histamine - H2, acetylcholine - M3 and gastrin – CCK-B as shown in Fig 21 (Urushidani and Forte, 1997). Parietal cells produce gastric acid (hydrochloric acid) in response to histamine (via H_2 receptors), acetylcholine (M_3 receptors) and gastrin (CCK receptors). The histamine receptors act by increasing intracellular cAMP, whereas the muscarinic (M3) and gastrin receptors increase intracellular Ca^{2+} levels. Both cAMP and Ca^{2+} act via protein kinases to increase the transport of acid into the stomach. Gastrin is more important indirectly by increasing histamine synthesis in enterochromaffin like (ECL) cells, as gastrin has no effect on the maximum histamin-stimulated gastric acid secretion. The production of gastric acid in the stomach is tightly regulated by positive regulators and negative feedback mechanisms. Four types of cells are involved in this process: parietal cells, G cells, D cells and enterochromaffine-like cells. Besides this, the endings of the vagus nerve (CN X) and the intramural nervous plexus in the digestive tract influence the secretion significantly (Fig 21).

The Fig 21 shows the regulatory mechanism of acid secretion (Watson et al., 2006). Gastric acid is secreted across the parietal cell canalicular membrane by the H^+, K^+ -ATPase (proton pump) into the gastric lumen. The main site of gastrin synthesis is the gastrin-containing G cell within the antro-pyloric mucosa. Gastrin is secreted by antral G cells into blood vessels in response to intraluminal dietary peptides. Within the gastric body, gastrin passes from the blood vessels into the submucosal tissue of the fundic glands, where it binds to gastrin-CCK-B receptors on parietal cells and enterochromaffin-like (ECL) cells. The vagus nerve stimulates postganglionic neurons of the enteric nervous system to release acetylcholine (ACh), which binds to M_3 receptors on parietal cells and ECL cells. Stimulation of ECL cells by gastrin (CCK-B receptor) or acetylcholine (M_3 receptor) stimulates release of histamine. Within the gastric antrum, vagal stimulation of postganglionic enteric neurons enhances gastrin release directly by stimulation of antral G cells (through gastrin-releasing peptide, GRP) and indirectly by inhibition of somatostatin secretion from antral D cells. Acid

secretion must eventually be turned off. Antral D cells are stimulated to release somatostatin by the rise in intraluminal H^+ concentration and by CCK that is released into the bloodstream by duodenal cells in response to proteins and fats. Binding of somatostatin to receptors on adjacent antral G cells inhibits further gastrin release.

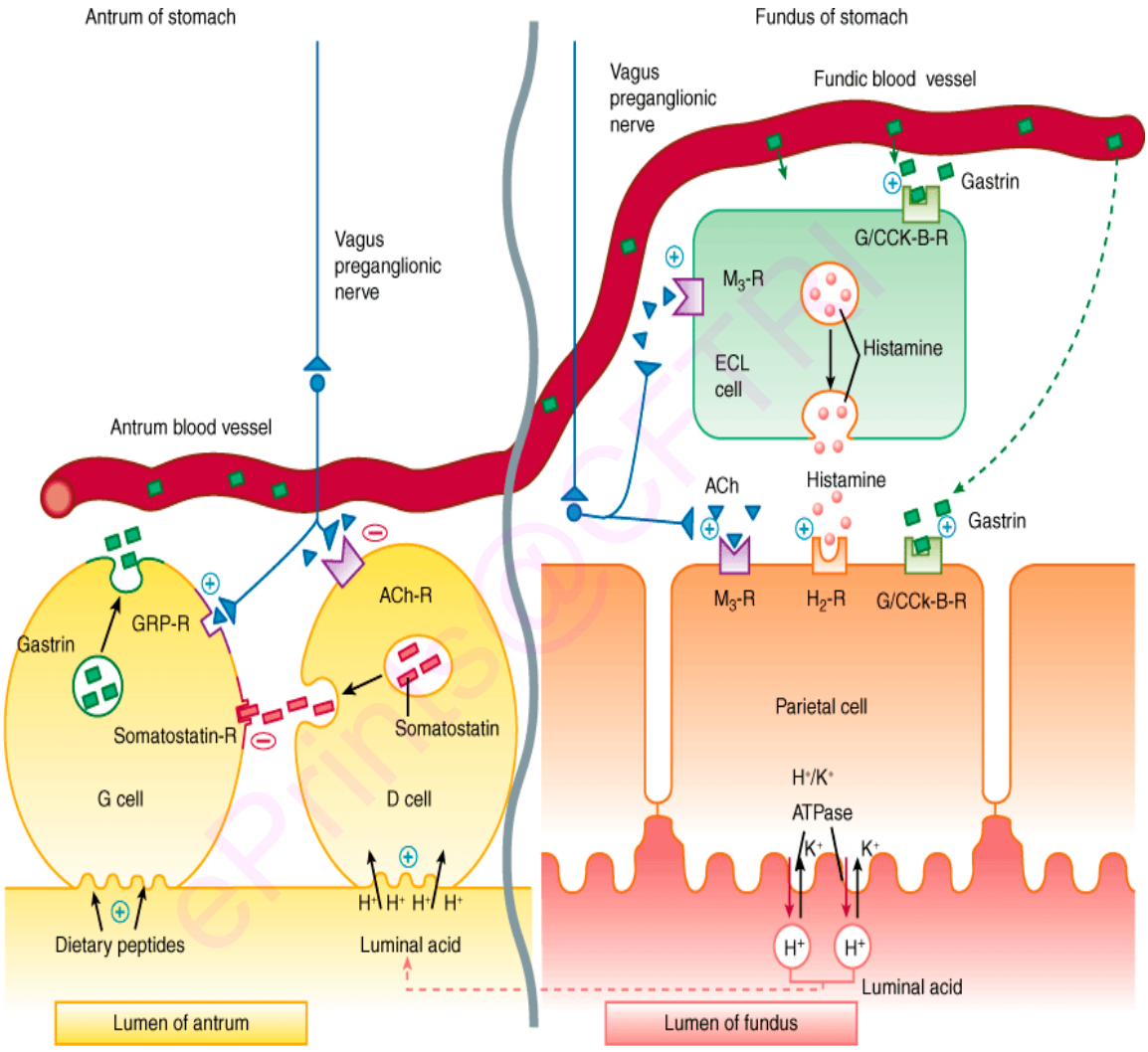


Fig 21. Molecular events in regulation of gastric acid secretion by the parietal cells of the gastric fundic glands. Parietal cells are stimulated to secrete acid (H^+) by gastrin (acting on gastrin/CCK-B receptor), acetylcholine (M_3 receptor), and histamine (H_2 receptor).

Gastric proton potassium ATPase

Molecular cloning experiments have identified the existence of two H^+, K^+ -ATPases, colonic and gastric. Recent functional and molecular studies indicate the presence of both transporters in the kidney, which are presumed to mediate the exchange of intracellular H^+ for extracellular K^+ (Kleinman et al., 1993). On the basis of these studies, a picture is evolving that indicates differential regulation of H^+, K^+ -ATPase at the molecular level in acid-base and electrolyte disorders (Silver & Manoocher, 1999). Of the two transporters, gastric H^+, K^+ -ATPase is expressed constitutively along the length of the collecting duct and is responsible for H^+ secretion and K^+ reabsorption under normal conditions and may be stimulated with acid-base perturbations and/or K^+ depletion. This regulation may be species specific. To date there are no data to indicate that the colonic H^+, K^+ -ATPase plays a role in H^+ secretion or K^+ reabsorption under normal conditions. However, colonic H^+, K^+ -ATPase shows adaptive regulation in pathophysiological conditions such as K^+ depletion, NaCl deficiency, and proximal renal tubular acidosis, suggesting an important role for this exchanger in potassium, HCO_3^-, and sodium (or chloride) reabsorption in disease states (Silver & Manoocher, 1999). Gastric H^+, K^+ -ATPase will be functionally distinguished by its ability to be inhibited by specific blockers. Gastric H^+, K^+ -ATPase is blocked by compounds such as vanadate (a P-type ATPase inhibitor) and the more specific inhibitors Sch-28080 and omeprazole. Gastric H^+, K^+ -ATPase differs from its relative the Na^+ pump by being insensitive to ouabain (Silver & Manoocher, 1999).

The gastric H^+, K^+ -ATPase is a member of the P-type ATPase superfamily, a large family of related proteins that transport ions, most usually cations, across biological membranes in nearly all species. The H^+, K^+ -ATPase transports one hydrogen ion (H^+) from the cytoplasm of the parietal cell in exchange for one potassium ion (K^+) retrieved from the gastric lumen as shown in Fig 20 & 22. As an ion pump the H^+, K^+ -ATPase is able to transport ions against a concentration gradient using energy derived from the hydrolysis of ATP. Like all P-type ATPases, a phosphate group is transferred from adenosine triphosphate (ATP) to the H^+, K^+ -ATPase during the transport cycle as shown in Fig 22. This phosphate transfer powers a

conformational change in the enzyme that helps drive ion transport as depicted in Fig 23.

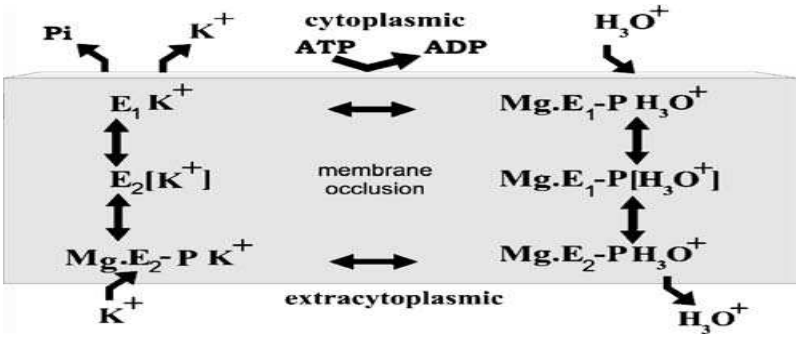


Fig 22. The catalytic cycle of the gastric H^+, K^+ -ATPase. *(Ref: Shin et al., 2009)*

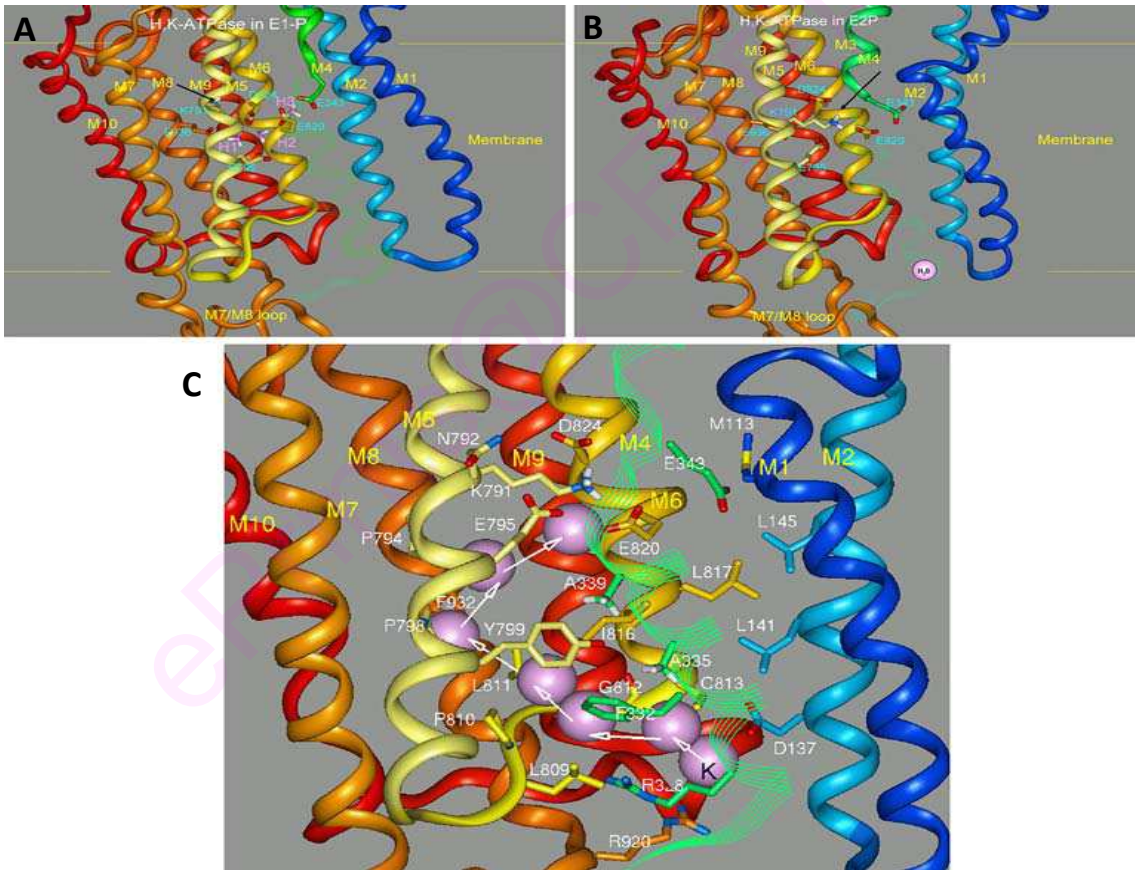


Fig 23. Ribbon diagram depicting the ion entry and exit pathways of the gastric H^+, K^+-ATPase. *(Ref: Shin et al., 2009).*

Fig A illustrates the conformation of the pump in the E1P form, and the intramembranal carboxylic acids are shown in stick form and numbered in blue, with the arrow highlighting the position of lysine791. Three hydronium ions are shown in binding sites designated H1, H2, H3. **Fig B** illustrates the E2P conformation where lysine 791 has displaced the hydronium in H2 to the luminal face, and an arrow now emphasizes the new orientation of lysine 791. The **Fig C** illustrates the entry path for K+ (illustrated as a series of violet spheres) between M4, M5, M6, and M8 in the E2P conformation.

The gastric H^+, K^+ -ATPase is a heterodimeric protein, the product of 2 genes. The gene *ATP4A* encodes the H^+, K^+ -ATPase α subunit of ~1000-amino acid protein that contains the catalytic sites of the enzyme and forms the pore through the cell membrane that allows the transport of ions. The gene *ATP4B* encodes the β subunit of the H^+, K^+ -ATPase, which is an ~300-amino acid protein with a 36-amino acid N-terminal cytoplasmic domain, a single transmembrane domain, and a highly glycosylated extracellular domain. The H^+, K^+ -ATPase β subunit stabilizes the H^+, K^+ -ATPase α subunit and is required for function of the enzyme. It also appears to contain signals that direct the heterodimer to membrane destinations within the cell, although some of these signals are subordinate to signals found in H^+, K^+ -ATPase α subunit.

Role of gastric mucin in gastroprotection

Mucins are a family of high molecular-weight heavily O-glycosylated glycoproteins that are either secreted or are membrane-bound form, play a major role in the mucosal defense against gastric acid, proteases, pathogenic microorganisms, and mechanical trauma. The high molecular weight mucins are responsible for the viscoelastic properties of the mucous barrier. They are widely expressed in epithelial tissues and are characterised by variable number tandem repeat peptide sequences rich in serine, threonine, and proline which carry large numbers of *O*-linked oligosaccharide chains. The mucin protein core consists of highly glycosylated regions (resistant to proteolysis) and regions shown to be non-glycosylated (susceptible to proteolysis). Cysteines in these 'naked' regions link mucin monomers by disulphide bridges to form large mucin oligomers of 2-40 kDa molecular mass. This may be repeated several times as repeated units ultimately leading to a very high molecular weight structure which is needed to protect larger surface area of the stomach as a protective lining. At present, 12 genes have been described as shown in Table 4. Two clusters have been reported, the secretory mucin genes MUC2, MUC5AC, MUC5B, and MUC6 on chromosome 11p15.5, and MUC3, MUC11, and MUC12 on chromosome 7q22.3 (Corfield et al., 2000).

Gastric mucin, which forms a protective layer over the surface epithelium of stomach and acts as a diffusion barrier as shown in Fig 24. Bicarbonate ions

secreted by the gastric epithelium are trapped in the mucus gel, establishing a gradient from pH 1-2 at the lumen to pH 6-7 at the cell surface. But the HCl secreted at the base of gastric glands by parietal cells traverse the mucus layer by producing viscous fingering pattern without acidifying it. HCl traverse the gastric mucin layer only at pH above 4, whereas below pH 4, HCl cannot pass through the mucin gel. Thus HCl secreted by gastric glands can penetrate (pH 5-7) through narrow fingers in to the lumen, whereas from the lumen side it is prevented from diffusion back to the epithelium by high viscosity of gastric mucus gel due to acidic condition (pH 2) on the luminal side (Bhaskar et al., 1992).

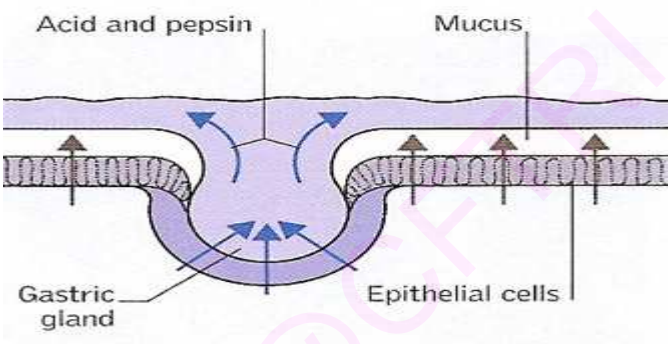


Fig 24. Mucus layer covering gastric mucosa to protect from gastric acid.

Helicobacter pylori infects the stomachs of nearly a half the human population, yet most infected individuals remain asymptomatic even though few strains of *H. pylori* reduced the levels of MUC5AC and MUC1 mucins in *in vitro* cell cultures (Byrd et al., 2000), which suggests that there is a host defense against this bacterium. Because *H. pylori* is rarely found in deeper portions of the gastric mucosa, where *O*-glycans are expressed that have terminal α1,4-linked *N*-acetyl glucosamine. These *O*-glycans have anti-microbial activity against *H. pylori*, inhibiting its biosynthesis of cholesteryl-α-D-glucopyranoside, a major cell wall component. Thus, the unique *O*-glycans in gastric mucin appeared to function as a natural antibiotic, protecting the host from *H. pylori* infection (Kawakubo et al., 2004). Mucin also acts as a receptor for *H. pylori* adhesion and this adhesive property may reduce the entry of *H. pylori* to epithelial cell surface. These findings also show that a battery of carbohydrates expressed in the stomach is closely associated with pathogenesis and also prevention of *H. pylori*-related diseases.

MUC gene	Chromosome	Mucin type	Major expression in normal gastrointestinal tract
MUC1	1q21	Membrane	*Salivary glands*: acini *Oesophagus*: surface stratified squamous epithelium, submucosal gland ducts *Stomach-fundus*: surface epithelium, glands; *body and antrum*: surface foveolar cells, mucous neck cells *Small intestine*: goblet cells and enterocytes in surface/villi and deep/crypts *Colorectum*: goblet cells and colonocytes in surface/villi and deep/crypts
MUC2	11p15.5	Secreted, gel forming	*Small intestine*: goblet cells in surface/villi and deep/crypts *Colon*: goblet cells in surface/villi and deep/crypts
MUC3	7q22	Membrane	*Salivary glands*: submaxillary gland acini *Stomach-body and antrum*: surface foveolar cells *Small intestine*: goblet cells and enterocytes in surface/villi *Colorectum*: goblet cells and colonocytes in surface/villi
MUC4	3q29	Membrane	*Oesophagus*: surface stratified squamous epithelium, submucosal gland ducts *Stomach-fundus*: surface epithelium, glands; *Stomach-body and antrum*: surface foveolar cells, mucous neck cells *Small intestine*: goblet cells and enterocytes in surface/villi and deep/crypts *Colorectum*: goblet cells and colonocytes in surface/villi and deep/crypts
MUC5AC	11p15.5	Secreted, gel forming	*Stomach-fundus*: surface epithelium; *body and antrum*: surface foveolar cells
MUC5B	11p15.5	Secreted, gel forming	*Salivary glands*: acin *Oesophagus*: submucosal gland acini and ducts *Stomach-fundus*: surface epithelium, *Colorectum*: goblet cells in deep/crypts
MUC6	11p15.5	Secreted, gel forming	*Stomach-fundus*: glands; *body and antrum*: mucous neck cells *Small intestine*: duodenal Brunner's gland acini
MUC7	4q13-q21	Secreted, non-gel forming	*Salivary glands*: acini
MUC8	12q24.3	Secreted, gel forming	Not expressed
MUC9	1p13.	Secreted, gel forming	Not expressed
MUC11	7q22	Secreted, gel forming	*Small intestine* *Colorectum*
MUC12	7q22	Membrane	*Colorectum*

Table 4. Mucin genes and their locations in human gastrointestinal tract. *(Ref: Corfield et al., Gut 2000.)*

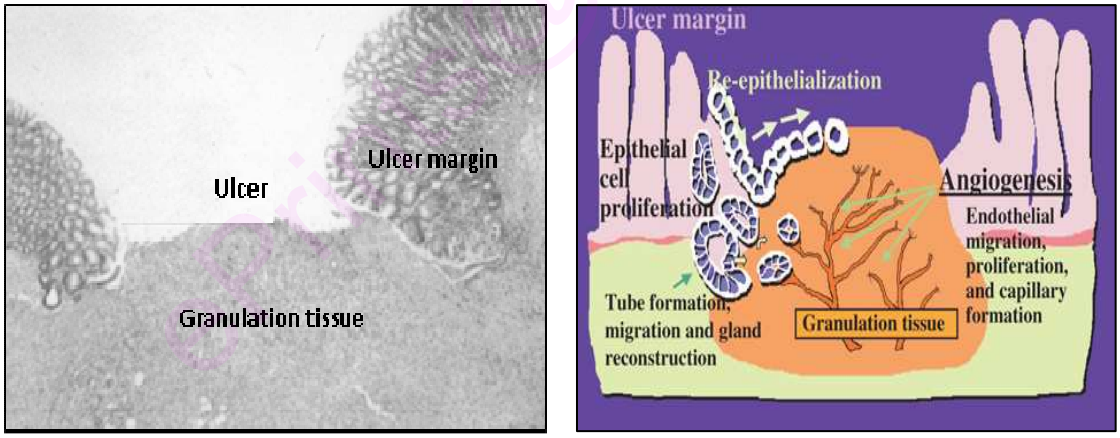
The gastrointestinal epithelium is continuously exposed to reactive oxygen metabolites that are generated within the lumen. In spite of this exposure, the gastric epithelium appears unaffected, due to antioxidant property of gastric mucin, which showed hydroxyl radical scavenging

property but, during that process the viscosity of the gastric mucin is reduced (Grisham et al., 1987) and this suggests efficient mechanisms for protection against these potentially cytotoxic oxidants. Thus mucin acts as a protective barrier against gastric acid, microbial infection and oxidative damages in the gastrointestinal barrier.

Mechanism of ulcer healing

Histologically, an ulcer consists of two major structures; a distinct ulcer margin formed by the adjacent non-necrotic mucosa - the epithelial component, and granulation tissue at the ulcer base, which consists of fibroblasts, macrophages and proliferating endothelial cells forming microvessels as shown in Fig 25 (Tarnawski, 2005).

Ulcer healing is a complex process, which involves cell migration, proliferation, reepithelialization, angiogenesis, and matrix deposition, all ultimately leading to scar formation. All these processes are controlled by growth factors, transcription factors and cytokines (Fig 25B) (Tarnawski, 2005).



Ref: Tarnawski, 2005. Digestive Diseases and Sciences

Fig 25. Histological picture of gastric ulcer (A) and diagrammatic representation of ulcer healing zone (B).

Mucosa of the ulcer margin forms a characteristic "healing zone" (Tarnawski, 2000). The epithelial cells lining glands of the ulcer margin undergo de-differentiation, express epidermal growth factor receptor (EGF-R) and actively proliferate. Proliferation is essential for ulcer healing, because it supplies epithelial cells crucial for re-epithelialization of the mucosal surface and reconstruction of gastric glands. These cells migrate from the ulcer

margin onto the granulation tissue to re-epithelialize the ulcer base. In addition, the epithelial cells from the base of the ulcer margin form tubes composed of ulcer-associated cell lineage, which invade granulation tissue migrate toward the surface, branch and undergo transformation into gastric glands within the ulcer scar. Growth factors are the major stimuli for cell proliferation, division, migration and re-epithelialization (Tarnawski, 2000). In addition to the initial pool of growth factors derived from the platelets, macrophages and injured tissue, ulceration triggers in cells lining mucosa of the ulcer margin, genes encoding for the growth factors (e.g. EGF, bFGF, HGF, VEGFand PDGF) and COX2, in a well synchronized spatial and temporal manner (Tarnawski, 2000). These growth factors produced locally, activate epithelial cell migration and proliferation via autocrine and/or paracrine actions to restore epithelial continuity, an essential step in ulcer healing.

Granulation tissue develops at the ulcer base within 48-72 hours after ulceration (Tarnawski, 2005). Granulation tissue consists of proliferating connective tissue cells, i.e. macrophages, fibroblasts and proliferating endothelial cells, which form microvessels through the process of angiogenesis. Migration of fibroblasts into the granulation tissue and their proliferation are triggered by growth factors: TGFβ, PDGF, EGF, FGF and cytokines: TNFα and IL-1 derived from inflammatory cells activated endothelial cell and macrophages. Granulation tissue supplies connective tissue cells (synthesizing extracellular matrix) for restoring the lamina propria and microvessels for the restoration of the microvasculature within ulcer scar.

Extracellular matrix (ECM) is secreted locally by fibroblasts epithelial, smooth muscle and endothelial cells and assembles into a network in the spaces surrounding cells (Basson, 2001). It sequesters water and minerals and binds growth factors. ECM consists of fibrous structural proteins such as the collagens and elastins, adhesive glycoproteins including fibronectin and laminin and an amorphous gel composed of proteoglycans and hyaluronan. The above components assemble and form an interstitial matrix and the basement membrane. The cell growth, differentiation and migration are regulated in part by extracellular matrix interaction with epithelial and endothelial cells via integration of multiple signaling.

The replacement of granulation tissue with a connective tissue scar involves changes in the composition of the ECM. The growth factors that stimulate synthesis of collagen and other connective tissue components also modulate the synthesis and activity of metalloproteinases, enzymes that degrade these ECM components. The net result of ECM synthesis versus its degradation is remodeling of the connective tissue– an important feature of ulcer healing. Activated MMPs are rapidly inhibited by specific tissue inhibitors of metalloproteinase (TIMP), which are produced by most mesenchymal cells, thus preventing uncontrolled action of these proteinases. Collagenases and their inhibitors are spatially and temporally regulated during gastric ulcer healing. They are essential in the remodeling of connective tissue necessary for tissue defect repair and scar formation.

The re-epithelialized mucosa of grossly "healed" experimental gastric ulcers may have prominent histological and ultrastructural abnormalities: reduced height, dilation of gastric glands, increased connective tissue and a disorganized microvascular network. These prominent abnormalities may interfere with the mucosal defense and cause ulcer recurrence when ulcerogenic factors are present (Tarnawski et al., 2000). Therefore, the quality of mucosal structural restoration may be the most important factor in determining future ulcer recurrence. Several studies indicate that topically acting ulcer healing drugs such as antacids and rebamipide in addition to antioxidants may improve quality of ulcer healing in experimental models and in human ulcers (Schmassmann et al., 1993; Naito et al., 1995).

Diagnosis of ulcer

The predominant symptom of uncomplicated ulcer is epigastric pain, which can be accompanied by other dyspeptic symptoms such as fullness, bloating, early satiety, and nausea. In patients with duodenal ulcer, epigastric pain occurs typically during the fasting state or even during the night and is usually relieved by food intake or acid-neutralizing agents. Roughly a third of these patients also have heartburn, mostly without erosive oesophagitis. Chronic ulcers can be asymptomatic. In particular, this absence of symptoms is seen in NSAID-induced ulcers, for which upper gastrointestinal bleeding or perforation might be the first clinical

manifestation of disease. The most frequent and severe complication of ulcers is bleeding. Perforation is less frequent than bleeding. Penetration of retroperitoneal organs is characterised by constant severe pain but fortunately is rare. Gastric outlet obstruction due to ulcer-induced fibrosis is also rare, and should raise suspicion of underlying malignant disease (Malfertheiner et al., 2009). No specific symptom helps differentiate between *H. pylori*-associated or NSAID-associated ulcers, but a careful history can identify surreptitious NSAID users and an appropriate *H. pylori* test can detect infected individuals.

Diagnostic method	**Sensitivity and Specificity[a]**	**Typical application**	**Remarks**
Invasive methods			
Histology	>95%	"Gold standard" in routine hospital diagnostics	Requires expert pathologist; also provides histological data on inflammation and atrophy
Culture biopsy	>95%	Alternative gold standard	Allows for testing of antimicrobial sensitivity; requires specific microbiological expertise
Rapid urease (CLO) test	>90%	Cost-effective and rapid test	Requires an additional test for confirmation of *H. pylori* infection
Noninvasive methods			
Urea breath test	>95%	Alternative gold standard	Very useful, reliable test to evaluate success of eradication treatment of *H. pylori*; limited availability due to requirement of expensive equipment
Fecal antigen test	>90%	Not widely used yet	Simple test but may not be reliable for evaluation of success of eradication treatment of *H. pylori*
Serology	80-90%	Mainly used for epidemiological studies	Insufficient reliability for routine screening; cannot prove ongoing infection due to immunological memory

[a] Global range, depending on regional variations and subjects.

Table 5. Diagnostic methods for detection of *H. pylori* infection.

Various tests have been developed for the detection of *H. pylori*, each with their specific advantages and disadvantages as described in Table 5. Endoscopy is essential for an accurate and differential diagnosis of ulcer disease and ulcer complications. The available tests are generally divided into invasive tests, based on gastric specimens for histology, culture, or other methods, and noninvasive tests, based on peripheral samples, such as blood, breath samples, stools, urine, or saliva for detection of antibodies, bacterial antigens, or urease activity (Kusters et al., 2006). Polymerase chain reaction (PCR) analysis of *H. pylori* infection in both invasive and noninvasive samples is a very sensitive test but prone to producing false-positive results.

Treatments for ulcer

Since Karl Schwarz's dictum of no acid, no ulcer, development of medical therapies has targeted gastric acid secretion and mucosal defense mechanisms (Schwartz, 1910). Many drugs have been used to treat ulcers, but few early treatments stood the test of time.

Drugs	Mechanisms	Use
H2-receptor antagonists (cimetidine, ranitidine, famotidine, nizatidine, roxatidine)	Acid inhibition	*H. pylori*-negative peptic ulcer; replaced by PPI because of inferiority in acid suppression
PPI (omeprazole, pantoprazole, lansoprazole, rabeprazole, esomeprazole)	Most potent acid inhibition	Standard treatment for all *H. pylori*-negative peptic ulcers; prevention of NSAID or aspirin ulcers; essential component in eradication regimen; given intravenously in bleeding ulcers
Prostaglandin analogues* (misoprostol)	Increase mucosal resistance; weak acid inhibition	*H. pylori*-negative gastric ulcer; prevention of NSAID ulcers
H. pylori eradication regimens (PPI plus two antibiotics)	Cure of *H. pylori* infection	Standard therapy in all *H. pylori*-positive ulcers
Bismuth salts (subcitrate, subsalicylate)	Weak antibacterial effect; increase of mucosal PGE2 synthesis	In quadruple therapy for *H. pylori* eradication
Several mucosal protectives used in some countries (ie, sucralfate, rebamipide, and others) do not have sufficient trial documentation to be included in the efficacy comparison with the listed standard therapies. PPI=proton-pump inhibitor. NSAID=non-steroidal anti-inflammatory drug. *Contraindicated in pregnancy.		

Table 6. Classes of drugs with proven effect on healing of peptic ulcer.

(Ref: Malfertheiner et al., 2009)

The most successful classes of drugs were those inhibiting gastric acid secretion, inhibit *H. pylori* growth and enhance mucosal protection (Table 6). Among them H2-receptor antagonists and proton pump inhibitors reduces acid secretion, prostaglandin analogues increases mucosal resistance, bismuth salts also increases mucosal resistance in addition to acts as a weak antibacterial compounds and final and more important are antibacterial compounds which can inhibit *H. pylori* growth. Table 6 summarizes list of available drugs which are currently in use for the treatment of gastric ulcers. The mechanism of each group of drugs with advantages and drawbacks are described as follows:

Mucosal protectants

These are the second group of drugs next to aced secretion inhibitors and antibiotics. These are directed at reinforcement of the mucosal barrier, and has found its major application in protection against NSAIDs and aspirin. Prostaglandins, nitric oxide and, more recently, lipoxin A4 have been shown to be important mediators of mucosal defense (Wallace, 2005). Misoprostol (Fig 26A), a prostaglandin analogue, has been the most widely used but its application is limited by abdominal side-effects, especially at higher doses and other classes of drugs, especially H2-receptor antagonists and proton pump inhibitors, are more effective for the treatment of acute peptic ulcers, Misoprostol is only indicated for use by people who are both taking NSAIDs and are at high risk for NSAID-induced ulcers, including the elderly and people with ulcer complications. Misoprostol is sometimes co-prescribed with NSAIDs to prevent their common adverse effect of gastric ulceration. At lower doses misoprostol stimulate increased secretion of the protective mucus that lines the gastrointestinal tract and increase mucosal blood flow, thereby increasing mucosal integrity.

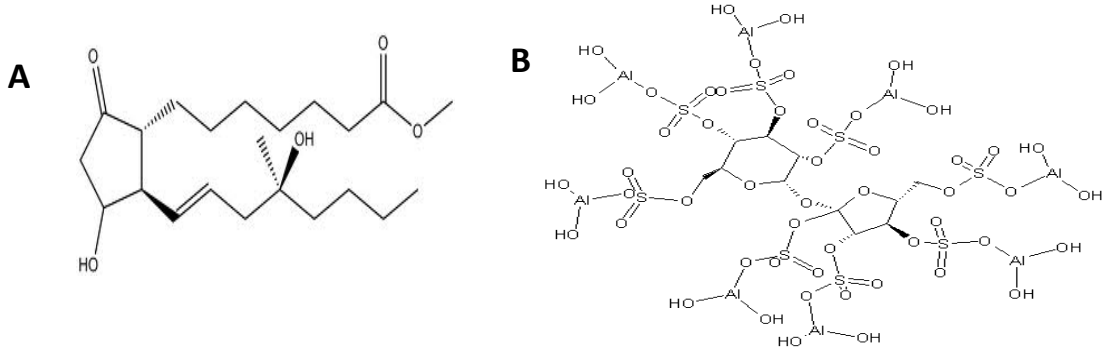


Fig 26. Structures of misoprostol (A) and sucralfate (B).

Sucralfate (Fig 26B) is a locally acting mucoprotective substance that in an acidic environment (pH < 4), reacts with hydrochloric acid in the stomach to form a cross-linking, viscous, paste-like material capable of acting as an acid buffer for as long as 6 to 8 hours after a single dose. It also attaches to proteins on the surface of ulcers, such as albumin and fibrinogen, to form stable insoluble complexes. These complexes serve as protective barriers at the ulcer surface, preventing further damage from acid, pepsin, and bile. In addition, it prevents back diffusion of hydrogen ions, and adsorbs both pepsin and bile acids. Recently, it has been indicated that sucralfate also stimulates the increase of prostaglandin E_2, epidermal growth factors (EGF), bFGF, and gastric mucus.

Bismuth salts also promote ulcer healing by improving mucosal repair. It causes an increase in mucus glycoprotein secretion and may also bind to the gastric mucus layer to act as a diffusion barrier to HCI. In addition, it has a cytoprotective effect and increases mucosal secretion of prostaglandins and bicarbonate. Bismuth salts with some intrinsic anti-*H. pylori* activity are used in ulcer therapy only in combination with antibiotics.

Histamine H2 -receptor antagonists

Histamine H2 -receptor antagonists (H2-RAs), competitively inhibit the action of histamine on the histaminic H2-receptors of parietal cells, and thus reduce the gastric acid secretion. Therefore, these drugs are used for treatment of active duodenal ulcer, active and benign gastric ulcer, pathogenic gastrointestinal hypersecretory conditions (e.g. Zollinger-Ellison Syndrome), and symptomatic relief of gastroesophageal refluxes. Four H2-receptor antagonists are cimetidine, famotidine, nizatidine, and ranitidine (Fig 27).

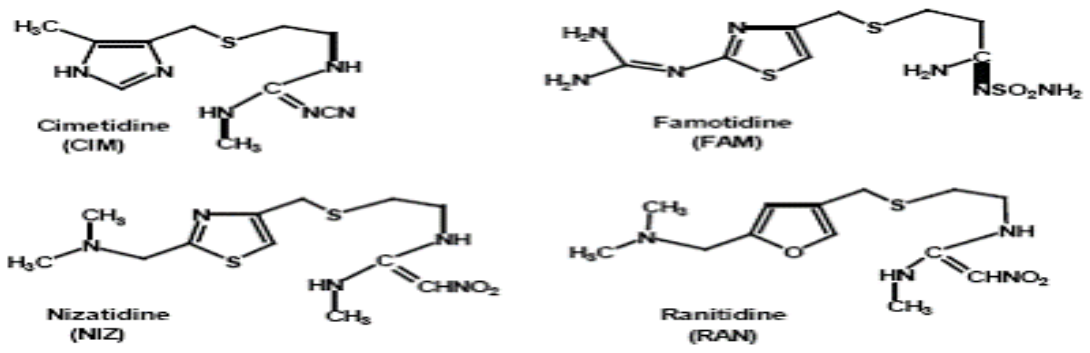


Fig 27. Structures of Histamine receptor blockers.

Proton pump inhibitors (PPI)

Earlier H2 receptor antagonists revolutionized treatment of gastroduodenal ulcer. They were gradually replaced with the more potent class of acid-inhibitory drugs, the PPIs, which became available in 1989. Gastric acid is secreted by parietal cells of the stomach in response to stimuli such as the presence of food in the stomach or intestine and the taste, smell, sight or thought of food. Such stimuli result in the activation of histamine, acetylcholine or gastrin receptors (the H_2, M_3 and CCK2 receptors, respectively) located in the basolateral membrane of the parietal cell as shown in Fig 28, which initiates signal transduction pathways (Fig 21) that converge on the activation of the H^+, K^+-ATPase — the final step of acid secretion. Inhibition of this proton pump has the advantage that it will reduce acid secretion independently of how secretion is stimulated, in contrast to other pharmacological approaches to the regulation of acid secretion; for example, the inhibition of acid secretion by H2 receptor antagonists can be overcome by food-induced stimulation of acid secretion via gastrin or acetylcholine receptors as depicted in Fig 28 whereas PPIs selectively block the H^+, K^+-ATPase of the parietal cell.

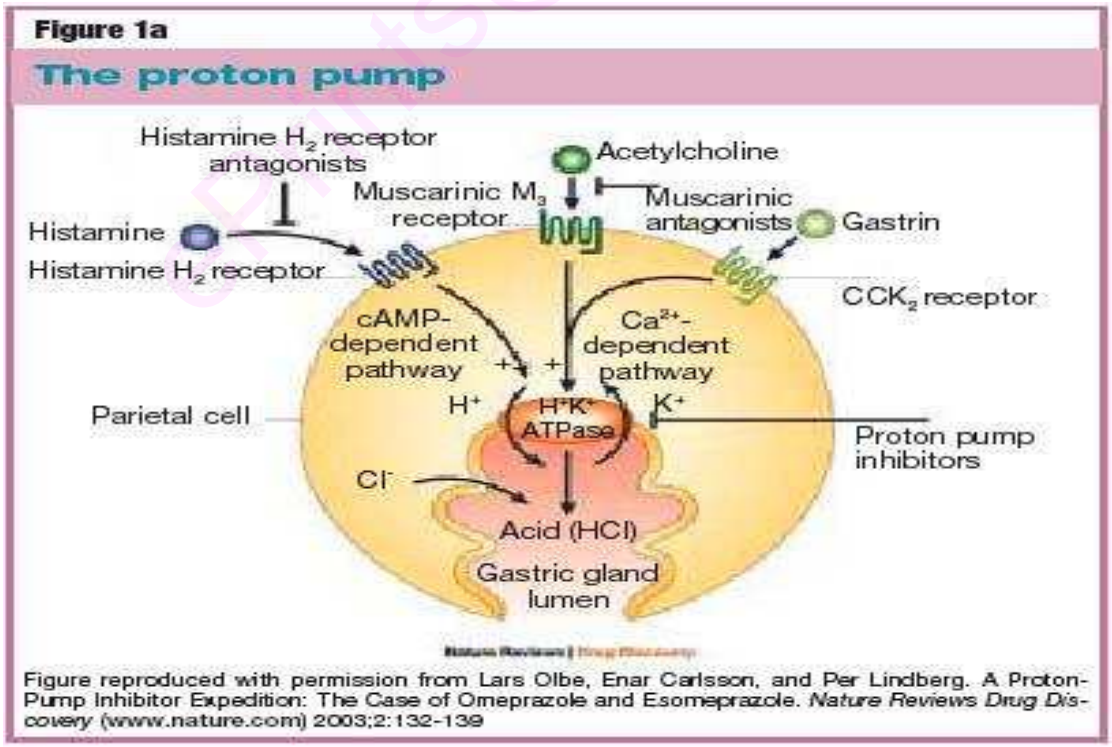


Figure reproduced with permission from Lars Olbe, Enar Carlsson, and Per Lindberg. A Proton-Pump Inhibitor Expedition: The Case of Omeprazole and Esomeprazole. *Nature Reviews Drug Discovery* (www.nature.com) 2003;2:132-139

Fig 28. Efficacy of proton pump inhibitors: since PPIs directly binds and inhibit proton pump as apposed to other acid secretion inhibitors (i.e. Histamine receptor antagonists and Muscaranic antagonists)

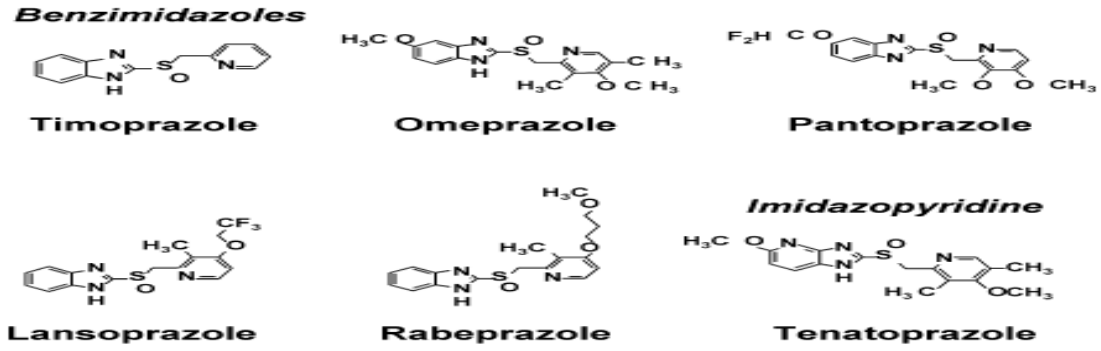


Fig 29. Structures of proton pump inhibitors. (*Ref: Sachs et al., 2006*).

Proton-pump inhibitors as shown in Fig 29 are prodrugs that are converted to their active form in acidic environments. Omeprazole is a weak base, and so specifically concentrates in the acidic secretory canaliculi of the parietal cell, where it is activated by a proton-catalysed process to generate a sulphenamide. The sulphenamide interacts covalently with the sulphydryl groups of cysteine residues in the extracellular domain of the H^+, K^+-ATPase — in particular Cys 813 — thereby inhibiting its activity. The specific concentration of proton-pump inhibitors such as omeprazole in the secretory canaliculi of the parietal cell is reflected in their favourable side-effect profile. The speed of ulcer healing is associated with degree of acid suppression, thus PPIs became the hallmark in ulcer therapy. However, after the healing phase ulcers were usually seen to recur, and for years standard practice was to keep patients on maintenance of acid suppression until the revolutionary introduction of *H. pylori* eradication therapy (Malfertheiner et al., 2009).

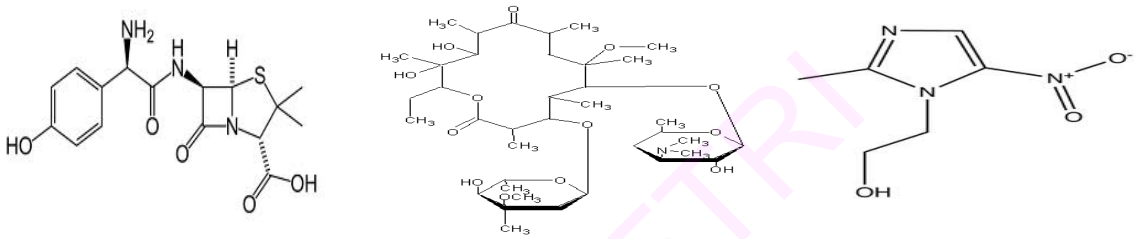
Anti-*H. pylori* compounds and eradication of *H. pylori*

Treatment for *H pylori*-associated ulcer disease is mainly directed at eradication of infection. Although *H. pylori* is sensitive to a wide range of antibiotics *in vitro*, they all fail as monotherapy *in vivo* as shown in Table 7 (Megraud, 1995). The lack of efficacy of monotherapy is related to the niche of *H. pylori*, residing at lower pH in a viscous mucus layer. Eradication is usually achieved with a combination of acid-inhibiting therapy and antibiotics. Antibacterial therapy alone does result in healing, but the process is accelerated by addition of acid suppressants (ie, PPIs).

Antimicrobials (% Resistance)	Commonly used compounds	Mode of action	Mechanism of resistance
Nitroimidazoles (20–95%)	Metronidazole	Reduction of prodrug by nitroreductases leads to formation of nitro-anion radicals and imidazole intermediates and subsequent DNA damage	Absence of imidazole reduction caused by reduced or abolished activity of electron transport proteins (eg, RdxA, FrxA, FdxB)
Macrolides (0–50%)	Clarithromycin, Erythromycin	Binds 23S rRNA ribosomal subunit, resulting in inhibition of protein synthesis	Point mutations in 23S rRNA genes
Penicillins (0–30%)	Amoxicillin	Binding of beta-lactam antibiotic to penicillin-binding proteins (PBP) inhibits cell division	Decreased binding of amoxicillin to PBP D (tolerance) or PBP1A (by point mutation in *pbp*1A gene), and reduced membrane permeability (resistance)
Tetracyclines (0–10%)	Tetracycline	Binding to ribosome prevents association with aminoacyltRNA and subsequent protein synthesis	Point mutations in 16S rRNA genes and reduced membrane permeability
Fluoroquinolones (0–20%)	Ciprofl oxacin, moxifl oxacin, levofl oxacin	Inhibition of DNA gyrase and topoisomerases, interfering with DNA replication	Point mutations in the DNA gyrase gene, *gyrA*
Rifamycins (0–2%)	Rifabutin	Binding to RNA polymerase, resulting in transcription inhibition	Point mutations in the RNA polymerase gene, *rpoB*
Nitrofurans (0–5%)	Furazolidone	Reduction of prodrug by nitroreductases, leads to formation of nitro anion radicals and subsequent DNA damage	Unknown
Proton pump inhibitor (Not reported)	Omeprazole, lansoprazole, pantoprazole	Inhibits the proton motive force of the bacterium, and destabilises its site of colonisation in the stomach	Unknown
Bismuth (Not reported)	Bismuth subcitrate & subsalicylate	Inhibits protein, ATP, and cell membrane synthesis	Unknown

Table 7. Mode of action, resistance mechanism and resistance rate of antimicrobials used for treatment of *H. pylori* infection.

Eradication rates depend on several factors: (i) drug regimen; (ii) resistance rate to the antibiotic used; (iii) compliance with the drug; (iv) duration of therapy; and (v) genetic variations in drug-metabolizing enzymes. Therefore triple therapy containing combination of two antibiotics and proton pump inhibitor or quadruple therapies containing bismuth in addition to triple therapy are recommended for successful eradication of *H. pylori.* The Maastricht Consensus Report provides recommendations on management of *H pylori* infection as shown in Table 8. The structures of commonly using antibiotics for *H. pylori* eradication are shown below (Fig 30).



Amoxicillin Clarithromycin Metronidazole

Fig 30. Structures of commonly used *H. pylori* growth inhibitors.

***Helicobacter pylori* eradication regimens**
First-line options (7–14 days)

- In populations with less than 15–20% clarithromycin resistance and greater than 40% metronidazole resistance:
  Proton-pump inhibitor (PPI) standard dose, clarithromycin 2×500 mg, and
  amoxicillin 2×1000 mg, all given twice a day
- Less than 15–20% clarithromycin resistance and less than 40% metronidazole resistance:
  PPI standard dose, clarithromycin 500 mg, and metronidazole 400 mg or
  tinidazole 500 mg, all given twice a day
- In areas with high clarithromycin and metronidazole resistance:
  Bismuth-containing quadruple therapy

Second-line option (10–14 days)

- Bismuth-containing quadruple therapy
- PPI plus metronidazole and amoxicillin, if clarithromycin was used in first-line treatment (in Latin America and China, furazolidone 2–4×100 mg is often preferred over metronidazole)

Rescue therapies (10–14 days)

- PPI twice a day plus amoxicillin 2×1000 mg with either levofloxacin 2×250 (500) mg, or with rifabutin 2×150 mg

Table 8. *Helicobacter pylori* eradication regimens.

Future directions of ulcer therapy

H. pylori infection is the major factor in most cases of ulcer disease. As with any established microbial disease, we need to understand the epidemiology and mechanisms of pathogenesis, but the major focus should be towards improving therapies and defining the long-term outcome of *H. pylori* eradication. To devise novel therapeutic agents effectively, we must increase our knowledge of the basic physiology of *H. pylori* and its ecology in the stomach (Lee, 1994). The key to developing better strategies for accelerating ulcer healing is the identification and characterization of the growth factors and mediators that play key roles in the ulcer repair process. Much progress in this effort has been made over the past two decades, but this has not yet been translated to a marketed agent for accelerating ulcer healing per se. Important area for future research is to better understand how we can improve the quality of ulcer healing. In other words, can ulcers be healed in such a way that they are less likely to recur?. Part of this latter approach will involve gaining a better understanding of the long-term consequences of ulcers on the gastric mucosa. For example, do ulcers alter mucosal defense in an "irreversible" manner such that the tissue is more prone to recurrent tissue injury?. It is possible that in the future we will be able to use a range of agents to heal ulcers, irrespective of the factor(s) that caused the ulcer to form, such that ulcers no longer recur (Wallace, 2001). Gene therapy specifically applied to accelerate the healing of chronic ulcers is another promising area of ulcer therapy, but it has not been explored to date except few experimental studies in rats (Jones et al., 2001). In addition to this, since gene expressions in non-target tissues/cells cause side effects, a selective gene delivery system targeted to the specific site must be developed (Fumoto et al., 2008). Increase in incidence of non-NSAID and *H. pylori* negative ulcers (idiopathic ulcers) is another worrying factor. Molecular techniques will continue to help us identify genetic factors that predict the development of idiopathic ulcers. The existence of host-related differences in the physiology of acid secretion might lead to the identification of genetic markers associated with peptic ulcer disease (Yuan et al., 2006). Such markers might, in the future, help to identify patients at high risk of or with susceptibility to peptic ulcer disease.

Role of dietary / phytochemicals in ulcer therapy

Historically, natural products have provided an endless source of medicine, and despite the diversification of drug discovery technology and reduced funding for natural product–based drug discovery, natural products from plants and other biological sources remain an undiminished source of new pharmaceuticals. Indeed, even though industrial funding specifically allocated for natural product–based drug discovery declined from 1984 to 2003, the percentage of natural product–derived, small-molecule patents has remained relatively unchanged. A comprehensive review of human drugs introduced since 1981 suggests that, out of 847 small-molecule drugs, 5% were natural products, ~27% were derived from natural products (usually semisynthetically) and the remaining 572 were synthetic molecules. However, 262 of the synthetic molecules had a natural product–inspired pharmacophore or could be considered natural-product analogs (Schmidt et al., 2007).

Natural compounds from dietary or plant sources with discrete bio-activities towards animal biochemistry and metabolism are being widely examined for their ability to provide health benefits. It is important to establish the scientific rationale to defend their use in pharmaceuticals.

These nutraceuticals or phytochemicals could provide health benefits as: (1) substrates for biochemical reactions; (2) cofactors of enzymatic reactions; (3) inhibitors of enzymatic reactions; (4) absorbents/ sequestrants that bind to and eliminate undesirable constituents in the intestine; (5) ligands that agonize or antagonize cell surface or intracellular receptors; (6) scavengers of reactive or toxic chemicals; (7) compounds that enhance the absorption and or stability of essential nutrients; (8) selective growth factors for beneficial gastrointestinal bacteria; (9) fermentation substrates for beneficial oral, gastric or intestinal bacteria; and (10) selective inhibitors of deleterious gastrointestinal bacteria. Such phytochemicals include terpenoids, phenolics, alkaloids and fibers (Dillard & German, 2000).

Plants and phytoconstituents are better choice to treat diseases than the allopathic drugs. Most of the drugs used in primitive medicine were originated from plants and are the earliest and principal natural source of medicines. The drugs from plants are fairly innocuous and relatively free

from toxic effects. The nature has provided us various medicinal plants which became the storehouse of remedies to cure all ailments of mankind. In modern era many plant-derived compounds have been used as drugs, either in their original or semi-synthetic form (Sen et al., 2009).

Research supporting beneficial roles for phytochemicals against cancers, coronary heart disease, diabetes, high blood pressure, inflammation, microbial, viral and parasitic infections, psychotic diseases, spasmodic conditions, ulcers, etc is based on chemical mechanisms using *in vitro* and cell culture systems, various disease states in animals and epidemiology of humans (Dillard & German, 2000).

The fruits and vegetables are rich sources of bioactive compounds including carotenoids, beta-carotene, lutein, lycopene, zeaxanthin, flavonoids, anthocyanins, quercetins and phenolic exhibiting various health beneficial effects and reduce the risk of many diseases including ulcer. Various medicinal plants are used traditionally in the treatment of peptic ulcer and they exhibit their action by various mechanisms like antioxidant, cytoprotective, antisecretory, mucoprotective, anti-inflammatory and antibacterial properties.

Role of phenolic compounds in ulcer therapy

Phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. The most important dietary phenolics are the phenolic acids (including hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolyzable and condensed tannins) and flavonoids, the latter being the most studied group. They have also been studied extensively as antioxidant protectants for humans (Repetto and Llesuy, 2002).

There are reports supporting the potential for amelioration of diseases simply by improving the dietary intake of nutrients with antioxidant properties, such as vitamin E, vitamin C, b-carotene and carotenoids, and plant phenolics, such as tannins and flavonoids (Fig 31). The widespread interest in the potential benefits of phytochemicals as phenolic antioxidants has led to considerable commercialization activities surrounding several commodity extracts.

Phenolic compounds from ginger, turmeric, garlic, mango ginger, green tea, grape, chilli and pepper are showing a good antiulcer potential.

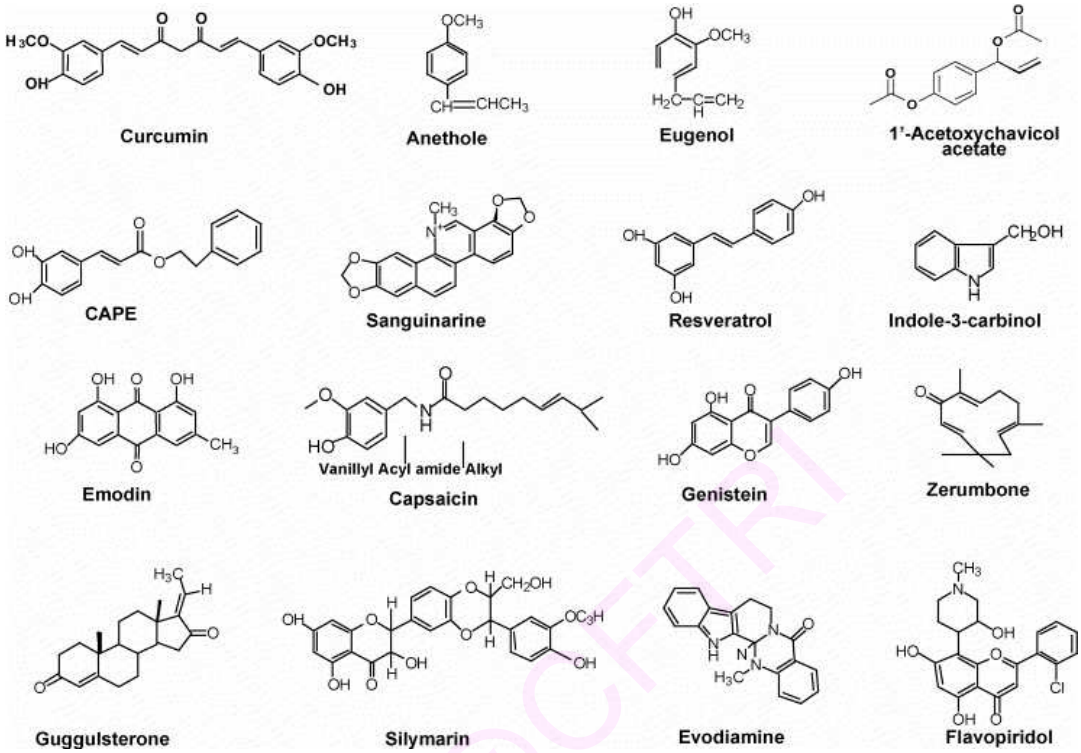


Fig 31. Chemical structures of the bioactive compounds from dietary sources. (Ref. Aggarwal & Shishodia, 2006).

They act as either anti-*H. pylori* compounds or inhibitors of other pathways of ulcer pathogenesis (Repetto and Llesuy, 2002; Swarnakar et al., 2005; Siddaraju & Shylaja, 2007a & b; Lee et., 2008). Phenolic acids such as caffeic, ferulic, cinnamic and protocatechuic acids have recently been shown from our laboratory to exert antioxidant, proton pump inhibitory and anti-microbial activities (Siddaraju & Shylaja 2007a,b). The antioxidant activity of phenolics appears to be an important factor contributing to antiulcer activity since free radicals and ROS are the main causative factors during ulcer pathogenesis (Das et al. 1997). However data still needs to be elucidated for the complete understanding of the potentials of dietary phenolics against ulcer.

Role of polysaccharides in ulcer

As a major class of biomolecules, carbohydrates are the most complex and least appreciated for their bioactivity when compared to other bioactive small

molecules. They vary considerably in its physical properties and chemical composition. Polysaccharides from plants have been the subject of studies for very long time, mainly focused on their physical properties, their chemical and physical modification and their application. Over last 20 years there has been increasing interest in the biological activity of the natural polysaccharide polymers. These studies became possible as a result of the scientific development of isolation, purification and characterization methods concomitant with the development of fairly simple *in vitro* tests for effects especially on immune system. The growing acceptance of the knowledge to be gained by people still using so-called traditional medicine in finding sources worthy of study has lead to new sources for interesting bioactive polysaccharides (Paulsen & Barsett, 2005).

The role of polysaccharides in gastroprotection is an emerging field of clinical importance. The biological activates of polysaccharides are influenced by their different solubility in water, molecular weights, degrees of branching and their different triple helical confirmations (Nangia-Makker *et al.*, 2002). Polysaccharides of different origin are reported for their gastroprotective efficacy against different stress and acid induced ulcer models in animals (Matsumoto et al., 2002; Nergard et al., 2006). Some of the polysaccharides act as antiadhesive agents against pathogenic bacteria including ulcer causing pathogen *H. pylori* (Lee et al., 2006). The polysaccharides are highly complex in structure and show complement fixation activities and induction of B cell proliferation *in vitro*. (Sakurai et al. 1999) Polysaccharides are composed of typical monosaccharides, Ara Gal, GalA, Rha, GlcA, Xyl, Man, Fuc and Glc. Possessing similar types of linkages. However, the relative amounts and linkage patterns differ.

Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galactosyluronic acid residues. In plant cells, pectin consists of a complex set of polysaccharides that are present in most primary cell walls and particularly abundant in the non-woody parts of terrestrial plants. Pectin is present throughout primary cell walls but also in the middle lamella between plant cells where it helps to bind cells together. The amount, structure and chemical composition of the pectin differs between plants, which a plant over time and in different parts of a plant. Pectin is a natural part of human diet, but does not contribute significantly to nutrition. Soluble fibers found in

these polysaccharides provide many health benefits including gastroprotection. Apples, oranges, grapefruit, apricot, bananas, cabbage, and carrots are considered as the highest pectin-containing food sources. Pectins can generally be divided into neutral and acidic polymers, but certain structural features are common between the different types of pectic substances.

It is generally accepted that three types of polysaccharides comprise pectin: a linear homopolymer known as homogalacturonan (HG), the branched polymer rhamnogalacturonan I (RG-I), and the substituted galacturonans of which the ubiquitous member is rhamnogalacturonan II (RG-II). Majority of the bioactive pectic polysaccharides contain a rhamnogalacturonan I backbone. Most of these polysaccharides contain attached arabinogalactan II side chains, and few have arabinogalactan type I chains attached. The basic structures of these polymers are shown in Fig 32. Although structural details of these polysaccharides have been presented, only in a few cases, studies have been carried out to ascertain which parts or structural details are really responsible for the bioactivity.

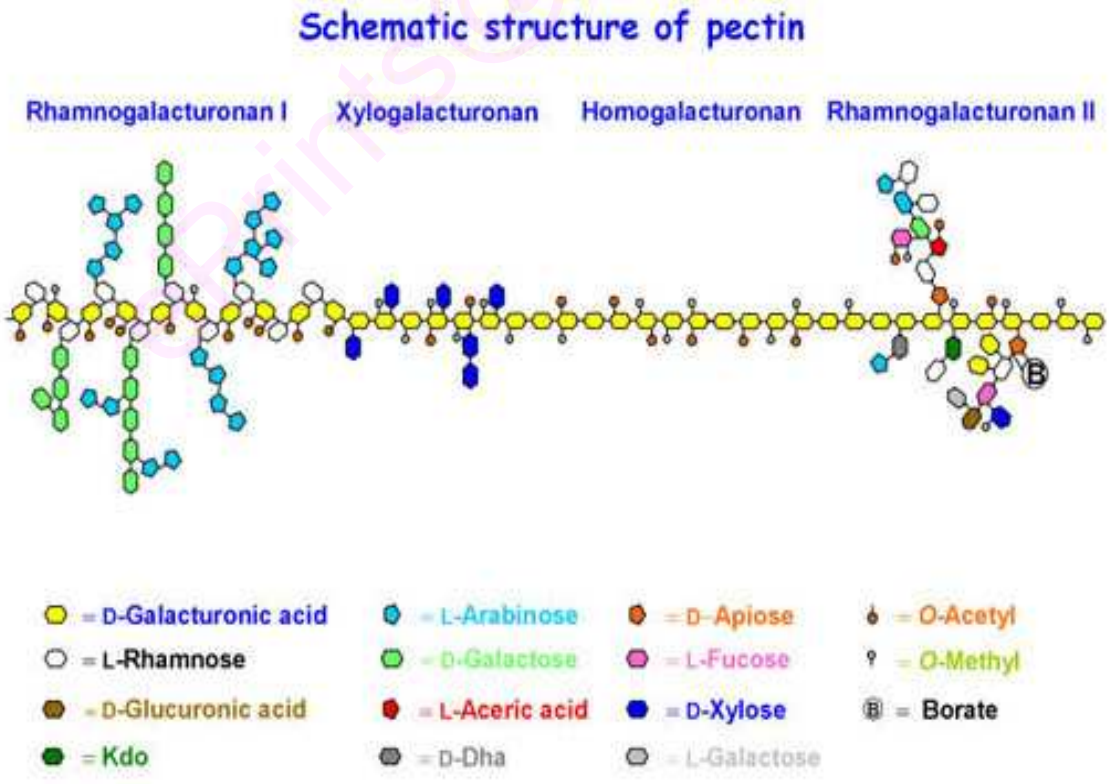


Fig 32. The basic structure of pectic polysaccharides.

The arabinans found in plants are basically composed of L-arabinofuranosides. Depending on the source they may be linear or branched, and primarily linked through positions 3 or 5. Linkages through C-2 are also observed, but generally they are less frequent than the 3-linkage.it is generally accepted that the core linkage of the arabinans is the 5-linkages and the branches occur at C-3 or C-2. But it is not obvious that the arabinans exist as such in nature. They are most probably linked to the galactans in the pectic complex and released either via enzymatic action or weak acid hydrolysis during extraction process. Rhamnogalacturonans are a group of closely related cell wall pectic polysaccharides that contain a backbone of the repeating disaccharide: "4)-α-D-Gal*p*A-(1,2)-α-L-Rha*p*-(1,". The term rhamnogalacturonan I is typically used to refer to this pectic polysaccharide. Rhamnogalacturonans-II is a low molecular mass (5–10 kDa) pectic polysaccharide that is solubilized by treating a cell wall with endopolygalacturonase. RG-II contains eleven different glycosyl residues. The backbone of RG-II contains at least 8 1,4-linked α-D-GalpA residues. Two structurally distinct disaccharides (C and D) are attached to C-3 of the backbone and two structurally distinct oligosaccharides (A and B) are attached to C-2 of the backbone.

Bupleurum falcatum pectins have been studied in great detail as they were shown to have an effect on the antiulcer activity, complement system and macrophage Fc-receptor up-regulating activity to enhance immune complex clearance (Yamada, 1995; Sakurai et al., 1998). The most potent fraction was Bupleuran 2IIc. The detailed structural studies revealed that the ramified region contained the bioactive parts, and on this section of the molecule the oligosaccharide β-D-4-O-methyl-Glc*p*A- or β-D-Glc*p*A-1,6-β-Gal*p*-β-D-1,6-β-D-Gal*p* was shown to be a possible structural unit for the recognition of carbohydrate receptors on the B-cells. The other group of investigators has also found similar oligosaccharide structures in polysaccharides from plants that are used in traditional medicine against ulcers (Paulsen & Barsett, 2005).

From the science performed mainly over the last ten years it is obvious that the role of pectic polysaccharide in health care has been substantiated. For some of the pectic substances, parts of the structure of the bioactive sites have been determined, but further studies of the relevant structures for the

individual active polymers must be performed in order to find a possible common structure for the activities observed. It is also appears that there are special structural features present in some of the polymers, which are not found in others, and which are important for their activity, and this may explain the different behavior of the polysaccharide in the same system (Paulsen & Barsett, 2005).

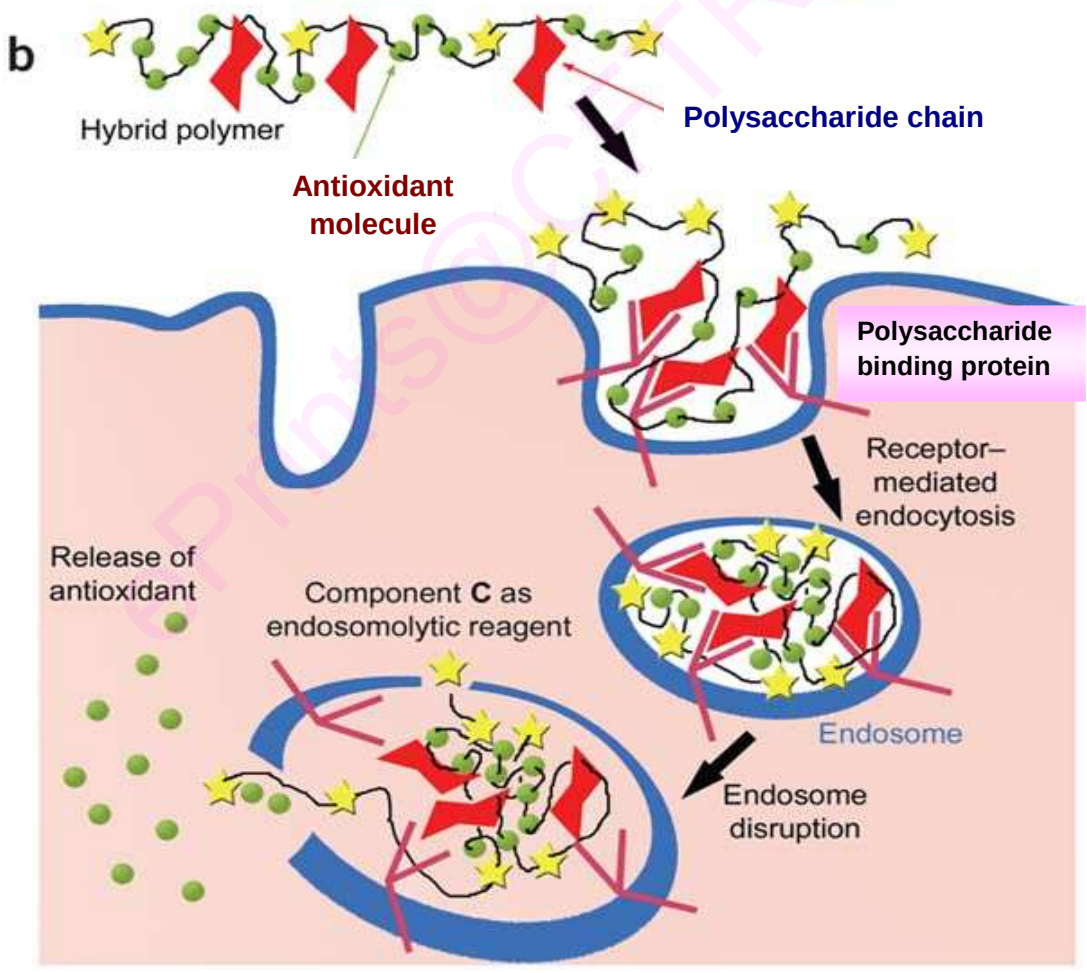
Phenolic compounds as cross-links of plant derived polysaccharides

Plant cell wall polysaccharides are partially cross-linked via phenolic compounds. As shown in the past, the most important phenolic compound to cross–link plant cell wall polysaccharides are ester-linked ferulic acid, but p-coumarate dimers were also shown to be potential cross linking compounds (Newby et al. 1980). Recently, ferulic acid dimmers were identified and quantified in a range of cereal grains. The isolation of 8-0-4-dehydrodiferulic acid–di-arabinoside from maize bran shows that diferulic acids are able to form intermolecular cross-links between arabinoxylans. The more recently identified sinapic acid dehydrodimers and ferulic acid dehydrotrimers provide additional contributions to building up a strong network of plant cell wall polysaccharides.

Cross linking of plant cell wall polymers, especially of wall polysaccharides, is of considerable interest not only in food chemistry, food technology and nutritional sciences but also in neighboring disciplines like agricultural chemistry and plant physiology (Ralph et al, 2004). Regarding the fields of food chemistry and food technology it is worth mentioning the influence of phenolic cross-links on the thermal stability of cell adhesion and maintenance of crispness of plant based food, on the gelling properties of sugar beet pectins and other food compounds as well as on the solubility properties of cereal dietary fibres derived from plant cell walls. Phenolic cross-links decrease enzymatic degradation of plant cell walls (Grabber et al, 2009). In this way cross-links may influence microbial degradation in the human gut thus controlling for example, the formation of short chain fatty acids and the bulk properties of these fibres. Hydroxycinnamic acids, especially ferulic acid, are the most important phenolic compounds to form cross-links in plant cell walls. Ferulic acid and p-coumaric acid are ester-

linked to arabinoxylans arabinoxylans in cereals and other grasses and ferulic acid is linked to pectins in some dicots and similarly, sinapic acid is thought to be bound to polysaccharides via ester-linkages.

In the light of the functional role of polysaccharide with bound phenol, investigators on designing phenol-carbohydrates were attempted for the safeguard of phenolics from degradation under physiological conditions and to deliver them to the target cell. Once the phenolics reach the target cell, it was found to be released to execute its antioxidative actions against oxidative stress condition in the cell during disease conditions. Fig 33 depicts the mechanism of uptake of phenolic carbohydrate and subsequent action of phenolic antioxidant.



Craig Fleming et al, Nature Chemical Biology 1, 270-274 (2005)

Fig 33. Role of phenolic-bound polysaccharide.

Phenolics may be internalized along with polysaccharide and may release phenolic acid to act on targets.

Previously we have isolated a series of polysaccharides from dietary sources and tested their antimetastatic activity (Sathisha et al., 2007). Among them arabinogalactan type pectic polysaccharide from the roots of *Decalepis hamiltonii* showed a potent activity against cancer metastasis. In the current study we have screened series of dietary/plant sources rich in phenolics and polysaccharides for antiulcer potentials. Based on these results we selected pectic polysaccharides and phenolics isolated from roots of *Decalepis hamiltonii* and seeds of *Nigella sativa.* These plants are known for their gastroprotective properties in traditional medicine.

***Decalepis hamiltonii* Wight & Arn. (Swallow root)**

Family : Apocynaceae
Sub family : Periplocoideae
Genus : Decalepis
Species : *D. hamiltonii*, Whight & Arn.
Also placed in: Asclepiadaceae, Periplocaceae

Fig 34. Swallow root plant (A) with its tuberous roots (B).

Decalepis hamiltonii is a monotypic genus, commonly called as Swallow root (English), makali beru (Kannada), maredu kommulu or barre sugandhi or maraud gaddalu by the Yanadi tribe of Andra pradesh. It is indigenous to Andhra Pradesh and extends throughout the Eastern Ghats of South India and it can also be seen in the hilly and forest areas of the Western Ghats. The plant grows between the rocks and places where there is a thick vegetation. Milky latex is present in entire plant. Each root is 5-10 cm in diameter and 5-15 roots arise from the root stock. A 2-3 year old plant produces 15-20 kg of roots (Vedavathy, 2004). The tuberous roots used as laxative, as an appetizer, and as health tonic. In the food industry the root extract is used as a substitute for vanillin and also used for the preparation of pickles. The roots are also used as a substitute for *Hemidesmus indicus* in Ayurvedic preparations. Therefore the roots of this plant are marketed on a

large scale (Raju & Ramana, 2009) and became endangered (Vedavathy, 2004). This plant becomes a commercially important because of increasing knowledge and scientific support of this plant as a nutraceutical and food plant in recent days.

The root (Swallow root) contains approximately 90% of fleshy matter and 10% woody core. The root can be stored for longer periods and it is resistant to microorganisms and insects. The Swallow root has a strong aromatic odor. The chemical compound responsible for the aroma of *D. hamiltonii* root is 2-hydroxy-4-methoxy benzaldehyde (HMBA), which is an isomer of vanillin (Nagarajan & Rao, 2003). *D. hamiltonii* is a rich source of bioactive compounds having diversified activities such as antioxidant, anti-inflammatory, antimicrobial, hepatoprotective and insecticidal properties (Thangadurai et al., 2002; Harish et al., 2005; Murthy et al., 2006; Srivastava & Shivanandappa, 2006; Srivastava et al., 2006; Ashalatha et al., 2010). Earlier we have reported from our laboratory on the antioxidant and gastroprotective properties of phenolic rich fractions (Naik et al., 2007; Nayaka et al., 2010) and anticancer particularly antimetastatic potential of pectic polysaccharide of *D. hamiltonii* (Sathisha et al., 2007). The bioactive role of polysaccharide from the roots of *D. hamiltonii* was least explored except few reports from our laboratory. Therefore in the current research work efforts were made to isolate the pectic polysaccharide of *D. hamiltonii* and understand its mode of action against multi-step pathogenesis of gastric ulcer.

***Nigella sativa L.* (Black cumin)**

A

B

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Ranunculales
Family	:	Ranunculaceae
Genus	:	Nigella
Species	:	*N. sativa L.*

Fig 35. Black cumin plant (A) with its seeds (B).

Black cumin (*Nigella sativa*) is an annual flowering plant belonging to the family Ranunculaceae, native to southwest Asia. It grows to 20-30 cm tall, with finely divided, linear (but not thread-like) leaves. The flowers are delicate, and usually coloured pale blue and white, with 5-10 petals. The fruit is a large and inflated capsule composed of 3-7 united follicles, each containing numerous seeds. The seed is used as a spice since antiquity by Asian herbalist and pharmacist. Black cumin is used in India and the Middle East as a spice and condiment and occasionally in Europe as both a pepper substitute and a spice. The popularity of the plant was highly enhanced by the ideological belief in the herb as a cure for multiple diseases. In fact, this plant has occupied special place for its wide range of medicinal value. Consequently, Black cumin has been extensively studied particularly, which justifies its broad traditional therapeutic value. The reason might be found in the complex chemical composition of the seeds. Black cumin seed has over 100 different chemical constituents, including abundant sources of all the essential fatty acids (Ramadan, 2007).

Black cumin is used in Indian medicine as a carminative and stimulant and is used against indigestion and bowel complaints. In India, it is used to induce post-natal uterine contraction and promote lactation. Several studies on Black cumin have shown its anticancer, antimicrobial (El-Kamali et al., 1998), antiulcer (El-Dakhakhny et al., 2000; Kanter et al., 2005), anti-inflammatory, diuretic and hypotensive activity. They have been also used to treat autoimmune diseases (Ramadan, 2007). Gastroprotection (Al-Mofleh et al., 2008) and anti-*H. pylori* activity (Salem et al., 2010) was also reported in aqueous extract of *N. sativa.*

The seeds contain a yellowish volatile oil (0.5–1.6%), a fixed oil (35.6–41.6%), proteins (22.7%), amino acids, reducing sugars, mucilage, alkaloids, organic acids, tannins, resins, toxic glucoside, metarbin, bitter principles, glycosidal saponins, melanthin resembling helleborin, melanthigenin ash, moisture and arabic acid (Duke, 1992; Al-Gaby, 1998). A novel alkaloid (nigellicine), an isoquinoline alkaloid (nigellimine) and an indazole alkaloid (nigellidine) were isolated from the Black cumin seeds. The active constituents of the seeds include the volatile oil consisting of carvone. Pharmacologically, active constituents of volatile oil are thymoquinone, dithymoquinone, thymohydroquinone and thymol. The majority of bioactive compounds of *N.*

sativa were from seed oil fractions, and very few reports were found on bioactivity of aqueous fractions and there are no reports on the polysaccharide of seed and its bioactivity. Therefore in the current work we have isolated the pectic polysaccharide of *Nigella sativa* seeds and studied its mechanism of action against multi-steps of gastric ulcer.

Need of the current study

Based on the literature, it is clear that ulcer incidences are increasing due to changes in the life style, environment, diet and exposure to other diseases including genetic variations. Despite increased documentation on occurrence of and recurrence of ulcers including morbidity and mortality and; parallel attention towards development of antiulcer drugs, satisfactory drugs with no or less side effects are not available. Also, there is a limitation for the use of these drugs to only particular physiological conditions. Pregnant women, alcoholics and patients suffering from other diseases particularly NSAIDs users on the regular basis have no choice of antiulcer drugs for their health maintenance since exciting antiulcer drugs either do not work effectively on them or pose side effects. In this scenario, in addition to the greatest surge in the literature on the role of alternative medicines, our efforts directed towards the screening of several commonly used dietary sources as effective, multi-step active and potent sources against ulcer. With the previously established information in the laboratory regarding the significant role of constituents of dietary sources particularly phenolics and pectic polysaccharides, it is pertinent to address the role, antiulcer potency and mechanism of action of selected dietary sources – Swallow root and Black cumin. Thesis entitle "Mechanism of action of multi–potent ulcer blockers in *in vitro* and *in vivo* models" has therefore been proposed and presented.

Mixtures of interacting compounds produced by plants may provide important combination therapies that simultaneously affect multiple pharmacological targets and provide clinical efficacy beyond the reach of single compound–based drugs. Developing innovative scientific methods for discovery, validation, characterization and standardization of these multicomponent botanical therapeutics is essential to their acceptance into mainstream medicine.

-By Schmidt et al., 2007.

CHAPTER 1

Screening and selection of dietary sources for multi-step ulcer blockers

1.1. Hypothesis

Can dietary sources be potential antiulcer sources ?

Dietary sources

Examined for antiulcer potentials *in vitro*

H^+, K^+ -ATPase inhibition

Antioxidant

***H. pylori* inhibition**

Selection of potential sources

1.2. Work plan

Dietary sources

Prepared aqueous extracts;
Examined for antiulcer potentials *in vitro*

H^+, K^+ -ATPase inhibition

Antioxidant

***H. pylori* inhibition**

Selection of sources with highest antiulcer index (AUI)

Swallow root (*Decalepis hamiltonii*)

Black cumin (*Nigella sativa*)

Selection of active antiulcer components

- **Pectic polysaccharide (SRPP/ BCPP)**
- **Phenolic compound (2-Hydroxy -4-methoxy benzaldehyde – HMBA)**

1.3. Introduction

Before the 19th century, gastric ulceration was uncommon, be it in East or West. Pathology of gastric ulcer was first described in 1935 by Jean Cruveilhier and those days, gastric ulcers were occasionally seen and duodenal ulcers were rare (Lam, 2000). In recent days it became a common global problem because of change in the life style. The incidence of gastric ulcer is increasing to 3.7 million with mortality rate of around 4600 per year in USA alone (www.cureresearch.com/p/peptic_ulcer/prevalence.htm). Gastric ulcer is erosion in the lining of the stomach, which can lead to hemorrhage, perforation, obstruction and death. It develops due to stress, intake of nonsteroidal anti-inflammatory drugs (NSAIDs), ethanol consumption and *Helicobacter pylori* infection, where increased acid secretion is known to aggravate the ulcer and prevent wound healing (Das & Banerjee, 1993; Okabe & Amagase, 2005; Tarnawski, 2005). Among various mechanisms involved in the formation of gastric lesions, free radicals generated during stress and infection are the major causative factors for the gastric lesion through oxidative damage (Phull et al., 1995; Demir et al., 2003). Thus the best approach to control gastric ulcer would be to inhibit oxidative damage, acid secretion and *H. pylori* infections in addition to reinforcing gastric mucosal protection.

The mechanism of free radical mediated damage during ulcer involves lipid peroxidation, which destroys cell membranes with the release of intracellular components, such as lysosomal enzymes, leading to further tissue damage. The radicals also promote mucosal damage by causing degradation of the epithelial basement membrane components, alteration of the cell metabolism; and DNA damage leading to gastric lesions (Schraufstatter et al., 1988; Dharmesh & Srikanta, 2009).

Free radicals can also affect parietal cells which harbors the rate limiting enzyme H^+, K^+ -ATPase or proton pump that participate in the final step of acid secretion (Sachs et al., 1976). Therefore, blocking the final process in hydrogen ion secretion and quenching of free radicals also have been known to reduce acid secretion. Proton pump inhibitors in addition to antioxidants therefore can provide faster onset of action in the control of acid secretion

and hence envisage greater relief of symptoms and increased healing rates (Salas et al., 2002).

H. pylori is a Gram-negative, helical bacteria that colonizes human gastric epithelium (Warren and Marshall, 1983). It is now accepted as a major cause of gastroduodenal disease and peptic ulcer colonizing ~70% of population worldwide. In addition, *H. pylori* is epidemiologically associated with the development of gastric adenocarcinomas (Hansson et al., 1993), gastric lymphoma and MALT lymphoma (Parsonnet et al., 1994). Eradication of *H. pylori* thus believed to contribute to the treatment and prevention of these diseases. It is possible that *H. pylori* inhibitors accelerate ulcer healing and may reduce relapse of the disease. Currently new triple therapies consisting of antibiotics and proton pump inhibitors show eradication rates much better than the individual drugs. However, problems arise due to *H. pylori* rapidly acquiring resistance to traditional antibiotics, in addition to side effects posed by synthetic proton pump blockers and mucoprotectants.

Clinical microbiologists have two reasons to be interested in antimicrobial plant/dietary extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics (Cowan, 1999). Therefore drugs of plant/dietary origin are gaining popularity and are being investigated for number of disorders, including gastric ulcer.

Phenolic compounds are secondary plant metabolites found in numerous plant species, and they play a key role in the biochemistry and physiology of plants. These compounds especially oxidation products of phenolic compound appear to be involved in the defence of plants against invading pathogens including bacteria, fungi and viruses. Dietary phenolic compounds have numerous beneficial properties by virtue of their ability to act as antioxidants, anticarcinogens, antimutagens and antiglycemic agents in addition to act as antimicrobial agents against human pathogens (Friedman et al., 2003; Friedman, 2007). Plants are richest source of phenolic antioxidant molecules and there are plenty of literatures on the usage of plant/dietary phenolics as antioxidants for different purposes including food preservation and prevention of health complications. There

are also a few reports on the proton pump inhibitory property of plant phenolics (Reyes-Chilpa et al., 2006). Traditional medicine systems for treating gastric disturbances mainly depend on plant natural products, but their composition, efficacy and mode of action is poorly understood. Further plant cell wall components – pectins which are used as major dietary fibre sources have been known to be documented with potential health beneficial properties including gastroprotection (Paulsen & Barsett, 2005). Current study therefore has been aimed at determining the effect of aqueous extracts of traditionally known medicinal/dietary plants against potential gastric ulcer causing factors such as free radicals, H^+, K^+ -ATPase and *H. pylori* in *in vitro* assay systems. Aqueous extracts were preferred since the selected dietary sources have been shown to exhibit health beneficial properties mainly in aqueous form itself. Antiulcer indices were calculated based on the *in vitro* assays and the sources exhibiting best antiulcer index such as *Decalepis hamiltonii* (Swallow root) and *Nigella sativa* (Black cumin) were selected for further studies which are reported in subsequent chapters.

1.4. Materials and methods

1.4.1. Chemicals

Agarose, fetal bovine serum (FBS), phenolic acid standards such as gallic, tannic, caffeic, p-coumaric, ferulic, gentisic, protocatechuic, syringic, vanillic and cinnamic acids and 1,1-diphenyl-2-picryl hydrazyl (DPPH), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), amoxicillin, omeprazole, lansoprazole, protease, thermoamylase, glucoamylase and PCR reagents were obtained from Sigma Chemical Co. (St. Louis, USA). DNA isolation kit was purchased from Genei, India. Standard 2-hydroxy-4-methoxy benzaldehyde was purchased from Fluka Chemicals, Switzerland. Folin-Ciocalteu reagent, ferric chloride, trichloroacetic acid, sodium carbonate, ammonium oxalate and ferrous sulphate were of the highest quality purchased from Qualigens Fine Chemicals (Mumbai, India). HPLC grade solvents employed for HPLC analysis were purchased from Spectrochem Biochemicals (Mumbai, India). Ham's F12 nutrient mixture was obtained from Himedia, Sterile syringe filters were from Millipore (India) Pvt. Ltd. Bangalore.

1.4.2. Isolation of *H. pylori*

H. pylori was isolated from endoscopic samples of gastric ulcer patients from Karnataka Cardio Diagnostic Centre (KCDC), Mysore, India and cultured on Ham's F-12 nutrient agar medium with 5% FBS at 37 ^{0}C for 3 days in a microaerophilic condition with 5% CO_2 and 100% humidity (Traci et al., 2001). *H. pylori* culture was confirmed by specific tests as shown in Table 1.1 such as urease, catalase, oxidase, gram staining, colony characteristics and morphology by scanning electron microscopy as described previously from our laboratory (Siddaraju and Dharmesh 2007a). In addition, confirmation of virulence of *H. pylori* was performed by PCR amplification of vacA gene of *H. pylori*.

For PCR analysis, bacterial DNA was isolated using bacterial genomic DNA isolation kit according to the manufacturer's protocol. The amplification of vacA gene was performed using vacA specific primers, forward 5' GTCAGCATCACACCGCAAC 3' and Reverse 5' CTGCTTGAATGCGCCAAAC 3'. The amplification reaction was carried out using Peq Lab, Advanced

Primus96 PCR system with a program consisting of an initial denaturation at 94 ^{0}C for 2 min and 35 cycles of denaturation at 95 ^{0}C for 1 min, annealing at 52 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min and final extension at 72 ^{0}C for 3 min (Atherton et al., 1995). The PCR product was then analyzed by electrophoresis on 1% agarose gel.

1.4.3. Plant collection and preparation of extracts

Nineteen dietary/plant sources that are commonly used in ethnomedicine were collected. A wide range of dietary source's parts including leaf, stem, root (rhizome), seeds and fruit peel were selected as shown in Fig 1.1 & Table 1.2 based on their traditional usage as either food or medicine. The plant materials were collected either from local vendor at Devaraja market, Mysore or BR Hills forest area and identified with the help of flora (Gamble and Fisher, 1957) and also by referring them to the herbarium collections maintained in the Department of Botany, Manasagangothri, University of Mysore, Mysore. For preparation of plant extracts, the plant material was shade dried and powdered. 10 g each powdered substance was extracted in 100 mL of distilled, boiling water and stirred for 20 min. The extracts were filtered through muslin cloth and centrifuged at 2000 *g*. The supernatants were directly used for phenol estimation, H^+, K^+ -ATPase inhibition and antioxidant assays. For *H. pylori* inhibition studies, the pH of the supernatants were neutralized and further filtered through 0.22 µm sterile filters. All extracts were stored in the dark at –20 ^{0}C until use.

1.4.4. Isolation of pectic polysaccharide from Swallow root (*D. hamiltonii)* and Black cumin (*Nigella sativa)*

Since Swallow root and Black cumin showed better antiulcer index with good antiulcer and antioxidant activities in *in vitro* assays, the polysaccharide fractions were isolated from these two sources to further analyze their efficacy as antiulcer components. Pectic polysaccharides were isolated following the ammonium oxalate extraction method (Phatak *et al.*, 1988) as depicted in the scheme 1.1. Briefly 100 g of finely powdered, defatted sample was extracted 3 times with 70% (v/v) ethanol for 1 h each. The samples were centrifuged at 8000 *g* for 15 min and the residue was treated with 0.5 U/g of protease in 500 mL of 0.1 M sodium phosphate buffer (PB) of pH 7.4 and incubated for 10 h at 37 °C with stirring. Contents were

centrifuged and the supernatant was discarded after successive washing, 3 times with phosphate buffer (PB). The residue was suspended in 500 mL of 0.05 M acetate buffer pH 4.6 along with thermoamylase (0.25 U/g) and boiled for 2 h. After ensuring complete degradation of starch as tested by iodine solution, the contents were cooled, centrifuged at 8000 *g* and the residue was resuspended in 0.05 M acetate buffer pH 4.6, treated with glucoamylase (0.7 U/g) and incubated for 10 h at 37 ^{0}C. Further, the contents were centrifuged at 8000 *g* and the supernatant was dialyzed and lyophilized to get water soluble polysaccharide. The pellet was resuspended in 500 mL of 0.05% (w/v) ammonium oxalate and incubated for 3 h at 70 ^{0}C with occasional stirring and centrifuged at 10,000 *g* for 15 min and the supernatant was precipitated with 4 volumes of absolute ethanol, kept in cold for 1 h and the pellet was washed twice with 50 mL of ethanol (45%). Finally, the pellet was resuspended in 100 mL of water, dialyzed extensively against water and lyophilized to get pectic polysaccharide. The remaining residue was processed further to get hemicellulose A, hemicellulose B and alkali insoluble fractions. Since only pectic polysaccharide fraction showed H^+, K^+ -ATPase inhibition, it was further used to test other antiulcer and antioxidant activities.

1.4.5. Estimation of total phenol content

The Folin-Ciocalteu method was used to determine the total phenolic content (Singleton and Rossi, 1965). Different doses of precisely weighed and dissolved samples were taken for the analysis. To the final volume of 100 µL sample in water, 500 µL of 0.2 N Folin-Ciocalteu reagent and 400 µL of 10% sodium carbonate solution in distilled water was added, mixed in a cyclo mixer. The absorbance was measured at 765 nm in a Shimadzu UV-Visible spectrophotometer (Shimadzu UV-160 spectrophotometer) after incubation for 2 h at room temperature. Gallic acid at concentration range of 5 to 25 µg/mL was used as a standard for the calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram of sample.

1.4.6. HPLC analysis of phenolic acids

Since Swallow root and Black cumin showed better antiulcer potentials, the aqueous extracts of these plants were further analyzed for the composition of

phenolic acids. Phenolic acids of extracts were detected by HPLC (model LC-6A, Shimadzu) on SphereClone 5µ ODS 2 column (4.6 mm x 150 mm, Phenomenex), using a UV-detector (operating at *k*max 280 nm). An isocratic solvent system consisting of water/acetic acid/methanol (80:5:15 v/v/v) was used as mobile phase at a flow rate of 1 mL/min (Siddaraju and Dharmesh, 2007a). Phenolic acid standards such as tannic, gallic, protocatechuic, gentisic, caffeic, vanillic, syringic, p-coumaric, ferulic, cinnamic acids and 2-hydroxy 4-methoxy benzaldehyde (HMBA) were employed for identification of phenolic acids present in test extracts by comparing the retention time under similar chromatographic conditions (Nayaka et al., 2010).

1.4.7. Determination of antioxidant activity

The antioxidant activity of extracts and pectic polysaccharides (SRPP & BCPP) were tested by employing free radical scavenging (DPPH assay) and reducing power assays.

1.4.7.1. DPPH assay

1,1, diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical that accepts an electron or hydrogen to become a stable 1,1, diphenyl-2-picrylhydrazine molecule. The reduction in DPPH was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH is generally employed as a substrate to evaluate antioxidant activity of plant extracts (Braca et al., 2003).

An aliquot (200 µL) of sample extract and pectic polysaccharides (SRPP & BCPP) of various concentrations were mixed with 100 mM tris-HCl buffer (800 µL, pH 7.4) and then added 1 mL of 500 µM DPPH in ethanol (final concentration of 250 µM). The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation.

$$\text{Scavenging effect (\%)} = \frac{\text{(Absorbance of control - Absorbance of sample)}}{\text{Absorbance of control}} \times 100$$

1.4.7.2. Reducing power assay

The presence of reductants (i.e. antioxidants) in the sample causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of the Perl's Prussian blue colour at 700 nm. The $FeCl_3/K_3Fe(CN)_6$ system offers a sensitive method for the semi-quantitative determination of dilute concentrations of antioxidants, which participate in the redox reaction.

The reducing powers of test extracts and pectic polysaccharides were determined according to the method of Yen and Chen (1995). The samples of various concentrations were mixed with equal volume of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide and incubated at 50 ^{0}C for 20 min. An equal volume of 10% trichloro acetic acid was added to the mixture and centrifuged at 3000 *g* for 10 min. The upper layer of solution was mixed with equal volume of distilled water and 300 µL of 0.1% $FeCl_3$ was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. The activity was expressed as absorbance units/mg GAE.

1.4.8. Inhibition of H^+, K^+ -ATPase

H^+, K^+-ATPase is a regulatory enzyme found in the plasma membrane of parietal cells involved in passage of protons into the lumen of stomach causing acidity in case of ulcers. Inhibition of this enzyme will reduce the ulcer aggravation.

The H^+, K^+ -ATPase enzyme of parietal cells was prepared from sheep stomach, as described in our previous paper (Siddaraju and Dharmesh, 2007a). Fresh sheep stomach was obtained from local slaughter house at Mysore, India. The mucosa of gastric fundus was cut off and the inner layer was scraped for parietal cells, homogenized in 16 mM Tris buffer (pH 7.4) containing 0.1% triton X-100 and centrifuged at 6000 *g* for 10 min. The supernatant (enzyme extract) was used for the assay. Protein content was determined according to Bradford's method using BSA as standard (Bradford, 1976).

The enzyme extract (350 µg/mL) was incubated with different concentrations of extracts, pectic polysaccharides and phenolics in a reaction mixture

containing 16 mM Tris buffer (pH 6.5) and the reaction was initiated by adding the substrate 2 mM ATP, in addition to 2 mM $MgCl_2$ and 10 mM KCl. After 30 min of incubation at 37 ^{0}C, the reaction was stopped by the addition of a mixture containing 4.5% ammonium molybdate and 60% perchloric acid. Inorganic phosphate formed was measured spectrophotometrically at 400 nm. Enzyme activity was calculated as µmoles of inorganic phosphate (Pi) released/h at various doses of samples. Percent inhibition of enzyme activity was calculated and expressed as IC_{50} values. IC_{50} is defined as amount of GAE of extracts or amount of polysaccharide needed to inhibit 50% of enzyme activity. Lansoprazole a known H^+, K^+ -ATPase inhibitor drug and other known phytochemicals (Table 1.6) were also tested and compared to understand structure–activity relationship.

1.4.9. Inhibition of *H. pylori* – Viable colony count method

Bactericidal activity of the plant extracts and pectic polysaccharides were determined by a viable colony count method (O'Mahony et al., 2005). One hundred microliters of a suspension of 10^8 bacteria/mL was added to 900 µL of plant extract or different concentrations of pectic polysaccharide solutions and incubated for 60 min at 37 ^{0}C. The control consisted of *H. pylori* incubated with sterile distilled water. Serial 10 folds dilutions were made, and 100 µL of each dilution was plated onto Ham's F-12 nutrient agar. These were incubated under microaerophilic conditions for 3 days, and colonies were counted (colony forming units per milliliter, CFU/mL). All experiments were performed three times. The effectiveness of the extracts at killing *H. pylori* was expressed as percentage inhibition of colony growth (i.e. percentage of bacteria killed) compared to that of the control. Amoxicillin a known antibacterial drug and other known phytochemicals were also tested at 50 µg/mL concentration.

1.4.10. Scanning electron microscopic (SEM) studies

H. pylori was incubated with different test extracts as described in viable colony count method (Section 1.4.9) for 1 h. Amoxicillin was used as standard *H. pylori* inhibitor and the cells without inhibitor were taken as control. After incubation, cells were fixed with 2% glutaraldehyde and dehydrated with series of ethanol treatments and examined under SEM (Model No. LEO 425 VP, Electron microscopy LTD, Cambridge, UK) with an

acceleration voltage of 20 KV. Multiple fields of visions were viewed at different magnifications (Kai et al., 1999).

1.4.11. Determination of minimal inhibitory concentration (MIC)

MIC values were determined by serial dilution of inhibitors (Eloff, 1998). This was performed in 96 well microplates by filling all the wells with 100 µL of media containing serially diluted inhibitors – SRPP/BCPP. Sterile control contain media alone, while the growth control contain both media and *H. pylori* without inhibitors were also included. After adding 25 µL of *H. pylori* suspension (10^5 cells/mL) to all the wells except sterile control wells, the plate was incubated in microaerobic condition at 37 ^{0}C overnight. Following morning the bacterial growth was measured by standard MTT assay (Mosmann, 1983). Stock solutions of inhibitors were prepared at a concentration of 2.5 mg/mL with PBS.

1.4.12. Determination of percent contribution to various potential antiulcer properties by constituent phenolic compounds

Aqueous extracts of Swallow root and Black cumin contain different phenolic compounds. These phenolics in pure form possessed differential abilities for either H^+, K^+ -ATPase inhibition, *H. pylori* inhibition or antioxidant activities. Mean % of these activities were calculated and indicated as antiulcer index (AUI). Relative % AUI has been considered to categorize very good, good, moderate and poor sources. Top two sources showing better AUI such as Swallow root and Black cumin were selected as the best antiulcer sources. Further, phenolic and polysaccharide fractions of these sources were employed to understand the precise contribution of these components to various potential antiulcer activities and was calculated and depicted.

Further, potency of individual component and the abundance were considered to calculate % contribution of individual phenolics towards H^+, K^+ -ATPase and *H. pylori* inhibitory and antioxidant activities and depicted (Fig 1.5).

1.4.13. Statistical analysis:

All the experiments were carried out in triplicates (n = 3) and the results are expressed as mean ± standard deviation (SD).

1.5. Results

1.5.1. Inhibition of *H. pylori*

H. pylori isolated from biopsy samples of gastric ulcer patient was confirmed by different tests as shown in Table 1.1. The virulence/pathogenic strain was further confirmed by PCR amplification of virulent vacA gene, specific to pathogenic strain of *H. pylori* (Fig 1.2) which induces vacuolation and increases membrane permeability of host cells. Results showed amplification of ~700 bp DNA of vacA gene. The *H. pylori* culture was further used for inhibitory studies. Inhibitory property of extracts against *H. pylori* growth was analyzed by colony count inhibition method. The results as shown in Table 1.3 indicated a varied anti-*H. pylori* potency among different dietary extracts. *Asparagus racemosus, Nigella sativa, Cassine glauca, Coscinium fenestratum, Coleus aromaticus, Cocculus hirsutus, Zingiber officinale* and *Decalepis hamiltonii* showed more than 90% inhibition of *H. pylori. Alpinia galanga, Murraya koenigii, Trigonella foenum-greacum* and *Passiflora edulis* showed moderate level of inhibition (~50 - 70%). Extracts from other sources showed very poor activity (less than 30%) against *H. pylori.*

Further, the ability of these extracts to kill *H. pylori* was analyzed by SEM after treating *H. pylori* with test extracts for 1 h. The SEM observations were correlated with the results of colony count inhibition assay. The extracts which showed more than 90% inhibition of *H. pylori* colony were also effective in producing marked changes in the morphology of bacterial cells as shown in Fig 1.3. These extracts modify the *H. pylori* to coccoid shape due to effective interaction, and the coccoid form appears to be an inactive form. Clumping of cells, lysis of bacterial cell wall and leaking of cell contents during the treatment with extracts were also observed suggesting potent *H. pylori* inhibitory effect. These changes were minimized in *H. pylori* treated with extracts showing moderate activity in colony count inhibition. These extracts could induce only coccoid shape but there was no lytic effect. Similarly the extracts which showed poor activity in colony count inhibition method were unable to induce any morphological changes in *H. pylori* suggesting the reliability of the employed assay methods.

1.5.2. Inhibition of H^+, K^+ -ATPase enzyme

All the extracts except *Alternanthera sessilis, Cassine glauca, Celosia argentea and Coleus aromaticus* inhibited the H^+, K^+ -ATPase isolated from the stomach of sheep with variable potency. The potency of the extracts determined as the concentration needed to inhibit 50% - IC_{50} of the activity of the enzyme is presented in Table 1.3. *Amorphophallus sylvaticus, Coscinium fenestratum, Zingiber officinale, Trigonella foenum-greacum, Decalepis hamiltonii and Solanum nigrum* exhibited a potential inhibition of H^+, K^+ -ATPase activity with the IC_{50} values ranging from 13 to 29 µg GAE/mL. *Alpinia galanga, Asparagus racemosus, Basella alba, Nigella sativa* and *Coriandrum sativum* exhibited a moderate inhibition of H^+, K^+ -ATPase enzyme with IC_{50} values of 34 – 47 µg GAE/mL. *Murraya koenigii* and *Cocculus hirsutus* exhibited a poorer activity whereas *Alternanthera sessilis, Cassine glauca, Celosia argentea* and *Coleus aromaticus* were unable to inhibit H^+, K^+ -ATPase activity suggesting poorer H^+, K^+ -ATPase inhibitory or antiulcer effect.

1.5.3. Phenol content and antioxidant potential

Wide range of phenol concentrations were found in these sources (Table 1.4). The values varied from 1.97 to 44 mg GAE/g as measured by the Folin–Ciocalteau method. *Cassine glauca, Murraya koenigii, Cocculus hirsutus* and *Decalepis hamiltonii* showed higher level (23 – 44 mg GAE/g) of phenolics followed by medium level (12 – 18 mg GAE/g) of phenolics in *Solanum nigrum, Zingiber officinale, Coriandrum sativum, Celosia argentea* and *Nigella sativa.* Remaining sources possessed lesser amount of phenolic content (less than 10 mg GAE/g). However contribution to activity from them can still be significant.

Antioxidant potency tested by DPPH-free radical scavenging and reducing power assays is shown in Table 1.4. Good antioxidant activity was exhibited by *Alpinia galanga, Nigella sativa, Coleus aromaticus, Cocculus hirsutus* and *Decalepis hamiltonii* extracts. They showed IC_{50} values ranging between 0.95 – 3.07 µg GAE/mL for DPPH-free radical scavenging effect. Also they exhibited potent reducing power with absorbance units range between 62 – 78 units/mg GAE. *Cassine glauca, Celosia argentea, Coriandrum sativum, Murraya koenigii, Zingiber officinale, Solanum nigrum* and *Passiflora edulis*

showed moderate level of free radical scavenging (IC_{50} of 4.2 – 8.2 µg GAE/mL) and reducing power (34 – 59 units/mg GAE) activity. The remaining sources such as *Alternanthera sessilis, Amorphophallus sylvaticus, Asparagus racemosus, Basella alba, Coscinium fenestratum* and *Trigonella foenum-greacum* showed poorer antioxidant activities with IC_{50} values between 8.2 to 22.8 µg GAE/mL for DPPH-free radical scavenging activity and reducing power activity of 27–33 units/mg GAE (Table 1.4).

1.5.4. HPLC analysis of phenolic acids of Swallow root and Black cumin

Composition of phenolic acids were determined in aqueous extracts of *D. hamiltonii* (Swallow root) and *N. sativa* (Black cumin), since these two sources exhibited highest antiulcer index and our previous studies had shown significant contribution of phenolics. The HPLC profile of aqueous extracts showed that Swallow root containing HMBA and vanillic acid as the major components in addition to relatively less amount of gallic, protocatechuic, gentisic, *p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric, ferulic, cinnamic acids and vanillin (Fig 1.4A). However Black cumin extract showed predominant tannic/gallic acid and protocatechic acid as phenolic acid components (Fig 1.4B).

1.5.5. Percent contribution to potential *in vitro* antiulcer activity by aqueous extracts of Swallow root and Black cumin

Aqueous extract of Swallow root and Black cumin have been shown to contain some phenolic compounds in predominant levels. The relative content of phenolic acids as revealed by HPLC analysis showed that, Swallow root aqueous extract contained 4.7 mg/g and 5.06 mg/g of vanillic acid and HMBA respectively amounting to the 25 & 28% of total phenolics respectively. Remaining 47% has been attributed to other phenolic acids such as gallic, protocatechuic, gentisic, caffeic, Syringic, p-coumaric, ferulic, cinnamic acid and vanillin, which are present in lower levels. Black cumin aqueous extract on the other hand contained predominant levels of tannic and gallic acid (2.6 mg/g) followed by protocatechuic acid (0.44 mg/g) amounting to 80 & 14% of total phenolic acids respectively.

In order to calculate their precise contribution to potential antiulcer activity, respective phenolic acids exhibiting various bioactivities such as free radical scavenging (antioxidant), H^+, K^+ -ATPase inhibitory and *H. pylori* inhibitory

properties and % abundance were considered. Total activity expressed by these phenolic acids was taken as 100% and relative % activity was calculated.

Table 1.7 showed that, in Swallow root, HMBA contributed significantly to H^+, K^+ -ATPase inhibitory and *H. pylori* inhibitory activities while >2 folds better antioxidant activity was due to vanillic acid (Fig 1.5A).

In case of Black cumin (Fig 1.5B)., although protocatechuic acid has better efficacy in inhibiting H^+, K^+ -ATPase and *H. pylori,* while almost equal or slightly poorer antioxidant activity when compared to that of tannic acid, high abundance of tannic acid predominated in contributing to all activities - H^+, K^+ -ATPase inhibition, *H. pylori* inhibition and antioxidant activity. However, the comparative efficacy of HMBA and tannic acid calculated by considering their abundance in addition to antiulcer potency indicated ~2.5 fold better contribution by HMBA when compared to tannic acid (Fig 1.5C). Data thus suggest that in addition to the potency, relative abundance of the constituent is important in dictating the bioactivity i.e. antiulcer activity.

1.5.6. H^+, K^+ -ATPase/*H. pylori* inhibition properties from known phytochemicals

Spices/dietary sources have been identified with several important bioactive principles such as cinnamic acid, quercetin, caffeic acid, curcumin, cinnamaldehyde, piperine, gallic acid, capsaicin, eugenol etc., These compounds have been implicated with potent antioxidant and various biological activities. Since the listed dietary components are extensively used by worldwide population it was warranted to test them for their antiulcer potentials. As per Table 1.6 cinnamic acid, quercetin and caffeic acid showed potent H^+, K^+ -ATPase inhibitory activity with IC_{50} ranging from 15-27 µg/mL, while curcumin and piperine showed modest activity (~50-70 µg/mL). Other compounds including gallic acid, capsaicin and eugenol showed poorer activity. On the contrary *H. pylori* inhibitory properties were best with eugenol (98% inhibition) followed by cinnamaldehyde/cinnamic acid (~90%). Rest of the compounds showed moderate *H. pylori* inhibitory effect (Table 1.6).

1.5.7. Antioxidant activity of pectic polysaccharides – SRPP and BCPP

The pectic polysaccharides isolated from Swallow root and Black cumin using classical procedure as shown in Scheme 1.1 gave 6% yield of SRPP and 2% yield of BCPP. Phenolics are generally found to be associated with polysaccharides. Current results also showed the presence of phenolics in pectic polysaccharide of Swallow root and Black cumin. SRPP contained 120 mg phenolics/g, while BCPP contained 26 mg phenolics/g (Table 1.8).

The antioxidant activity determined by free radical scavenging and reducing power assays indicated the good antioxidant potency of SRPP since it contained higher levels of phenolics also. It exhibited free radical scavenging activity with an IC_{50} of 40 µg/mL and reducing power ability of 3200 U/g (Fig 1.6B,C, Table 1.8). BCPP with moderate amount of phenolics (26 mg/g) exhibited ~10 folds less free radical scavenging activity (IC_{50} of 432 µg/mL) and ~2 folds less reducing power activity (1152 U/g) (Fig 1.8B,C, Table 1.8). Although there was only ~5 folds difference in phenolics was observed in BCPP, 10 folds reduction in activity when compared to SRPP was noted suggesting that nature of phenolic acids present in SRPP are more potent than those of BCPP. The phenolic content of pectic polysaccharides thus reflected the antioxidant activity of pectic polysaccharides.

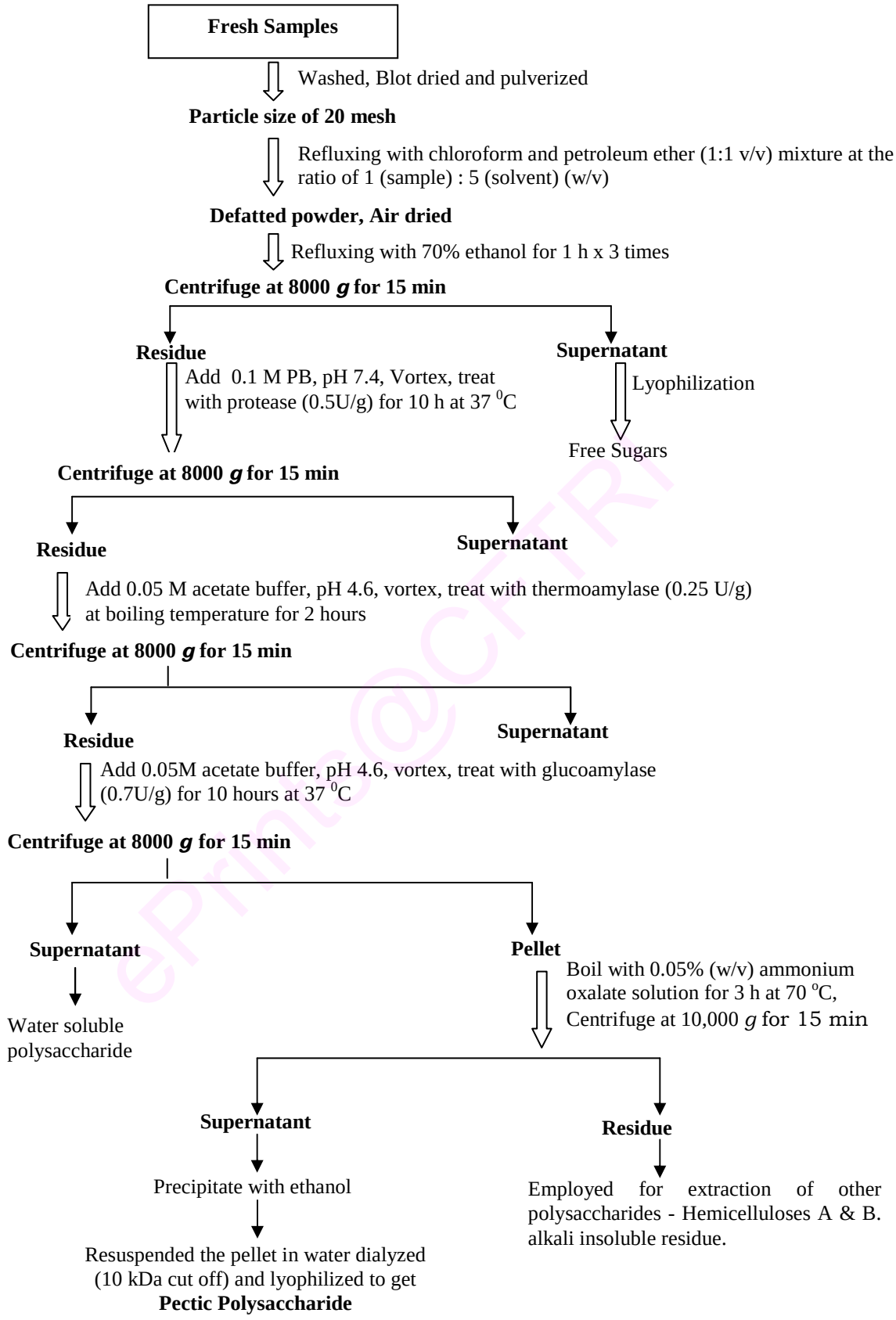
1.5.8. Inhibition of H^+, K^+ -ATPase by SRPP and BCPP

The polysaccharide fractions of Swallow root such as water soluble polysaccharide, hemicelluloses A & B, alkali insoluble residue and pectic polysaccharide were tested for their efficacy in inhibiting H^+, K^+-ATPase *in vitro.* The results (Fig 1.6A) showed that, only pectic polysaccharide fraction of Swallow root showed inhibitory effect with an IC_{50} of 77 µg/mL. Other polysaccharide fractions of Swallow root were unable to inhibit the H^+, K^+ -ATPase activity. Therefore, only pectic polysaccharide fractions of Swallow root and Black cumin was tested for H^+, K^+-ATPase inhibiting activity. The results (Fig 1.8A) showed ~2 folds less activity with IC_{50} of 170 µg/mL of BCPP than SRPP in inhibiting H^+, K^+-ATPase enzyme.

1.5.9. Inhibition of *H. pylori* growth by SRPP

Initially, anti-*H. pylori* activity was assayed by a viable colony count method. SRPP showed up to 95% inhibition at a 200 µg/mL concentration, which is equivalent to that of a susceptible antibiotic amoxicillin at 50 µg/mL concentration. MIC values determined by a broth dilution method, indicated significant anti-*H. pylori* activity of SRPP at 150 µg/mL (Table 1.8, Fig 1.7B). Whereas BCPP did not show any inhibitory effect on *H. pylori*.

Further, the effect of SRPP on *H. pylori* was substantiated by morphological changes observed by SEM. Results indicated that control *H. pylori* without any inhibitor treatment were uniform rod shaped cells (Fig 1.7A), whereas the cells treated with SRPP (200 µg/mL) changed from a helical form to coccoid and became necrotic (showed in arrows in (Fig 1.7C). A similar coccoid form was observed with *H. pylori* treated with amoxicillin (Fig 1.7B) and this form has been known to result in a loss of infectivity (Kusters et al., 1997). A coccoid form with blebs in the bacterial surface, appearance of vacuoles, granules and an area of low electron density in the cytoplasm (shown in arrow marks) were observed in SRPP treated samples indicating the lysis of *H. pylori*. Substantiating to this results viable colony test indicates the loss of >95% viability upon treatment with SRPP, highlights the antimicrobial nature of SRPP.



Scheme-1.1: Preparation of pectic polysaccharide

Fig 1.1. Images of plants used in the study

Tests	Results
Urease	+ve
Catalase	+ve
Oxidase	+ve
Gram staining	Gram-negative
Motility	Motile
Colony characteristic	White mucilage type
Response to antibiotics	
Erythromycin	Resistant
Nalidixic acid	Resistant
Polymixin	Resistant
Penicillin	Resistant
Vancomycin	Resistant
Amoxicillin	Susceptible
Clarithromycin	Susceptible
Metronidazole	Susceptible

Table 1.1. Characteristic biochemical tests used for *H. pylori* identification.

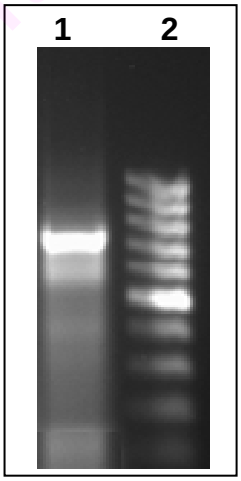


Fig 1.2. PCR amplified product of vacA gene of *H. pylori:*
Lane 1 – vacA amplified DNA band of ~700 bp, lane 2 – 100 bp DNA ladder.

Scientific name	Common English name	Local name (Kannada)	Family	Part of plant used
Alpinia galanga (L.) Willd.	Greater galangal	Rasmi	Zingiberaceae	Rhizome
Alternanthera sessilis DC.	Sessile joyweed	Honagone	Amaranthaceae	Leaf
Amorphophallus sylvaticus Roxb.	Amorphophalus	Suvarna gedde	Araceae	Tuber
Asparagus racemosus Willd.	Asparagus	Shathavari	Liliaceae	Root
Basella alba L.	Indian spinach	Basale	Basellaceae	Leaf
Basella alba L.	Indian spinach	Basale	Basellaceae	Stem
Cassine glauca Kuntze.	Ceylon tree	Mookurichi	Celastraceae	Leaf
Celosia argentea L.	Silver cockscomb	Anne soppu	Amaranthaceae	Leaf
Cocculus hirsutus L.	Broom creeper	Dagadi balli	Menispermaceae	Leaf
Coleus aromaticus Benth.	Coleus root	Dodda pathre	Lamiaceae	Root
Coriandrum sativum L.	Coriander	Kothambari	Apiaceae	Leaf
Coscinium fenestratum Gaertn.	False calumba root	Marada arisina	Menispermaceae	Stem
Decalepis hamiltonii Wight & Arn.	Swallow root	Makali beru	Asclepiadaceae	Root
Murraya koenigii L.	Curry leaf	Karibevu	Rutaceae	Leaf
Nigella sativa L.	Black cumin (Fennel)	Kari geerige	Ranunculaceae	Seed
Passiflora edulis Sims.	Passion fruit	-	Passifloraceae	Peel
Solanum nigrum L.	Black nightshade	Ganike soppu	Solanaceae	Leaf
Trigonella foenum-greacum L.	Fenugreek	Menthya soppu	Fabaceae	Leaf
Zingiber officinale Rosc.	Ginger	Shunti	Zingiberaceae	Rhizome

Table 1.2. List of plants used in the study.

	Plants	% *H. pylori* inhibition	ATPase inhibition IC_{50} (µg GAE/mL)
1	*Alpinia galanga*	59 ± 4.1	34.62 ± 4.2
2	*Alternanthera sessilis*	25 ± 3.2	88.86 ± 7.9
3	*Amorphophallus sylvaticu*	27 ± 4.2	15.91 ± 2.0
4	*Asparagus racemosus*	93 ± 8.0	45.03 ± 5.1
5	*Basella alba* (leaf)	24 ± 4.0	29.08 ± 2.3
6	*Basella alba* (stem)	19 ± 2.1	45.11 ± 5.0
7	*Cassine glauca*	98 ± 6.5	280.25 ± 10.9
8	*Celosia argentea*	18 ± 2.0	89.62 ± 9.0
9	*Cocculus hirsutus*	94 ± 11.4	69.64 ± 6.9
10	*Coleus aromaticus*	93 ± 8.4	206.48 ± 18.5
11	*Coriandrum sativum*	34 ± 4.0	42.29 ± 3.6
12	*Coscinium fenestratum*	97 ± 5.5	13.20 ± 1.8
13	*Decalepis hamiltonii*	97 ± 9.9	29.45 ± 3.0
14	*Murraya koenigii*	69 ± 9.3	88.67 ± 6.9
15	*Nigella sativa*	97 ± 10.2	37.52 ± 3.0
16	*Passiflora edulis*	65 ± 6.9	22.49 ± 3.0
17	*Solanum nigrum*	23 ± 3.4	25.83 ± 2.5
18	*Trigonella foenum-greacum*	68 ± 8.2	18.64 ± 2.1
19	*Zingiber officinale*	93 ± 10.4	14.58 ± 1.9

*Values are expressed as mean ± SD (n=3).

Table 1.3. Anti-*H. pylori* and proton pump inhibitory effects of dietary plants:

Bactericidal activity of plants against *Helicobacter pylori*, determined using viable colony count method. '% inhibition of growth' indicates the percentage of bacteria that were killed by the plants. ATPase inhibitory activity is expressed as IC_{50} values. IC_{50} is defined as amount of phenolics of plant extracts required to inhibit 50% of the enzyme activity.

	Plants	Phenolics (mg GAE/g)	DPPH assay IC_{50} (µg GAE/mL)	Reducing power assay (U/mg GAE)
1	*Alpinia galanga*	6.51 ± 1.0	2.07 ± 0.2	69.89 ± 6.4
2	*Alternanthera sessilis*	6.07 ± 0.5	10.76 ± 0.9	26.96 ± 3.0
3	*Amorphophallus sylvaticu*	1.97 ± 0.2	9.34 ± 7.8	36.20 ± 3.0
4	*Asparagus racemosus*	4.80 ± 0.5	13.63 ± 1.0	29.22 ± 2.4
5	*Basella alba* (leaf)	6.57 ± 0.6	22.85 ± 1.9	27.94 ± 2.2
6	*Basella alba* (stem)	2.61 ± 0.1	12.61 ± 1.4	40.63 ± 3.8
7	*Cassine glauca*	44.0 ± 3.5	4.45 ± 0.3	56.73 ± 5.9
8	*Celosia argentea*	14.67 ± 0.9	5.36 ± 0.4	50.08 ± 4.3
9	*Cocculus hirsutus*	31.83 ± 3.1	2.75 ± 0.3	65.17 ± 4.8
10	*Coleus aromaticus*	8.45 ± 1.0	3.07 ± 0.3	67.90 ± 7.4
11	*Coriandrum sativum*	18.25 ± 2.0	7.14 ± 0.8	43.38 ± 3.9
12	*Coscinium fenestratum*	3.34 ± 0.4	12.82 ± 0.9	32.79 ± 3.0
13	*Decalepis hamiltonii*	34.43 ± 4.0	0.95 ± 0.1	62.70 ± 4.9
14	*Murraya koenigii*	32.27 ± 4.1	5.73 ± 0.4	34.50 ± 2.8
15	*Nigella sativa*	12.39 ± 2.0	2.35 ± 0.1	78.08 ± 8.7
16	*Passiflora edulis*	4.27 ± 0.5	8.27 ± 0.7	59.01 ± 5.4
17	*Solanum nigrum*	18.08 ± 1.6	4.23 ± 0.2	53.26 ± 4.9
18	*Trigonella foenum-greacum*	11.55 ± 1.3	10.96 ± 1.4	27.14 ± 2.2
19	*Zingiber officinale*	8.89 ± 7.5	6.70 ± 0.5	36.73 ± 4.1

*Values are expressed as mean ± SD (n=3).

Table 1.4. Phenol content and antioxidant potencies of dietary plants: Amount of phenolics was expressed as gallic acid equivalents (GAE). Free radical scavenging activity was expressed as IC_{50} values in terms of GAE of extracts required to scavenge 50% of DPPH free radical. Reducing power activity was expressed as absorbance units at 700 nm.

Plants	% Relative activity				% Antiulcer Index	Grade
	H. pylori inhibition	ATPase inhibition	DPPH assay	Reducing power		
Decalepis hamiltonii	98.8	44.91	100	80.3	81.00	Very good
Nigella sativa	98.73	35.25	40.42	100	68.6	Good
Coscinium fenestratum	99.58	100	7.41	41.99	62.29	Good
Zingiber officinale	95.08	90.72	14.17	47.04	61.75	Good
Alpinia galanga	60.65	38.2	45.89	89.51	58.56	Moderate
Cocculus hirsutus	96.49	18.99	34.54	83.46	58.37	Moderate
Coleus aromaticus	94.94	6.4	30.94	86.96	54.81	Moderate
Passiflora edulis	66.8	58.81	11.48	75.57	53.16	Moderate
Cassine glauca	100	4.71	21.34	72.65	49.67	Moderate
Trigonella foenum-greacum	69.34	70.96	8.66	34.75	45.92	Moderate
Asparagus racemosus	94.79	29.37	6.97	37.42	42.13	Moderate
Amorphophallus sylvaticu	27.42	83.13	10.17	46.36	41.77	Moderate
Solanum nigrum	23.62	51.2	22.45	68.21	41.37	Moderate
Murraya koenigii	71.02	14.91	16.57	44.18	36.67	Poor
Coriandrum sativum	34.6	31.27	13.3	55.55	33.68	Poor
Celosia argentea	18.35	14.75	17.72	64.13	28.73	Poor
Basella alba - Leaf	24.75	45.48	4.15	35.78	27.54	Poor
Basella alba - Stem	19.09	29.32	7.53	52.03	26.99	Poor
Alternanthera sessilis	25.31	14.88	8.82	34.52	20.88	Poor

Table 1.5. Grading of plants for total antiulcer activity based on antiulcer index.
The values of plants showing best activity in each assay are considered as 100% to calculate % relative activity of the remaining plants in respective assays. The average of % relative activities of all the four assays is considered as "antiulcer index". The plants are arranged in the descending orders of their antiulcer index. The plant showing highest antiulcer index is considered as a best source having antiulcer potential.

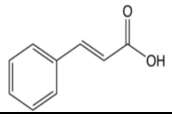
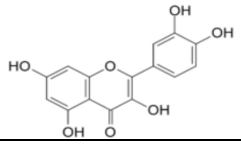
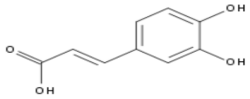
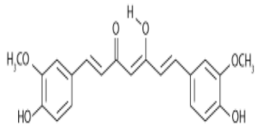
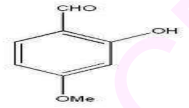
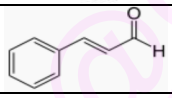
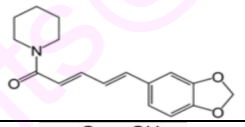
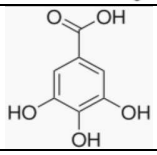
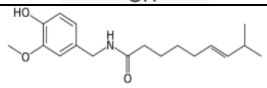
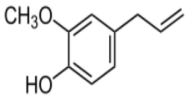
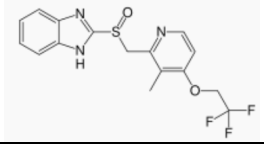
Known phytochemicals	Structure	ATPase inhibition (IC_{50} µg/mL)	% H. pylori inhibition at 50 µg/mL concentration
Cinnamic acid (3-phenyl-2-propenoic acid)		15.0 ± 2.1	90 ± 8
Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7- trihydroxy- 4H-chromen- 4-one)		25.6 ± 2.3	80 ± 8
Caffeic acid (3,4-Dihydroxy cinnamic acid)		27.1 ± 2.9	80 ± 9
Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione)		51.9 ± 4.5	87 ± 4
HMBA 2-Hydroxy-4-methoxy benzaldehyde		49.3 ± 5.5	84 ± 6
Cinnamldehyde (3-phenyl-2-propenal)		63.6 ± 5.9	90 ± 8
Piperine (1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidin)		71.4 ± 8.0	70 ± 7
Gallic acid (3,4,5-trihydroxybenzoic acid)		132.0 ± 12.0	70 ± 9
Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide)		242.3 ± 18.9	85 ± 5
Eugenol (4-Allyl-2-methoxyphenol)		306.6 ± 28.4	98 ± 8
Lansoprazole (2-([3-methyl-4-(2,2,2-trifluoroethoxy) pyridin-2-yl] methylsulfinyl)-1H-benzo[d]imidazole)		19.3 ± 2.2	Not tested

Table 1.6. Proton pump and *H. pylori* inhibitory effect of known phytochemicals.

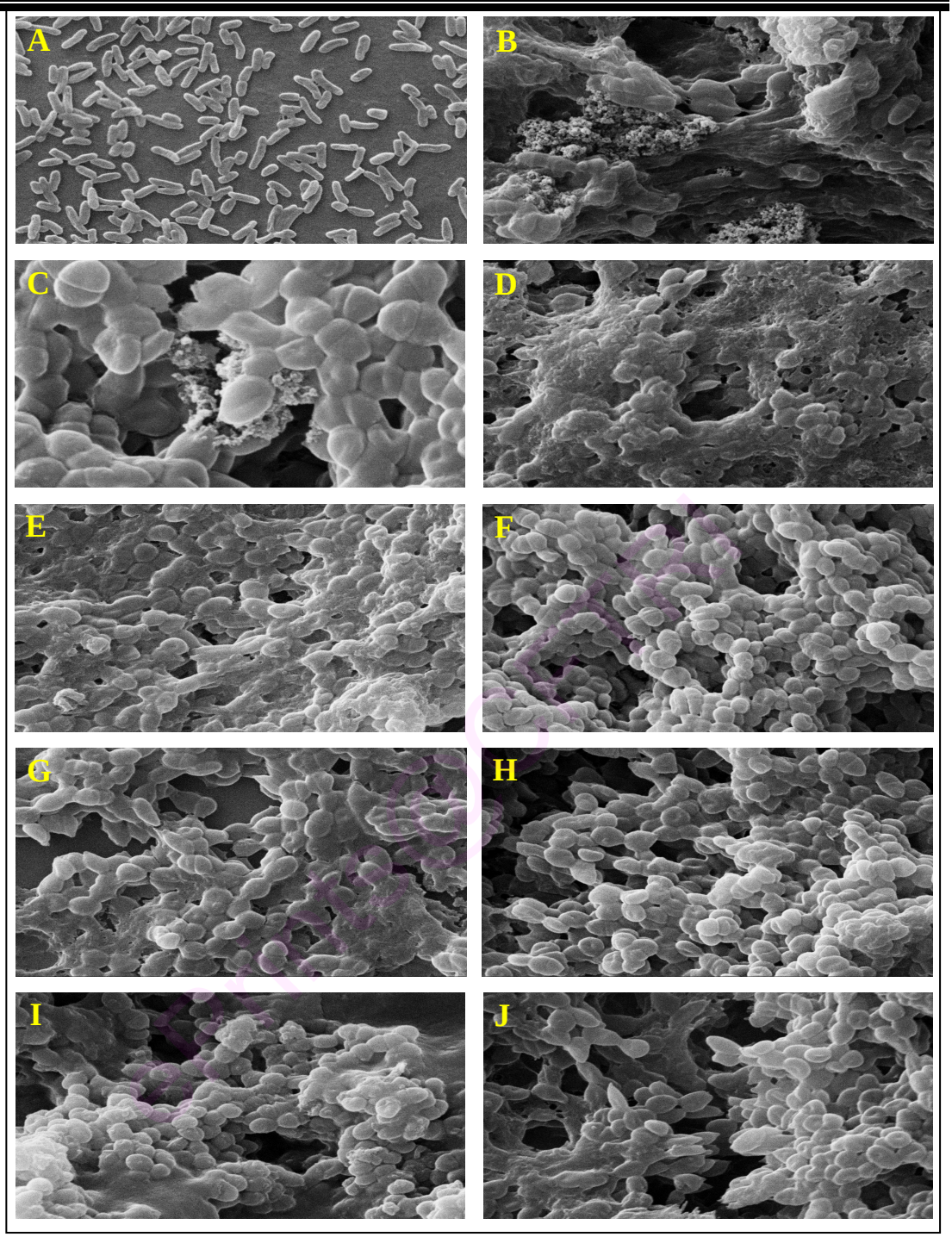


Fig 1.3. Electron microscopic pictures (10K magnification) of *H. pylori* treated with plant extracts exhibiting good *H. pylori* inhibitory activity:

A – Control, **B**- Amoxicillin, **C**- *A. racemosus*, **D**- *N. sativa*, **E**- *C. glauca*, **F**- *C. fenestratum*, **G**- *C. aromaticus*, **H**- *C. hirsutus*, **I**- *Z. officinale*, **J**- *D. hamiltonii.*

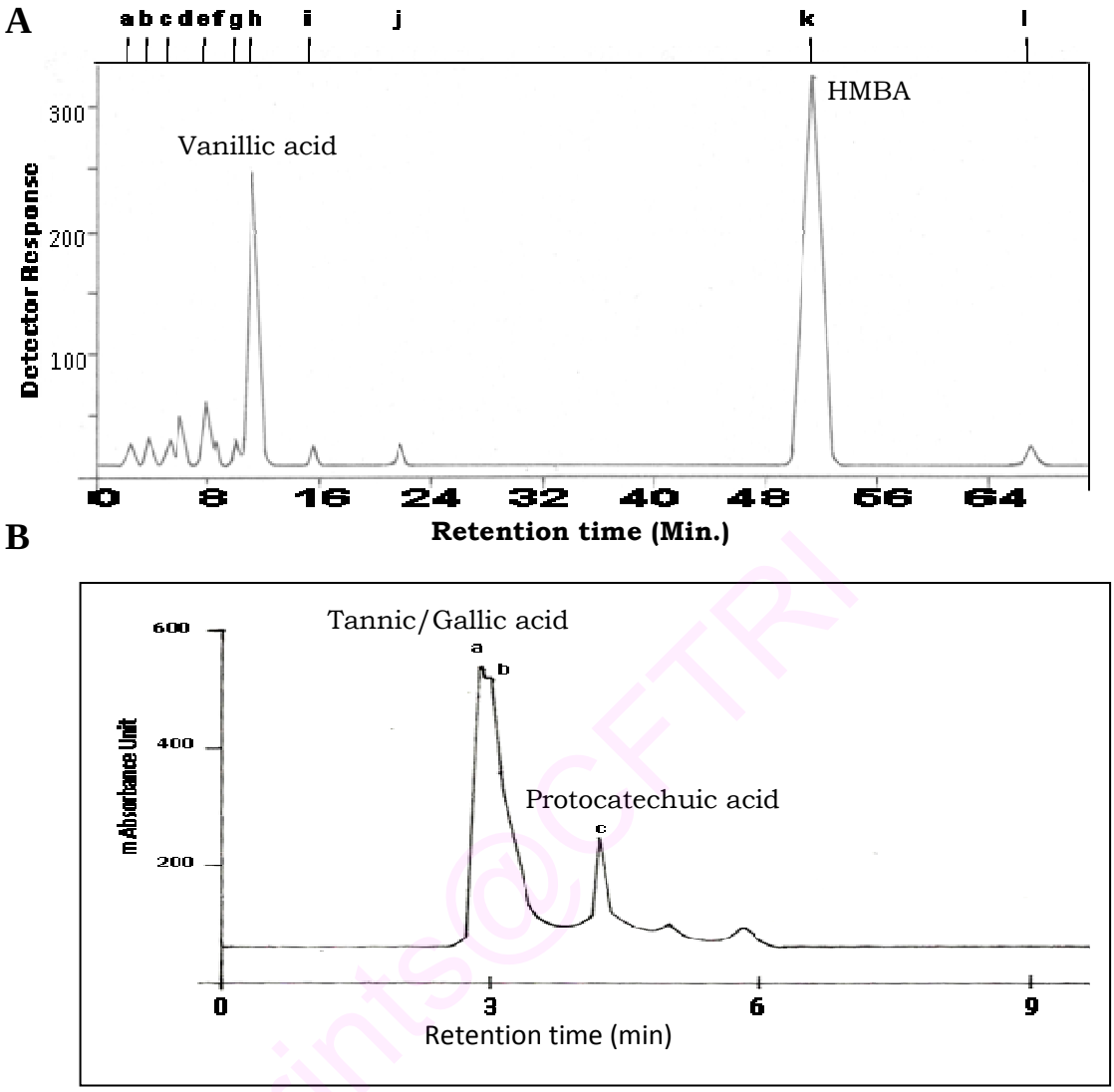


Fig 1.4. HPLC profile of aqueous extract of *D. hamiltonii* (A) and *N. sativa* (B).

In **Fig A** aqueous extract of *D. hamiltonii* (Swallow root) showed major peaks of HMBA (k) and vanillic acid (h) in addition to small amounts of other phenolic acids such as a – Gallic acid, b – protocatechuic acid; c – gentisic acid; d – *p*-hydroxybenzoic acid; e – vanillin; f – Caffeic acid; g – syringic acid; i - *p*-coumaric acid; j – ferulic acid and l –cinnamic acid.
In **Fig B** aqueous extract of *N. sativa* (Black cumin) showed major peaks of tannic acid (a), gallic acid (b) and protocatechic acid (c).

	H^+, K^+ -ATPase inhibition (IC_{50} µg/mL)	% inhibition of *H. pylori*	Free radical scavenging (IC_{50} µg/mL)
Phenolics of Swallow root			
Vanillic acid	69	38	49.5
HMBA	49	84	213
Phenolics of Black cumin			
Tannic acid	132	70	1.1
Protocatechuic acid	47.1	42	1.35

Table 1.7. *In vitro* antiulcer activities of major phenolic compounds of Swallow root and Black cumin aqueous extracts.

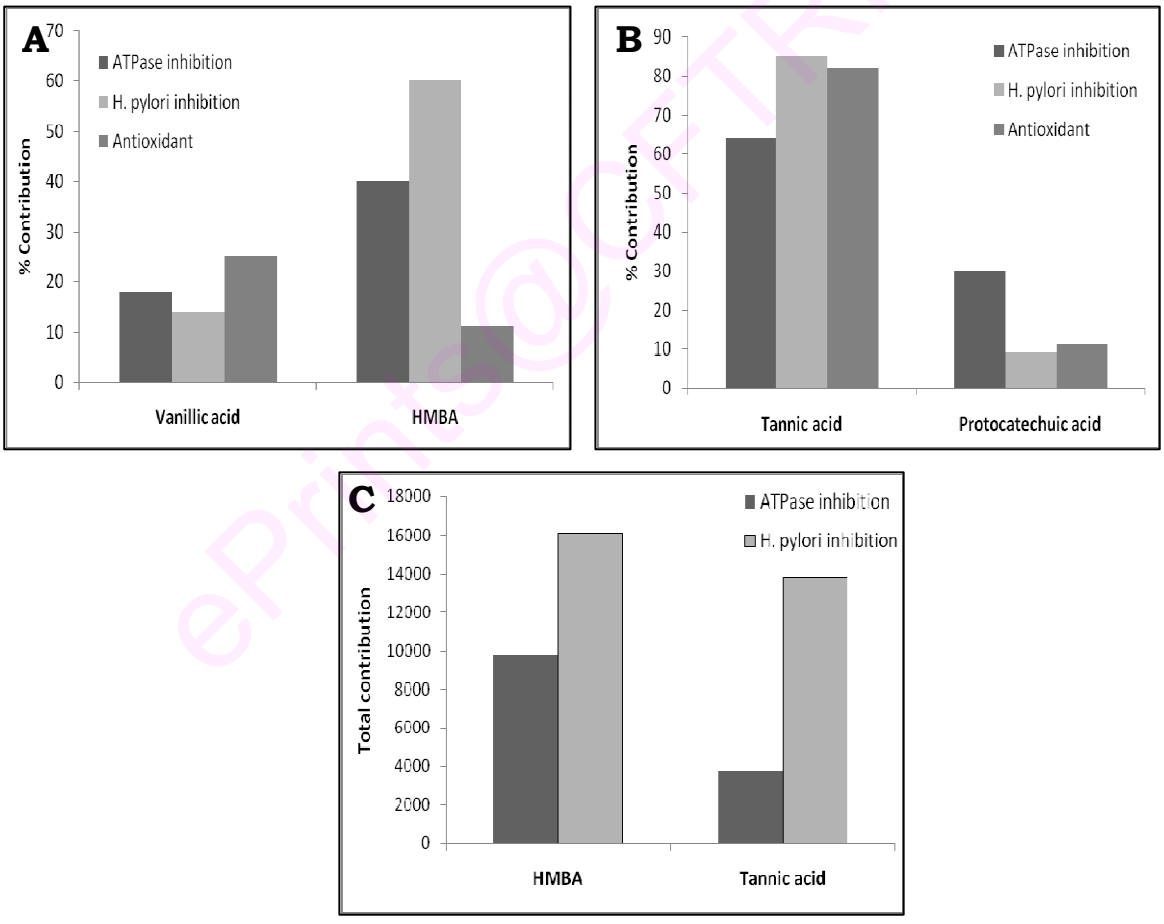


Fig 1.5. Relative % contribution of major phenolic compounds of Swallow root (A) and Black cumin (B) aqueous extracts to *in vitro* antiulcer activities. And comparison of total contributions of HMBA and tannic acid to *in vitro* antiulcer activity (C) by considering their abundance in their respective sources.

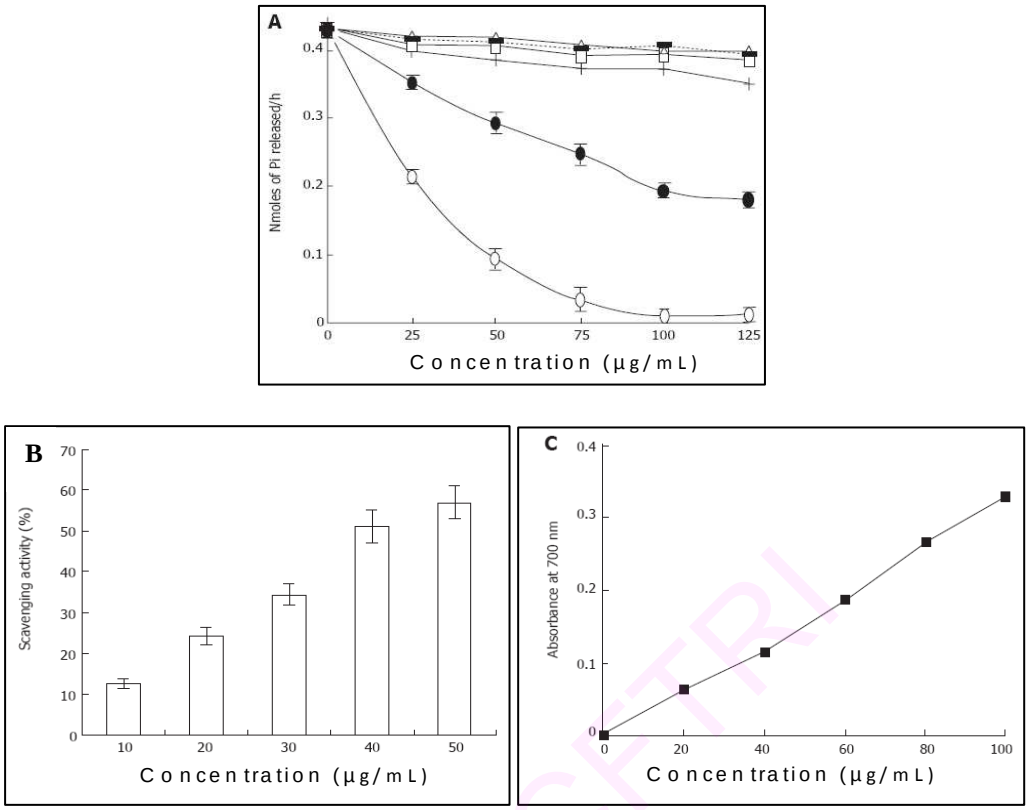


Fig 1.6. H^+, K^+ -ATPase inhibition (A), Free radical scavenging (B) and Reducing power (C) activities of SRPP *in vitro;*
Inhibition of H^+, K^+ -ATPase only by SRPP (●) and not by other fractions of polysaccharide of Swallow root; water soluble polysaccharide (■), Hemicellulose A (+), Hemicellulose B (□), alkali insoluble residue (◊) and inhibition by lansoprazole (o) a known proton pump blocker is also depicted in the figure. Dose dependent antioxidant activity evaluated as free radical scavenging ability (**B**) and reducing power ability (**C**) indicates potential antioxidant activity by bound-phenolics of SRPP.

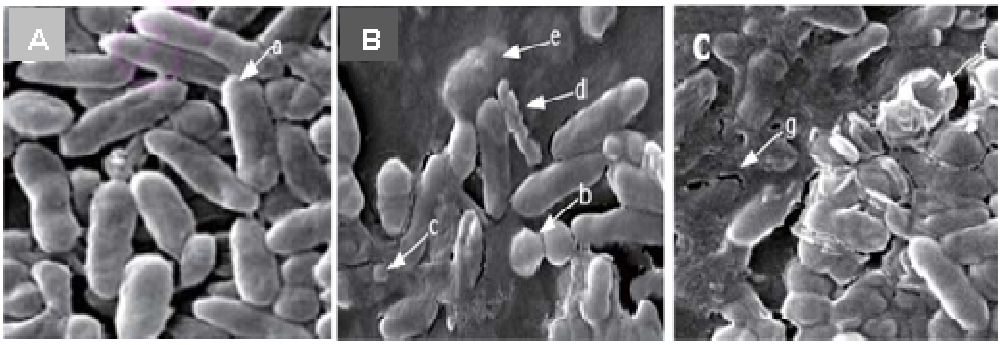


Fig 1.7. Inhibition of *H. pylori* growth by SRPP: SEM pictures of *H. pylori* at 15 K magnification showing uniform rod shaped cells in control group (**A**), Amoxicillin treatment at 50 µg/mL (**B**) showed coccoid form (b), blebbing (c), fragmented (d) and lysed (e) cells. SRPP treatment at 200 µg/mL (**C**) also showed similar changes in addition to cavity formation (f) with disrupted structures (g).

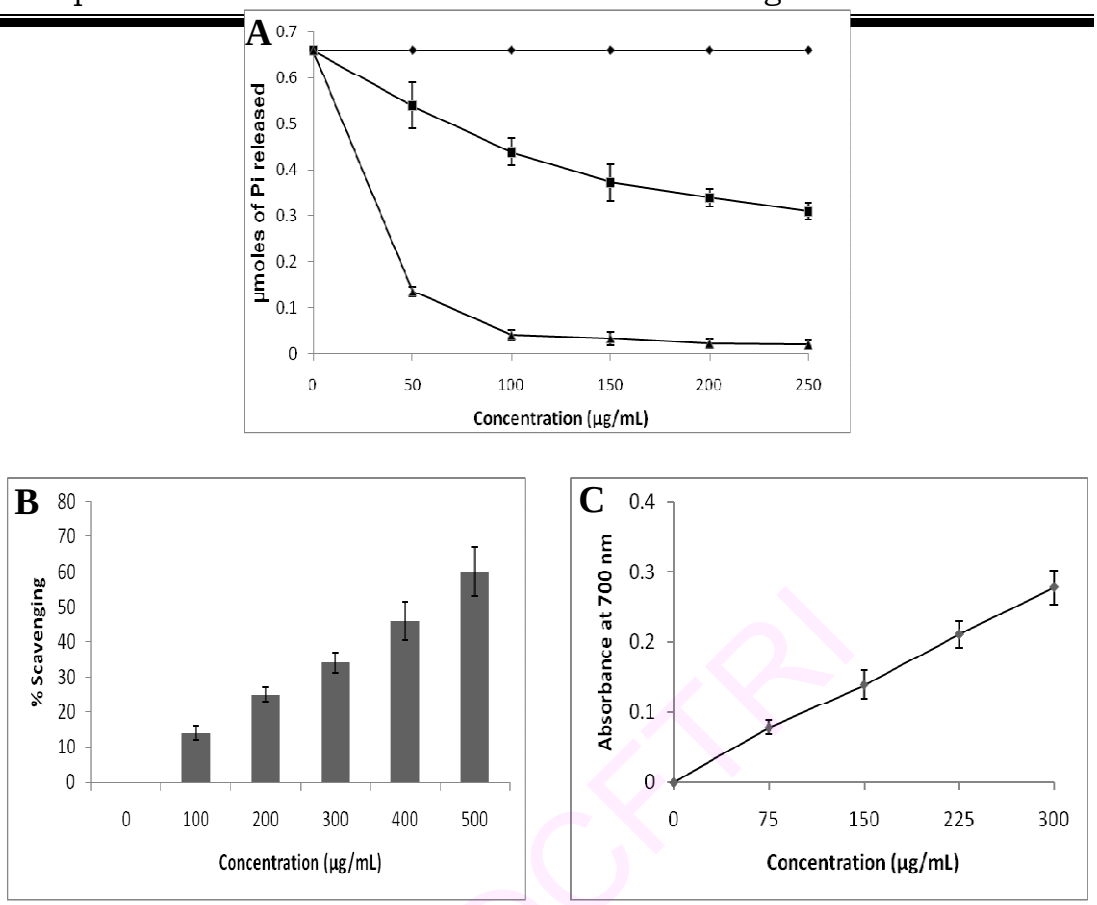


Fig 1.8. H^+, K^+ -ATPase inhibition (A), Free radical scavenging (B) and Reducing power (C) activities of BCPP *in vitro;* Inhibition of H^+, K^+ -ATPase by BCPP (■) and a standard proton pump inhibitor- lansoprazole (▲). Enzyme control (♦) contains no inhibitors. Dose dependent antioxidant property of BCPP was evaluated by free radical scavenging activity (**B**) and reducing power assays (**C**).

	SRPP	**BCPP**	**GRPP**
Phenolics (mg GAE/g)	120	26	5.76
Free radical scavenging - IC_{50} (µg/mL)	40	432	205
Reducing power (Units/g)	3200	1152	987
H^+, K^+ -ATPase inhibition - IC_{50} (µg/mL)	77	170	27.2
H. pylori inhibition - MIC (µg/mL)	150	Nil	60

Table 1.8. Phenolic content and antioxidant / antiulcer potencies of SRPP and BCPP in comparison with a known antiulcer pectic polysaccharide- GRPP (Ginger pectic polysaccharide).

1.6. Discussion

As per our previous studies, compounds/sources exhibiting anti-*H. pylori*, antioxidant and H^+, K^+ -ATPase inhibitory properties were known to possess antiulcer potential *in vivo* (Siddaraju & Dharmesh, 2007a;2007b; Naik et al, 2007). During the present investigation this observation was explored further to index antiulcerogenic potential of commonly used dietary sources including some of the known dietary spice's active principle. The study has an impact on revealing the potential sources that can be employed for gastric health. Further, studies were extended to determine the potential compounds, particularly phenolics that are responsible for gastric protection. Antiulcer Index (AUI) was calculated based on all the three *in vitro* assays carried out during the study and categorized the sources as either very good, good, moderate or poor sources based on the average of % relative activity from all the three assays.

Phytochemicals, especially phenolics of commonly used dietary sources such as fruits and vegetables are suggested to be the major bioactive compounds for health benefits. Recently plant food components particularly pectic polysaccharides are being implicated with several health benefits including potential gastro protective properties (Kiyohara et al., 1994; Gao et al., 2002; Yang et al., 2005; Cipriani et al., 2006; Nergard et al., 2006). Current study therefore focused on the determination of antiulcer potentials in boiling water extracts of selected dietary/plant sources, which contained generally phenolics as well as polysaccharides.

Results of the study indicated that extracts of different plant sources exhibited differential properties. For example, *Asparagus recemosus, Cassine glauca, Cocculus hirsutus, Coleus aromaticus, Coscinium fenestratum, Decalepis hamiltonii, Nigella sativa* and *Zingiber officinale* exhibited potential anti-*H. pylori* activity with >90% inhibition when compared to that of the control. However some of these sources particularly, *Cassine glauca*, *Coleus aromaticus* were very poor in exhibiting H^+, K^+ -ATPase inhibitory properties which is about 4 to 20 folds lesser than other potent anti-*H. pylori* sources (Table 1.3). Contrarily also, sources like *Amorphophalus sylvaticus, Basella alba* (stem), *Solanum nigrum, Passiflora edulis* and *Alpinia galanga* which showed potent H^+, K^+ -ATPase inhibition

with IC_{50} of less than 30 µg GAE/mL, exhibited poorer anti-*H. pylori* activity (Table 1.3) indicating that differential structure may be required for effective intervention in killing *H. pylori* or modulating stress/ulcerogen induced upregulation of H^+, K^+ -ATPase level during ulcer pathogenicity. Similarly some of the sources which showed higher anti-*H. pylori* activity such as *Asparagus recemosus, Trigonella foenum greacum* etc., also showed poorer antioxidant property (Table 1.3 and 1.4). Therefore it is important to arrive at the total antiulcer potential. In the current study, the relative percent activity in each assay was calculated and average from all the assays was considered and depicted as % antiulcer Index (AUI). Based on this, sources were categorized as very good (>80 – 100%), good (60-80%), moderate (30-60%) and poor (<30%) sources (Table 1.5). Accordingly *Decalepis hamiltonii* showed the highest (80%) antiulcer index followed by *Nigella sativa* (68%) and these two were considered as good antiulcer sources to understand structure-function relationship to evaluate *in vivo* efficacy and determining also the mode of action of antiulcer components (Table 1.5, Fig 1.3D,J).

Further, current chapter also addressed the role of already known active antioxidant phytochemicals for potential antiulcer properties. As provided in Table 1.6, well known spice's active antioxidant principles such as capsaicin, curcumin, cinnamaldehyde, eugenol, quercetin, 2-hydroxy-4-methoxy benzaldehyde (HMBA), cinnamic acid and gallic acid found to be present in commonly using spices –*Capsicum annuvum* (Chilly), *Curcuma longa* (Turmeric), *Elattaria cardamom* (Clove), *Allium cepa* (Onion), *D. hamiltonii* (Swallow root), *Z. officinale* (Ginger), *N. sativa* (Black cumin) etc, showed almost a reciprocal relationship between antioxidant activity and H^+, K^+ -ATPase inhibition. Studies thus could indicate that antioxidant activity is higher and positively correlated with the number of hydroxyl groups, while the phenolic acids with fewer hydroxyl groups appear to inhibit H^+, K^+ -ATPase inhibition as evidenced by gallic acid and cinnamic acid activities as antioxidant and H^+, K^+ -ATPase/*H. pylori* inhibitors respectively (Table 1.6).

Results of the current study also supports the fact that cinnamic acid followed by quercetin, curcumin, HMBA and piperine showed better H^+, K^+ -ATPase inhibitory property although, there is no inverse relationship between antioxidant and H^+, K^+ -ATPase inhibitory property suggesting that antioxidant domain per se may not be responsible for H^+, K^+ -ATPase

inhibitory activity in all and at the same time along with antioxidant nature, additional interaction of the molecule with H^+, K^+ -ATPase enzyme may also influence the activity. Data also suggest that apart from fewer hydroxyl groups, other functional groups in curcumin, capsaicin and quercetin may contribute to H^+, K^+ -ATPase inhibitory property.

Careful structure-function analysis revealed that cinnamic acid is the better inhibitor of H^+, K^+ -ATPase (Table 1.6) (Siddaraju & Dharmesh, 2007b; Nanjundaiah et al., 2009). However, alteration in the chemical structure such as change of -COOH group of cinnamic acid to -CHO group in cinnamaldehyde reduced the activity by 4-folds suggesting that, for H^+, K^+ -ATPase inhibition, -COOH group is important besides other associated 3–phenyl-2-propionic acid group. Similarly it is observed that addition of -OCH_3 group decreased the activity by ~4-folds as evidenced by significant reduction in the activity of hydroxycinnamates, where potent H^+, K^+ -ATPase inhibitory activity of cinnamic acid with an IC_{50} of 15.1 µg/mL was reduced by ~ 2 folds with an IC_{50} of 33.6 µg/mL and 37.4 µg/mL in ferulic and syringic acid respectively (Siddaraju & Dharmesh, 2007b). Further compounds - curcumin and HMBA containing -OCH_3 group also showed reduction in H^+, K^+ -ATPase inhibitory activity substantiating the interfering role of -OCH_3 group in inhibiting H^+, K^+ -ATPase activity. Further, lack of -COOH group despite presence of benzodioxol and pentadienyl group reduced the activity by ~4-5 folds as in the case of piperine with an IC_{50} of 71 µg/mL when compared to that of cinnamic acid. Addition of multiple hydroxyl group to the benzene ring reduced the activity by ~ 10 folds as in the case of gallic acid, which is therefore a poorer H^+, K^+ -ATPase inhibitor. In quercetin however, despite the presence of multiple hydroxyl groups in benzopyran ring, an additional phenyl group has offered a potent H^+, K^+ -ATPase inhibitory activity with an IC_{50} of 25 µg/mL. Addition of methyl group further reduced the activity by several folds as depicted in capsaicin (Table 1.6).

Data overall suggest that the nature of functional group is important for the activity. Among phenolic acids, hydroxy cinnmates are better than hydroxy benzoates in inhibiting H^+, K^+ -ATPase activity, which is a crucial enzyme for the generation of acidity in the lumen of the stomach by pumping H^+ into the gastric lumen. Stress or ulcerogen induced H^+, K^+ -ATPase activity thus resulted in hyperacidity and ulcers; inhibitors of H^+, K^+ -ATPase therefore

reduce this event envisaging potential gastric protection. Presence of a methoxy group may be abolishing the H^+, K^+ -ATPase inhibition, which could be due to interruption in the red-ox flow between phenolic acids and the enzyme. Results are substantiated by our previous study where, similar mechanism was observed in the inhibition of aldose reductase enzyme from cataracted eye lens and the observation by previous investigators (Matsuda 2002; Jung 2007; Chethan et al., 2008) that the hydroxylation in the 4' position is crucial for enzyme inhibitory effect.

Our earlier studies had indicated that there is a reciprocal relationship between antioxidant and H^+, K^+ -ATPase or *H. pylori* inhibitory properties. In the current study we could still dissect out further and could reveal that compounds which are good antioxidants still may show better anti-*H. pylori* activity although they differ in their potency. Eugenol which was very poor in exhibiting H^+, K^+ -ATPase activity showed the best anti-*H. pylori* activity revealing further that functional groups required for *H. pylori* or H^+, K^+ -ATPase inhibition are different. This could be attributed to differential interaction of these compounds with the target protein.

It may be important to indicate here also that, as per the known proton pump blockers, particularly, omeprazole, methyl group at position 4 of the pyridine ring donates electrons through resonance to the pyridine nitrogen. This not only increases the percentage of cationic pyridine (which sequesters the drug at the site of action), it also increases nucleophilic character of any molecules of the PPI with and unionized pyridine nitrogen. This in turn facilitates the intramolecular nucleophilic attack at C2 of the benzimidazole ring, leading to the formation of the active sulfonamide and sulfonic acid forms. Methyl groups at positions 3 and 5 also enhance the nucleophilic character of the unionized pyridine nitrogen through positive induction, a sigma bond effect. The rate of formation of these active uncharged (pyridine) and charged (benzimidazole) forms is directly correlated with the onset of relief from symptoms. It is possible that H^+, K^+ -ATPase inhibitors mentioned above may participate in the faster onset of antisecretory action than omeprazole since atleast some of the dietary compounds showed better IC_{50} values than omeprazole.

Since the extracts tested also contained both antioxidants as well as polysaccharides, studies were also conducted on polysaccharides of selected sources – *Decalepis hamiltonii* (Swallow root) and *Nigella sativa* (Black cumin) which showed highest antiulcer index, to enumerate the role of polysaccharides on *in vitro* indexing of antiulcer potential. Accordingly various polysaccharide fractions such as water soluble polysaccharide, hemicelluloses-A, hemicelluloses-B, alkali insoluble residue and pectic polysaccharides were isolated from Swallow root and tested for their efficacy in inhibiting H^+, K^+ -ATPase activity. As shown in Fig 1.6A only pectic polysaccharide fractions of Swallow root (SRPP) exhibited H^+, K^+ -ATPase inhibitory activity.

Therefore as shown in Fig 1.6 & 1.8, only pectic polysaccharide fractions of Swallow root (SRPP) and Black cumin (BCPP) were examined for potential antiulcer and antioxidant properties *in vitro* employing the assays that were used to assess antiulcer potentials. Data presented in Table 1.8 and Fig 1.6, 1.7 & 1.8 suggested that both SRPP and BCPP contained significant levels of phenolics (120mg GAE/g of SRPP and 26 mg GAE/g of BCPP) (Table 1.8). Ginger pectic polysaccharide (GRPP) has been taken as a positive control in the current study. GRPP contains phenolics of only 5.76 mg GAE/g of GRPP, which is very less than that of SRPP and BCPP, but inhibited H^+, K^+ -ATPase activity with IC_{50} of 27.2 µg/mL, whereas SRPP and BCPP inhibited H^+, K^+ -ATPase activity at IC_{50} of 77 µg/mL and 170 µg/mL respectively (Table 1.8).

Further, SRPP and BCPP exhibited free radical scavenging activity with an IC_{50} of 40 and 432 µg/mL respectively as opposed to 205 µg/mL of GRPP suggesting increased antioxidant potency of SRPP (Table 1.8). In other words results showed ~2 folds increased activity in SRPP in inhibiting H^+, K^+ -ATPase than BCPP (Table 1.8). However ginger was more potent (3 folds) better than SRPP suggesting that, it is not the total phenolic content, but probably the nature of phenolics which is important for the activity. Further, when anti-*H. pylori* activity was determined, SRPP inhibited *H. pylori* growth at MIC of 150 µg/mL, while BCPP did not show any activity. GRPP however was more potent which inhibited *H. pylori* growth at MIC of 60 µg/mL. In other words, a 2-3 folds better H^+, K^+ -ATPase and *H. pylori* inhibitory activities in GRPP than SRPP although 5 fold better antioxidant activity was observed in SRPP (Table 1.8). Data thus suggest that, the same component

may not be responsible for both the activity. Further, since there is no direct correlation between phenolics and the H^+, K^+ -ATPase & *H. pylori* inhibitory activities, suggest that apart from phenolics other non phenolic components in the polysaccharide may also play a role.

Although in both antioxidant and H^+, K^+ -ATPase/*H. pylori* inhibiting activities, BCPP is poorer (2 to 10 folds) than SRPP, it is intriguing to notify the contradictory results between SRPP and GRPP. Five times more potency in antioxidant activity and approximately 2-3 folds less potent in inhibiting *H. pylori* and H^+, K^+ -ATPase by SRPP than GRPP suggested that the components present in them vary. Phenolics with more potency in antioxidant nature and less potency in H^+, K^+ -ATPase and *H. pylori* inhibitory activity may be present in *D. hamiltonii*.

Enumerating literature reveals that, although potent and encouraging results are found in aqueous extracts, its *in vivo* bioavailability and efficacy depends on the stability and chemistry of antioxidants. More and more literature showing very good promising *in vitro*-active compounds may be inefficient in clinical trials, (Ex: anticancer property of β-carotene). Promising *in vivo* efficacy with polysaccharides enlightened us to carry on in-depth multi mechanistic/molecular studies employing them. Also not much work is available in terms of understanding the mechanism of antiulcer properties of pectic polysaccharides. In subsequent chapters focus has been thus on delineating the efficacy and mechanism of action of pectic polysaccharides against gastric ulcer. The prooxidant effect of antioxidants in polysaccharides is much less since antioxidants are protected by sugar residues of polysaccharides. Secondly targeting of antioxidants to the site of action is much easier and selective with polysaccharide and this would enhance the efficiency of the polysaccharide to act at pathogenesis site and stabilize the compound with polysaccharide so that the activity may remain longer.

Correlation studies between known active principle and potential anti-*H. pylori*, H^+, K^+ -ATPase inhibitory and antioxidant activities revealed that stronger antioxidants need not possess anti-*H. pylori* and H^+, K^+ -ATPase inhibitory property, which in turn indicate that the antimicrobial and H^+, K^+ -ATPase inhibitory activity may go through different mechanisms than

antioxidant activity route. Antioxidant mechanism may enhance antiulcer potential of the sources selected, since ulcerogenic pathway is initiated by oxidative stress.

Thus future attentions in subsequent chapters are dealing with the *in vivo* efficacy and mechanism of action and structure-function relationship of potential antiulcer pectic polysaccharide – SRPP and BCPP. Studies help in highlighting the role of pectic polysaccharide in prevention and healing of gastric ulcers. They are also found to be useful in offering general health such as lowering of cholesterol, against metastasis, preventing many chronic diseases, ability to participate and modulate the signaling cascade of chronic disease pathogenic steps.

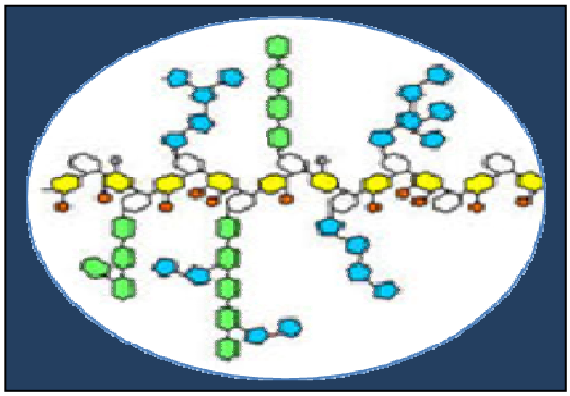
Among phenolic components, HMBA and tannic acid has been identified as predominant phenolics contributing potential antiulcer activity based on *H. pylori* and H^+, K^+ -ATPase inhibitory capacities (Table 1.7, Fig 1.5A,B). Further, abundance of these compounds - HMBA & tannic acid from respective sources – Swallow root and Black cumin were considered to calculate their total contribution to antiulcer activities. Approximately 2.6 fold increase in activity was observed with HMBA when compared to tannic acid (Fig 1.5C). Hence, structure-function studies, *in vivo* antiulcer efficacy and mechanism of anti-*H. pylori* activity of HMBA were studied and presented in chapter 2, 3 and 4 respectively.

1.7. Summary and conclusions

- Chapter 1 addresses screening of commonly used dietary sources for potential antiulcer property.
- A wide range of *in vitro* antiulcer potentials/antiulcer index (AUI) was observed among different dietary sources and they were categorized as very good, good, moderate and poor sources based on their *in vitro* antiulcer potential. Among them top two sources - Swallow root *(Decalepis hamiltonii)* and Black cumin *(Nigella sativa)* showing good antiulcer index (AUI) were selected for further studies.
- Among phenolics, 2-hydroxy-4-methoxy benzaldehyde (HMBA) of Swallow root and tannic acid of Black cumin were identified as potent antioxidant and H^+, K^+ -ATPase/*H. pylori* inhibitory compounds and HMBA predominated due to higher abundance although showed poorer antioxidant activity, contributed significantly to H^+, K^+ -ATPase/*H. pylori* inhibitory and antiulcer activities.
- Among polysaccharide fractions, pectic polysaccharide of Swallow root (SRPP) and Black cumin (BCPP) exhibited potent antioxidant (IC_{50} of 40 µg/mL SRPP & 432 µg/mL BCPP) and H^+, K^+ -ATPase inhibitory (77 µg/mL SRPP & 170 µg/mL BCPP) properties. *H. pylori* growth inhibitory activity was observed only in SRPP at MIC 150 µg/mL.
- Both phenolic and pectic polysaccharides thus appear to participate in potential antiulcer properties.
- Among the known spice principles cinnamic acid showed potent antiulcer activities *in vitro.*

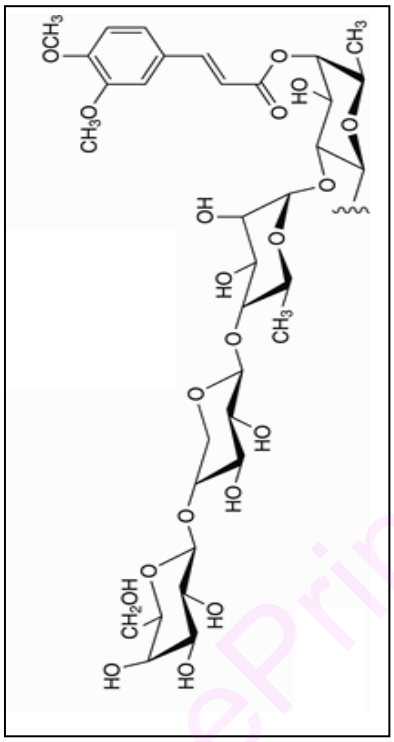
CHAPTER 2

Isolation and characterization of active antiulcer compounds from Swallow root *(Decalepis hamiltonii)* and Black cumin *(Nigella sativa)*



2.1. Hypothesis

Can multi-functional activity of SRPP and BCPP be due to different domains of pectic polysaccharides ?

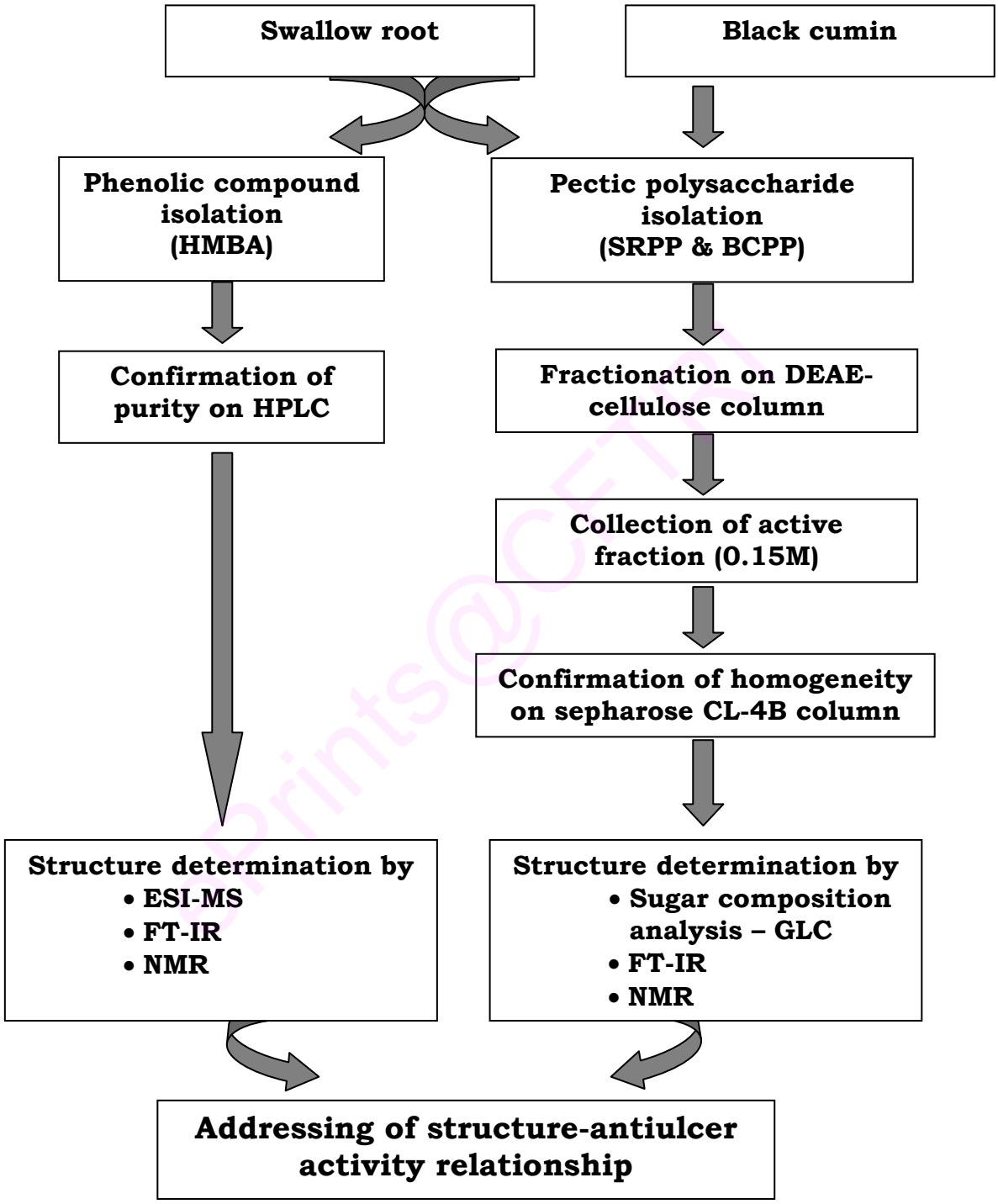


Phenolic-bound polysaccharide

Can polysaccharides

- **Prevent gastric ulcers**
- **Heal gastric ulcers**
- **Inhibit H^+, K^+ -ATPase**
- **Inhibit *H. pylori***
- **Exhibit antioxidant activity**

2.2. Work plan



2.3. Introduction

Ulcer is a common global problem with increasing incidence and prevalence. This increasing incidence and prevalence have been attributed to several reasons encompassed during day-to-day life such as stress (Miller, 1987), exposure to bacterial infection (Ernst & Gold, 2000), use of non-steroidal anti-inflammatory drugs (NSAIDS) (Langman et al., 1991) etc. Indeed NSAIDs are used daily by approximately 30 million people worldwide, constituting a world market in excess of $2 billion and its serious side effects are ulceration and gastric bleeding by inhibiting cyclooxygenase activity that is required for mucosal protection (Khanna et al., 2006).

Dietary/plant sources are potential sources of pharmacologically active polysaccharides. Plant polysaccharides have been known to promote the proliferation and differentiation of human cells (Gloaguen et al., 2008; Cho et al., 2010). Among the plant polysaccharides, pectins have long been used by traditional pharmacopeias (Paulsen & Barsett, 2005), and recently they attracted a lot of interest particularly in understanding their wide variety of functions in relation to their anionic or complicated, long or heterogeneous structures. Pectic polysaccharides are polydisperse macromolecules with substantial heterogeneity in terms of molecular mass as well as chemical structure. Compositions of pectins are strongly dependent on the origin of the source, its localization and the overall environment.

The structure-function relationships of pectins are currently under investigation, but definite data are still lacking about the actual bioactive pattern of pectin polymers. Bioactivity of pectins have been frequently correlated with their acidity, even though this characteristic is not an absolute requirement. On the other hand, increased degree of polymerization as well as extensive branching and ramification seem to enhance their biological activities (Nergard et al., 2006; Gloaguen et al., 2008). Rhamnogalacturonan from *Hibiscus sabdariffa* was shown to stimulate the proliferation and differentiation of human keratinocytes (Brunold et al., 2004). Deters and coworkers recently suggested that terminal arabinose residue of rhamnogalacturonan type-I side chains (Deters et al, 2005), as well as rhamnose content should justifies the observed bioactivity of pectins. More precisely, rhamnogalacturonans were shown to bind to outer

membrane receptors, triggering signal transduction cascade and ultimately nuclear activation (Deters et al, 2005).

In the previous chapter, chapter 1, aqueous extracts containing polysaccharides and phenolics were prepared from different dietary sources and screened for their efficacy against gastric ulcers employing multiple *in vitro* assays. Results highlighted Swallow root *(D. hamiltonii)* and Black cumin *(N. sativa)* as a potent gastroprotective sources having higher antiulcer indices.

The current chapter, Chapter 2 therefore addresses isolation and characterization of both phenolics and polysaccharide fractions of Swallow root and Black cumin that could exhibit antiulcer property. Probable phenolic compounds responsible for the activity, their precise contribution to *in vitro* antiulcer activity; isolation of various polysaccharide fractions of selected sources – Swallow root and Black cumin and their *in vitro* antiulcer activity are depicted in chapter 1. Attention has been paid in the current chapter to isolate, purify and characterize the active fraction of pectic polysaccharide of SRPP and BCPP. Further, among the phenolic compounds, since HMBA of Swallow root contributed significantly to the activity (Fig 1.5C of chapter 1), proposal has been made to isolate and purify HMBA and to determine its functionality. Significant H^+, K^+ -ATPase and *H. pylori* growth inhibitory activity of HMBA directed us to isolate, purify and characterize this compound to determine its antiulcer activity *in vivo* (Chapter 3), anti-*H. pylori* activity and probable mode of action (Chapter 4).

There are numerous information on the bioactivity of plant polysaccharides such as anti-inflammatory, immunomodulatory, anticancer, antimetastatic, antiadhesive, etc., (Sakurai et al. 1999; Gao et al., 1989; Shin et sl., 1997; Nangia-Marker et al., 2002). There are only few reports on the gastroprotective effects of polysaccharides isolated from different sources like plants, fungi and animals (Matsumoto et al., 2002; Nergard et al., 2006; Lee et al., 2006). Extensive studies in the laboratory also enabled us to highlight potent pectic polysaccharides having antimetastatic and gastroprotective properties (Sathisha et al., 2007; Srikanta et al., 2007 & 2010).

Pectic polysaccharides belong to a family of complex polysaccharides that contain 1,4-linked α-D-galacturonic acid residues in the backbone. There

are numerous reports on bioactivity of pectic polysaccharides (Paulsen & Barsett, 2005), however, there are only a few reports that delineates the structure of the bioactive domains, structure-function relationship and precise mechanism of action. It is not uncommon in pectic polysaccharides and other bioactive molecules that there are special features in polymers/components that can envisage special function (Paulsen & Barsett, 2005).

The current chapter thus deals with isolation, purification and characterization of active antiulcer polysaccharides – SRPP and BCPP; and abundant phenolic compound – HMBA. This chapter describes the structure-function relationship of SRPP, BCPP and HMBA to gastroprotective, antioxidant, H^+, K^+ -ATPase inhibitory and *H. pylori* growth inhibitory properties both *in vitro* and *in vivo.* Data on bioactivity studies compiled in other chapters have been considered for elucidation of structure-function relationship. Studies highlighted the role of both phenolics and polysaccharides of Swallow root and Black cumin that can participate as intervention tools at several steps of ulcer pathogenicity. Precise structural prerequisite of SRPP, BCPP and HMBA with respect to *H. pylori* induced ulcer pathogenic steps have been detailed in chapter-4.

2.4. Materials and methods

2.4.1. Chemicals

Carbohydrate standards such as rhamnose, arabinose, xylose, mannose, galactose, glucose and galacturonic acid, amberlite IR-120 H^+ resin, protease, thermoamylase, glucoamylase, KBr, Sepharose CL-4B gel, dextran standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-cellulose was purchased from Genei, Bangalore. 2-Hydroxy-4-methoxy benzaldehyde (HMBA) was purchased from Fluka Chemicals, Buchs, Switzerland. Other chemicals such as ammonium oxalate, ammonium carbonate, sodium hydroxide, sodium phosphate buffer, perchloric acid ($HClO_4$), acetic acid, sodium acetate, glutaraldehyde, sodium chloride, calcium chloride, sodium azide, glycine, sulphuric acid, sodium hydride, acetonitrile, methanol, alcohol, dichloromethane, sodium borohydride and solvents used were of the analytical grade purchased from local chemical companies, Mysore, India. All chemicals and solvents used for analysis were of HPLC grade. Some of the solvents like phenol, ethanol were distilled before use.

2.4.2. Isolation and characterization of pectic polysaccharides from Swallow root and Black cumin

2.4.2.1. Isolation of pectic polysaccharides

Pectic polysaccharides from Swallow root and Black cumin were isolated following the standard protocol (Phatak *et al.*, 1988) as described in Scheme 1.1 of chapter 1. Pectic polysaccharides isolated from Swallow root and Black cumin have been designated as Swallow root pectic polysaccharide (SRPP) and Black cumin pectic polysaccharide (BCPP) respectively. Yield of these polysaccharides were calculated considering the total recovery of pectins from the crude Swallow root and Black cumin powder. The isolated pectic polysaccharides were further fractionated on DEAE-cellulose column and confirmed its purity on Sepharose CL-4B column as follows. Sugar composition analysis and uronic acid contents were determined since it is the characteristic sugar component of pectic polysaccharide.

2.4.2.2. Fractionation of SRPP and BCPP on DEAE-cellulose column chromatography

Since pectic polysaccharides are negatively charged components, their resolution was studied on anion exchange column, DEAE-cellulose column chromatography. DEAE-cellulose was washed with water to remove fine particles. It was then regenerated successively with HCl (0.5 N) and NaOH (0.5 N). After each treatment, the pH was adjusted to neutrality by washing thoroughly with water. Silver nitrate and phenolphthalein tests were conducted to ensure the removal of excess chloride and hydroxyl ions respectively. The regenerated exchanger was suspended in ammonium carbonate (0.5 M, pH 9.3), packed in a column (3.5 cm x 26 cm) and excess carbonate was washed off with water.

SRPP and BCPP (0.5 g each) were dissolved in 20 mL of water and loaded on to DEAE-cellulose column in separate experiments and the elution was carried out with water, followed by increasing gradients of ammonium carbonate (0.05 to 0.20 M AC) and subsequently sodium hydroxide (0.1 and 0.2 M NaOH) solutions at a flow rate of 60 mL/h. Fractions (8 mL) were collected, assayed for total sugars by phenol-sulfuric acid method as described under section 2.4.2.4. After ensuring the peaks, pooled peak fractions were examined for H^+, K^+ -ATPase inhibitory activity as per the protocol mentioned under section 1.4.8 of chapter 1. In both the cases 0.15 M fraction was the major component amounting 63% and 72% in SRPP and BCPP respectively (Table 2.1) with H^+, K^+ -ATPase inhibitory activity. Thus 0.15 M SRPP and 0.15 M BCPP fractions were taken for further structural characterization studies.

2.4.2.3. Determination of homogeneity and molecular weight by gel permeation chromatography

Gel permeation chromatography was performed for 0.15 M fractions of Swallow root pectic polysaccharide (0.15 M SRPP) and Black cumin pectic polysaccharide (0.15 M BCPP) separately on Sepharose CL-4B column (1.6 cm x 92 cm). SRPP-0.15 M and BCPP-0.15 M were dissolved in distilled water (10 mg/mL), centrifuged at 6000 *g* for 10 min at room temperature and the supernatant was loaded (1 mL) on to the column. The elution was carried out by using NaCl (0.1 M) containing sodium azide (0.05%) at a

constant flow rate of 16 mL/h. Fractions (1.5 mL) were collected and analyzed for the presence of total sugar by phenol-sulfuric acid method and homogenous fraction thus showed were pooled, dialyzed, lyophilized and used for structural characterization.

To determine the relative molecular weight of the active fractions (0.15 M SRPP and 0.15 M BCPP) Sepharose CL-4B column was calibrated with standard dextrans of known molecular weights (T-10, T-20, T-40, T-70, T-150, T-500, and T-2000) and the respective elution volume (V_e) was recorded. The void volume V_o was determined using pre-dialyzed Blue dextran (20,00,000 Da). A calibration curve was obtained by plotting log molecular weight against V_e/V_o. The relative molecular weight of the samples (0.15 M SRPP and 0.15 M BCPP), were calculated from the standard plot.

2.4.2.4. Determination of total carbohydrate content

Total carbohydrate content was determined in both intact pectic polysaccharide and eluted fractions. Pectic polysaccharides were dissolved in water at the concentration of 1 mg/10 mL in water. Total carbohydrate content was estimated (Rao & Pattabiraman, 1989) in 0.5 mL aliquot of the sample in test tubes. To samples, 0.3 mL of phenol (5%) and 1.8 mL of concentrated sulphuric acid were added and the contents were mixed thoroughly. After cooling the tubes at room temperature (~20 min), the absorbance was read at 480 nm against a reagent blank. Sugar content was determined against the calibration graph, prepared by using D-glucose at 4-20 µg/mL of concentration range. To determine the carbohydrate content of the fractions to monitor the emergence of pectic polysaccharide, 0.5 mL aliquot in each fraction was examined for total sugar content.

2.4.2.5. Uronic acid estimation

Uronic acid was estimated as per the protocol described by Bitter & Muir, (1962). 0.5 mL of the samples (10 mg/100 mL) were taken in test tubes and kept in ice cold water bath for 10 min. To this, concentrated sulphuric acid (3 mL) was added slowly. Contents were mixed thoroughly and kept in boiling water bath for 20 min and cooled. 0.1 mL of carbazole solution (0.1% prepared by dissolving re-crystallized carbazole in alcohol) was added. The tubes were kept in dark for 2 h and the absorbance was recorded at 530 nm.

Uronic acid content was determined against the calibration graph prepared by using D-galacturonic acid at 10-50 µg/mL concentration range.

2.4.2.6. Sugar composition analysis by GLC method

In the GLC analysis the hydrolysis of polysaccharides to reducing sugars and the concomitant conversion to alditol acetates (borohydride reduction and acetylation) is a standard method to analyze polysaccharides containing aldoses, ketoses, deoxyaldoses, acetamidohexoses and other related sugars (Sawardekar et al., 1965). Determination of the sugar composition of the polysaccharides involves the identification and quantification of sugar constituents. Depolymerization of the polysaccharide is a prerequisite, for which various methods have been developed and acid hydrolysis is the most common and widely accepted protocol.

2.4.2.6.1. Hydrolysis and preparation of alditol acetates of neutral sugars

The respective purified abundant fractions of pectic polysaccharides of SRPP and BCPP – 0.15 M SRPP and 0.15 M BCPP, 10 mg each were suspended in water and hydrolyzed by prior solubilization with 72% sulphuric acid at ice cold temperature followed by dilution to 8% acid by adding water and heating in a boiling water bath at 100 °C for 10-12 h. The mixture was neutralized with barium carbonate, filtered, deionized with Amberlite IR-120 H^+ resin and concentrated using flash evaporator.

The neutralized and deionised sample was concentrated to about 0.5 mL; sodium carbonate was added to a concentration of 0.07 M to decompose uronic acids. Sodium borohydride (20 - 30 mg) was added and the test tubes were stoppered and taped with adhesive plaster around to hold the stoppers. They were left overnight. Next day, excess borohydride was destroyed with acetic acid (2 N). The excess borate and other salts were removed by co-distilling with methanol for 4 times (1 mL each) and then evaporated to dryness. Dry and distilled acetic anhydride and pyridine (0.5 mL each) were added and kept at 100 °C for 2 h after tightly stoppering the tubes. Excess reagents were removed by co-distilling with water (1 mL, x3) followed by toluene (1 mL, x3). After thorough drying, the contents were taken in chloroform and filtered through glass wool and dried by passing nitrogen

gas. Samples were re-solubilised in chloroform and subjected to Gas Liquid Chromatographic (GLC) analysis.

2.4.2.6.2. Gas liquid chromatography

Shimadzu GLC (Model-CR4A) fitted with flame ionization detector was used for analysis. OV-225 (1/8" x 6') was the column used with column, injector and detector block temperatures maintained at 200, 250 and 250 °C, respectively. Nitrogen with the flow rate of 40 mL/min was used as the carrier gas.

Standard sugars were also subjected to alditol acetate derivatization and were run on GLC. For individual sugar standards and samples, retention time and peak area was noted separately. Number of peaks observed in test samples were assigned to various known standards comparing the retention time in min with that of the known standard sugars. Proportions of different sugars were calculated based on the relative percent abundance.

2.4.2.7. Fourier Transform-Infrared spectroscopy (FT-IR)

Infrared waves are absorbed by the vibrating chemical bonds in the polysaccharide giving characteristic FT-IR spectra in the frequency range 4000 to 400 cm^{-1}. FT-IR spectroscopy was carried out to detect the functional groups, configuration of sugar residues and the substitution pattern of sugars. Pectic polysaccharides being polymeric in nature contained several functional groups and FT-IR spectroscopy is useful for characterization of pectic polysaccharides.

Each pectic polysaccharide samples (6 mg), in duplicates were mixed thoroughly with solid crystalline KBr (Spectroscopic grade) and pressed into a 1 mm pellet (Manuel *et al.*, 1998). IR spectral studies were performed in an absorbance mode at a resolution of 4 cm^{-1} with wave number range 4000 – 400 cm^{-1} using a Perkin-Elmer spectrum 2000 spectrometer (Connecticut, USA).

2.4.2.8. NMR spectral analysis

NMR spectroscopy is the rapid and non-destructive method to study the structure of polysaccharides. It requires no modification or degradation of the sample. ^{13}C and ^{1}H NMR together can give the details on molecular complexity and fine structure of the polysaccharides. ^{13}C-NMR gives details

about the composition, linkage and confirmation of polysaccharides with respect to carbon atom, while ^{1}H-NMR provides an idea about relative abundance of functional groups that are linked to carbon skeleton. The analysis also ascertains the purity of the polysaccharide sample (Michael *et al*, 1985).

The active fraction of SRPP and BCPP (0.15 M SRPP and 0.15 M BCPP) were dissolved in 1 mL of D_2O. After ensuring complete dissolution, spectra were recorded with a Bruker AMX 400 spectrometer at 500/700 mHz. The signals were assigned to respective sugars including the precise linkages based on previously published literatures (Polle et al., 2002; Prasanna et al., 2004; Zhao et al., 2007).

2.4.3. Isolation and characterization of 2-hydroxy-4-methoxy benzaldehyde (HMBA) from Swallow root

2.4.3.1. Isolation of HMBA

Fresh roots of Swallow root (*D. hamiltonii)* were procured from a local vendor at Devaraja market, Mysore, India. HMBA was isolated using the modified method of Nagarajan and Rao (2003). Briefly, 1 kg of fresh roots were cleaned, washed to remove the soil and central hard woody portions were separated out. The roots were cut into small pieces of 0.5 – 1.0 cm, suspended in 3 L of water and subjected to steam distillation for 3 h. The steam condensate was kept at 4 ^{0}C overnight; white needle like crystals obtained were further extracted with dichloromethane. The extract was passed through a funnel containing anhydrous sodium sulphate to remove water content, concentrated in flash evaporator and used for further characterization and bioactivity studies.

2.4.3.2. Characterization of HMBA

2.4.3.2.1. HPLC/ESI-MS analysis

HMBA dissolved in ethanol (1 mg/mL) was subjected to HPLC (model LC-6A, Shimadzu) analysis on a SphereClone 5µ ODS 2 column (4.6 mm x 150 mm, Phenomenox) using UV-detection system. Chromatographic run was done with mobile phase consisting of an isocratic solvent mixture of water : acetic acid : methanol (80 : 5 : 15 v/v/v) with a flow rate of 1 mL/min. The chromatograms were monitored at 280 nm. The standard HMBA (20 µL of 1

mg/mL) was also run on the same HPLC column under similar conditions for comparison. The identity of the compound was determined by comparing with the retention time of standard HMBA which was eluted at retention time of 48.41 min.

Sample was also analysed by ESI-MS - electron spray ionization mass spectrometry for the confirmation of their chemical identity and mass. Mass of the HMBA was obtained with Finnigan MAT 95 mass spectrometer by injecting the sample dissolved in ethanol.

2.4.3.2.2. Fourier Transform-Infrared spectroscopy (FT-IR)

Further, the confirmation of chemical groups was done by analysing the sample by Infrared spectrometry using Perkin-Elmer FT-IR spectrometer as described earlier **(Section 2.4.2.7)**. Briefly, the sample was prepared in the form of thin pellets by mixing HMBA with dry potassium bromide. Potassium bromide discs containing 1% (w/w) of film material were scanned at 4 mm/s with a resolution of 4 cm^{-1} over 4000 -400 cm^{-1}, averaging over 128 scans for each type of film.

2.4.3.2.3. NMR spectral analysis

^{13}C and ^{1}H NMR spectra of HMBA was analyzed. The 50 mg sample dissolved in DMSO (1mL) was used for recording the spectra with a Bruker AMX 400 spectrometer at 500/700 mHz. The spectral data are reported as ppm downfield from tetramethylsilane (TMS) (δ=0).

2.4.4. Structure-activity relationship

Based on the results of biophysical techniques such as NMR, FT-IR, ESI-MS etc., followed by biochemical analysis, structure of antiulcer components – SRPP, BCPP and HMBA were proposed. In order to correlate the structure to the function, list of bioactive antiulcer potentials both *in vitro* and *in vivo* presented in various chapters (Chapter 1, 3 & 4) were made (Table 2.6). Correlation between various sugar residues and bioactivity of various sources were carried out. Similarly in case of HMBA, the relation between functional groups and bioactivity was determined. Comparison between the components present in some of the selected sources such as Swallow root and Black cumin pectic polysaccharides and ginger pectic polysaccharide also has been made. Ginger pectic polysaccharide used as a positive

reference sample since it is found to be one of the potent sources as per our previous studies. Precise structure, responsible for the activity has been deduced. Structure-activity relationship has been established. Domains responsible for various activities have been deduced.

2.4.4. Statistical analysis:

All the quantitative experiments were carried out in triplicates (n = 3) and the results are expressed as mean ± standard deviation (SD). Correlation coefficient (R value) was calculated based on the following formula

$$Correl(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}$$

2.5. Results

2.5.1. Isolation, fractionation and composition analysis of pectic polysaccharides from Swallow root and Black cumin

The pectic polysaccharides isolated from roots of Swallow root (SRPP) and seeds of Black cumin (BCPP) by ammonium oxalate extraction method yielded ~6% and 2% respectively. These polysaccharides were further fractionated on DEAE-cellulose ion exchange column, eluted with different molar solutions of ammonium carbonate (AC) and sodium hydroxide. The results show that both Swallow root and Black cumin polysaccharides yielded major fraction eluted with 0.15 M ammonium carbonate (Fig 2.1A & Fig 2.2A, Table 2.1) with the yield of 63% and 72% respectively.

Various fractions of SRPP and BCPP eluted on DEAE-cellulose column (0.05 M, 0.1 M, 0.15 M and 0.2 M ammonium carbonate as well as 0.1 M and 0.2 M sodium hydroxide) were examined for potential H^+, K^+ -ATPase inhibitory property. Activity was resided in 0.15 M fractions of SRPP and BCPP (as indicated by plus (+) symbol on top of the active peaks in Fig 2.1A & Fig 2.2A). Therefore, steps were taken to purify active fractions to subject detailed structural analysis.

Attempt to resolve 0.15 M-SRPP and 0.15 M-BCPP fractions on gel filtration column (Sepharose CL-4B) revealed the presence of only one peak indicating the homogenous nature of these polysaccharide fractions (Fig 2.1B & 2.2B). The results also revealed the molecular weight of ~700 kDa for SRPP and ~500 kDa for BCPP, calculated according to the calibration curve.

Fractions of 0.15 M-SRPP and 0.15 M-BCPP were further subjected to sugar composition analysis. SRPP shows arabinose (50%) and galactose (32%) as major sugar residues followed by rhamnose (16%) and xylose (2%) with uronic acid content of 141 mg/g. BCPP however, contained arabinose (42%), rhamnose (29%) and galactose (24%) as major sugar residues with less uronic acid content (30 mg/g) when compared to that of SRPP (Table 2.2).

2.5.2. FT-IR analysis of SRPP and BCPP

The FT-IR spectral analysis of SRPP and BCPP was performed employing an infrared spectrophotometer (Perkin – Elmer 2000 spectrophotometer). Fig. 2.3 and Table 2.3 depicts the IR spectral profiles of 0.15 M-SRPP and 0.15

M-BCPP in the frequency of 4000 – 400 cm^{-1}. Both the fractions showed the profile characteristic of pectic polysaccharide with absorption at 1731 & 1696 cm^{-1} which corresponds to ester carbonyl group stretch and; 829 & 835 cm^{-1} indicated α-conformation of pyranose ring (Zhao et al., 2007; Yashoda et al., 2005). The intense peak at 1102 cm^{-1} and 1101 cm^{-1} corresponds to galacturonic acid residues (Yashoda et al., 2005). The region between 1200 and 830 cm^{-1} showed several absorption peaks, characteristics of carbohydrates with C-C-O, C-H and C-O-C stretching modes indicative of pectic polysaccharide backbone (Prasanna et al., 2004).

Absorption peak at 2924 cm^{-1} indicates CH stretching associated with ring carbon atoms and skeletal mode absorption of the glycosidic linkage at 954 & 958 cm^{-1}. Absorption at 1610 & 1600 cm^{-1} indicates the presence of carboxylic groups (C=O). The intense peak at 1019 cm^{-1} of BCPP corresponds to arabinan side chain (Yashoda et al., 2005). A minor absorption at 1084 cm^{-1} appeared to be due to β-(1-6) and (1-3) linked galactans. IR spectral analysis thus indicates the nature of SRPP and BCPP as a polymer of pectic polysaccharide type.

2.5.3. Structural elucidation of SRPP by ^{13}C-NMR analysis

^{13}C-NMR spectrum of SRPP (Fig 2.4, Table 2.4) reveals signals characteristics of pectic polysaccharide. The spectrum gave strong signal at δ 100.56 which is characteristics of α–(1-4) linked galacturonic acid units representing polysaccharide backbone { -1,4)-α–D-Gal*p*A-(1- }. Signals at δ 82.34, 78.01, 80.67 and 67.34 can be assigned to ring carbons of C-2, C-3, C-4 and C-5 of α-L-Ara*f* unit, { -5)- α-L-Ara*f*-(1- }. A sharp signal at δ 109.27 corresponds to anomeric carbon (C-1) of α-L-Ara*f* unit. A signal at δ 75.34 can be assigned to C-5 of β-D-galactopyronosyl unit which is linked to α-L-rhamnopyranosyl unit at side chains, { -4)-β-D-Gal*p*-(1-4)-α-L-Rha*p* }. A signal at δ 73.06 is attributed to C-2 of β-D-galactopyronosyl unit branched from α-Gal*p*A backbone residue of the polysaccharide at O-6 position, { -4,6)-β-D-Gal*p*-(1- }. A sharp signal at δ 184.62 is due to –C=O of acetyl groups, which indicates that α–D-Gal*p*A of the polymer backbone is acetylated and is also a characteristic feature of pectic polysaccharide (Fig 2.6).

2.5.4. Structural elucidation of BCPP by ^{1}H-NMR analysis

^{1}H-NMR spectrum of BCPP (Fig 2.5 and Table 2.5) indicates that it is a rhamnogalacturonan type-I (RG-I) pectic polysaccharide. The main polysaccharide backbone is a repeating units of α–D-galacturonic acid and α–L-rhamnopyranosyl units which are linked by α-(1-2) and α–(1-4) linkages respectively { -4)-α–D-Gal*p*A-(1-2)- α–L-Rha*p*-(1- }, and this is clearly indicated by strong signals at δ 4.25 and 1.22 in ^{1}H-NMR spectrum (Polle et al., 2002; Zhao et al., 2007). Signals at δ 5.06 and 4.4 correspond to anomeric proton and H-4 respectively of α–D-Gal*p*A residue (Zhao et al., 2007). The ^{1}H-NMR spectrum also indicated that the α–L-rhamnopyranosyl residue of polymer backbone substituted at position 4 with various side chain residues such as arabinan and galactan with varied degree of polymerization.

The signal at δ 4.19 and 3.97 can be assigned to β-(1-4) linked galactopyranosyl residues, indicating galactan side chain. A signal at δ 3.73 can be assigned to H-5 of α–L-arabinofuranosyl residue which is linked to β-D-galactopyranosyl unit of galactan side chain. A sharp signal at δ 4.11 is attributed to H-2 of α-(1-5) linked arabinofuranose of arabinan side chain { -5)-α–L-Ara*f*-(1- }. The presence of galactan and arabinan side chains were strongly supported by FT-IR spectrum of BCPP showing intense absorption peaks at 893 cm^{-1} and 1019 cm^{-1} respectively (Fig 2.3B). A signal at δ 2.20 indicated that a backbone residue, α–D-Gal*p*A is acetylated at O-3 position (Zhao et al., 2007).

2.5.5. Structures of SRPP and BCPP

Overall the structural features revealed by column chromatography, sugar composition analysis, FT-IR and ^{13}C-NMR analysis indicates that the isolated component of Swallow root (SRPP) is a arabinogalacturonan type of pectic polysaccharide (Fig 2.6) with molecular weight of ~700 kDa, containing galacturonan (A) and arabinogalacturonan (B) chains in the backbone structure. SRPP contains arabinogalactan as a side chain (C). SRPP also contains galactan side chains (D) with few galactose residues substituted with rhamnose residues.

On the other hand, the isolated component of Black cumin is rhamnogalacturonan type-I pectic polysaccharide with molecular weight of

~500 kDa having rhamnogalacturonan backbone (A) with galactan (B) and arabinan (C) side chains with varied degree of polymerization as shown in Fig 2.7. Few galactan side chain of polymer is also substituted with one or few arabinose (D) residues indicating the presence of arabinogalactan side chain also.

2.5.6. Structural elucidation of HMBA

HMBA was isolated from fresh roots of Swallow root *(D. hamiltonii)* by hydrodistillation method with a yield of ~ 0.09%. HMBA was dissolved in ethanol and tested for purity by HPLC. Results showed the presence of a single peak at retention time of 48.41 min (Fig 2.8A), similar to that of standard HMBA. The identity of HMBA was confirmed by the mass spectral fragmentation pattern (m/z). The strong molecular ion peak (m/z, 152) and a stronger M-1 ion peak (m/z, 151) with % abundance of ~20% and 100% respectively were observed, which are characteristic of aromatic aldehydes (Nagarajan et al., 2001) (Fig 2.8B).

The FT-IR spectral analysis (Fig 2.8C) showed several absorption peaks between 1450 to 1600 cm^{-1} corresponds to C=C stretching of benzene ring. The C-H stretching at 2840 cm^{-1} and C=O stretching at 1637 cm^{-1} indicating aldehyde group. The absorption peak at 1022 cm^{-1} corresponds to C-O stretching indicates the hydroxyl group. The 1300 cm^{-1} absorption peak corresponds to C-O-CH_3 indicates methoxy position.

The two-dimensional NMR (^{1}H and ^{13}C) study of HMBA shows signals as in Fig 2.9, characteristic of HMBA. All these data suggest that the isolated compound indeed is 2-hydroxy-4-methoxy benzaldehyde – HMBA (Fig 2.9).

2.5.6. Structure-activity relationship

In vivo and *in vitro* ulcer preventive/healing activity of SRPP, BCPP and HMBA addressed in chapters 1, 3 & 4 of this thesis and antiulcer potential and sugar composition of ginger pectic polysaccharide from previous work carried out in our lab was taken for the establishment of structure–activity relationship.

Correlation coefficient was calculated with respect to various sugars present in the selected pectic polysaccharides and different bioactivities listed in Table 2.6. Data indicated that there is a good correlation between

arabinose/galactose/uronic acid and phenolics with ulcer healing ability, mucosal recovery, antiadhesive property as well as anti-*H. pylori* activity with R value of ~1. Rhamnose did not show correlation with any bioactivity except *H. pylori* inhibition with moderate R value of ~0.5.

Phenolics and uronic acid showed correlation with antioxidant activities such as free radical scavenging and reducing power abilities with R value of 0.7 to 0.99.

The observed data thus suggest that arabinogalacturonan structure with phenolics together may be responsible for ulcer healing, mucosal recovery, anti-adhesive and antioxidant activities. A moderate correlation, (R ~0.5) between rhamnose and anti-*H. pylori* activity as opposed to no correlation of this activity with other sugars may suggest that it is possible that rhamnose can inhibit *H. pylori* growth. High potency of uronic acid similar to that of phenolics may suggest that antioxidant property could be due to both phenolics and uronic acid content. Interestingly display of moderate correlation to reducing power ability (R ~0.7) by arabinose and galactose and poor correlation with free radical scavenging activity suggest that by virtue of reducing power ability only antioxidant nature is executed.

Observed data is substantiated by overall increased antioxidant/ulcer healing properties of SRPP which has a typical arabinogalacturonan structure with enriched phenolic content as opposed to that of BCPP as evidenced by total phenol estimation. Further, rhamnose showing moderate correlation with anti-*H. pylori* activity suggest that rhamnose somehow by interacting with *H. pylori* surface molecules may inhibit *H. pylori* growth/H^+, K^+ -ATPase activity and; antioxidant activity could be due to phenolics which are covalently bound to SRPP and BCPP. Thus this chapter enabled us to understand the precise role of carbohydrate and phenolics in preventing gastric ulcers.

It is also intriguing to observe that potent *H. pylori* inhibition by SRPP and BCPP could probably due to antioxidant effects only. Thus antioxidant component in SRPP and BCPP could be responsible for anti-*H. pylori* activity, which in turn protect against *H. pylori* induced gastric ulcers.

Overall data may thus suggest that rhamnogalacturonan type-I pectic polysaccharide found in BCPP and arabinogalacturonan type pectic

polysaccharide found in SRPP may show potential ulcer prevention/healing, mucosal recovery, inhibition of *H. pylori* growth and H^+, K^+ -ATPase activity, and antioxidant activities.

Although with varied degree, significant correlation of phenolics and uronic acid content for antioxidant activity and H^+, K^+ -ATPase inhibitory activity may suggest that negatively charged uronic acids may also participate in H^+, K^+ -ATPase inhibitory property. Thus pectins with phenolics may help in multi-step prevention and healing of ulcers.

With regard to HMBA, it has shown poorer antioxidant activity with an IC_{50} of 213 µg/mL as opposed to a potent antioxidant gallic acid, which showed free radical scavenging at IC_{50} of 1.1 µg/mL. However HMBA exhibited potent H^+, K^+ -ATPase and *H. pylori* inhibition similar to or little less than that of respective standard such as cinnamic acid.

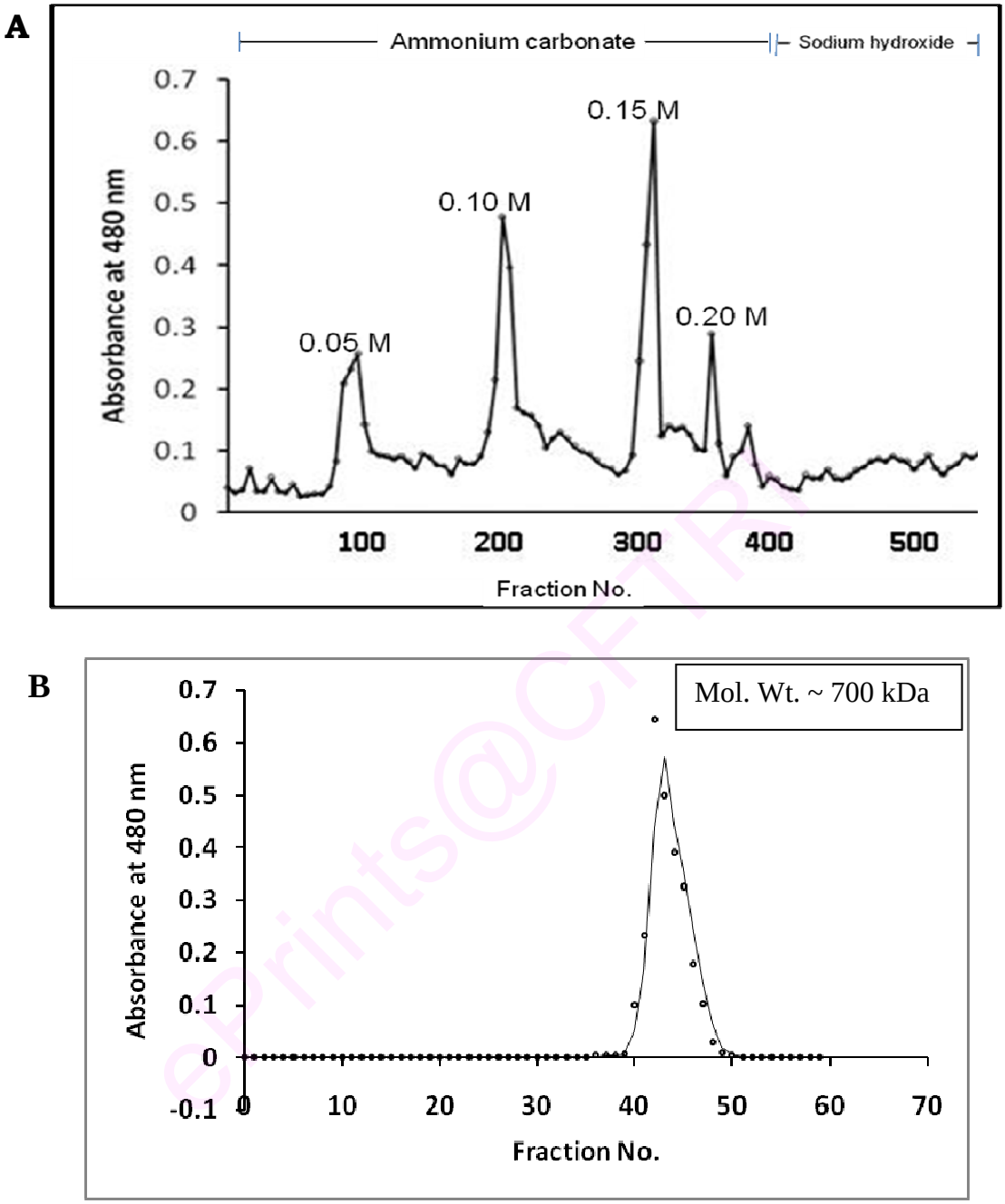


Fig 2.1. Elution profile of SRPP on DEAE-cellulose (A) and Sepharose CL-4B (B) columns: SRPP was fractionated on DEAE-cellulose column (A), eluted with different molar solutions of ammonium carbonate and sodium hydroxide. Fractions (5 mL/tube) were assayed for carbohydrate content by phenol-sulfuric acid method as well as for H^+, K^+ -ATPase inhibitory activity. Presence or absence of the H^+, K^+ -ATPase inhibitory activity is represented as (+) or (-) on top of the each peak. Only 0.15 M fraction showing H^+, K^+ -ATPase inhibitory activity was tested for homogeneity and determination of molecular weight on Sepharose CL-4B column (B).

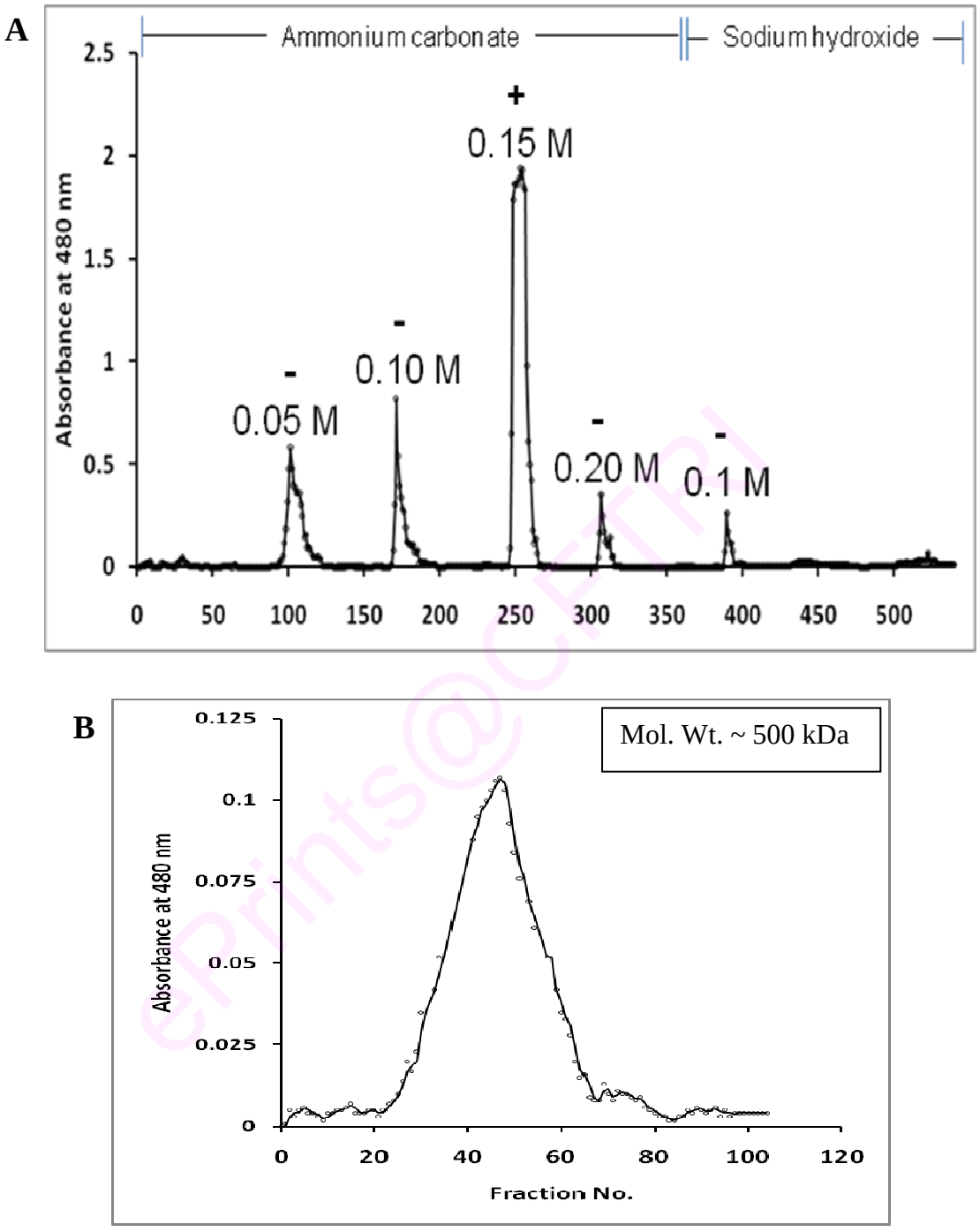


Fig 2.2. Elution profile of BCPP on DEAE-cellulose (A) and Sepharose CL-4B (B) columns: BCPP was fractionated on DEAE-cellulose column (A), eluted with different molar solutions of ammonium carbonate and sodium hydroxide. Fractions (5 mL/tube) were assayed for carbohydrate content by phenol-sulfuric acid method as well as for H^+, K^+ -ATPase inhibitory activity. Presence or absence of the H^+, K^+ -ATPase inhibitory activity is represented as (+) or (-) on top of the each peak. Only 0.15 M fraction showing H^+, K^+ -ATPase inhibitory activity was tested for homogeneity and determination of molecular weight on Sepharose CL-4B column (B).

		Relative % abundance	
	Fractions	**SRPP**	**BCPP**
Ammonium carbonate	0.05 M	11	13
	0.1 M	25	4
	0.15 M	63	72
	0.2 M	1	7
Sodium hydroxide	0.1 M	0	4
	0.2 M	0	0

Table 2.1. Relative percent abundance of polysaccharide fractions separated on DEAE-cellulose column.

Pectic polysaccharides	**Rham**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**Uronic acid (mg/g)**	**% Yield of polysaccharide**
SRPP	16	50	2	-	32	-	141	6
BCPP	29	42	2	0	24	3	30	2

Table 2.2. Relative percent sugar composition of pectic polysaccharides

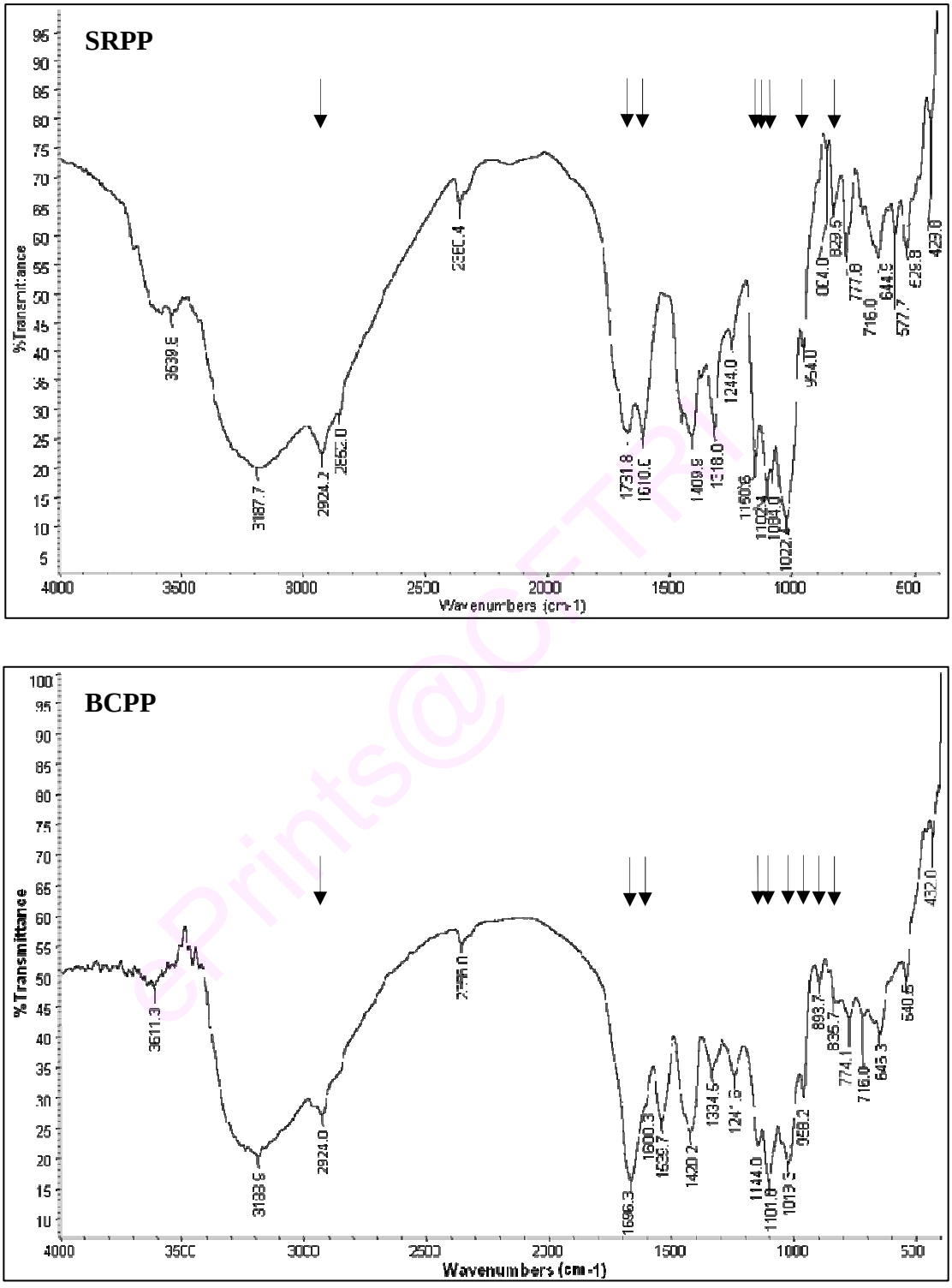


Fig 2.3. FT-IR spectrum of SRPP (A) and BCPP (B).

SRPP (Frequency cm^{-1})	**BCPP (Frequency cm^{-1})**	**Characteristic features**
2924 cm^{-1}	2924 cm^{-1}	CH stretching associated with carbon atoms
954 cm^{-1}	958 cm^{-1}	Glycosidic linkage, high galactose
-	893 cm^{-1}	β – glycosidic linkage between neutral sugar residues in the side chain galactan
1084 cm^{-1}	-	β- (1-6) and β- (1-3) linked galactan
1102 cm^{-1}	1101 cm^{-1}	Galacturonic acid residues
	1019 cm^{-1}	Arabinan side chain
1731 cm^{-1}	1696 cm^{-1}	Ester carbonyl group
1610 cm^{-1}	1600 cm^{-1}	Presence of carboxyl group
829 cm^{-1}	835 cm^{-1}	α-confirmation
800-1200 cm^{-1}	800-1200 cm^{-1}	Highly coupled C-C-O, C-H and C-O-C stretching modes indicative of pectin back bone
1150 cm^{-1}	1144 cm^{-1}	Sulfonamide group

Table 2.3. FT-IR Spectroscopic analysis and characteristic features of SRPP and BCPP.

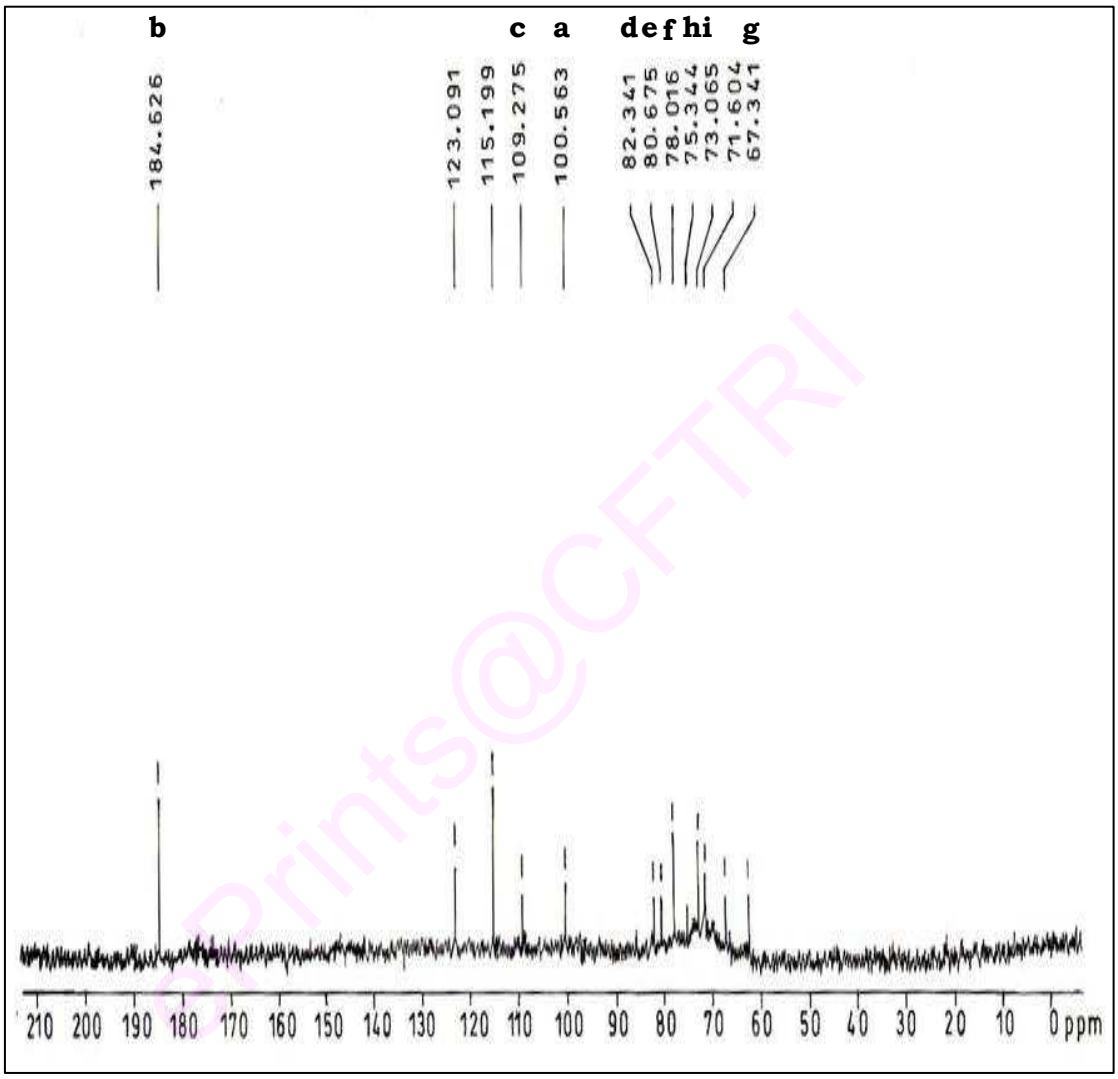


Fig 2.4. ^{13}C-NMR spectrum of SRPP

	^{13}C-NMR chemical shift (signals in ppm)	**Characteristic features**	**Reference**
a	100.56	-1,4)-α–D-Gal*p*A-(1-, Represents backbone residue	Polle et al., 2002
b	184.62	Acetylated α–D-Gal*p*A residues (-C=O of acetyl group)	Prasanna et al., 2004
c	109.27	Anomeric carbon (C-1) of α 1-4 linked Ara*f* units {-5)- α-Ara*f*-(1-4}	Polle et al., 2002
d, e, f, g	82.34, 80.67, 78.01 and 67.34	Assigned to C-2, C-4, C-3 and C-5 of α-Ara*f* unit { -5)- α-L-Ara*f*-(1- }	Polle et al., 2002
h	75.34	Corresponds to C-5 of β-D-Galactopyronosyl unit linked to α-L-Rhamnopyranosyl unit at side chains { -4)-β-D-Gal*p*-(1-4)-α-L-Rha*p* }	Polle et al., 2002
i	73.06	Attributed to C-2 of β-D-Galactopyronosyl unit branched from α-Gal*p*A backbone residue at O-6 { -4,6)-β-D-Gal*p*-(1- }	Polle et al., 2002

Table. 2.4. Characteristic features of ^{13}C-NMR spectrum of SRPP

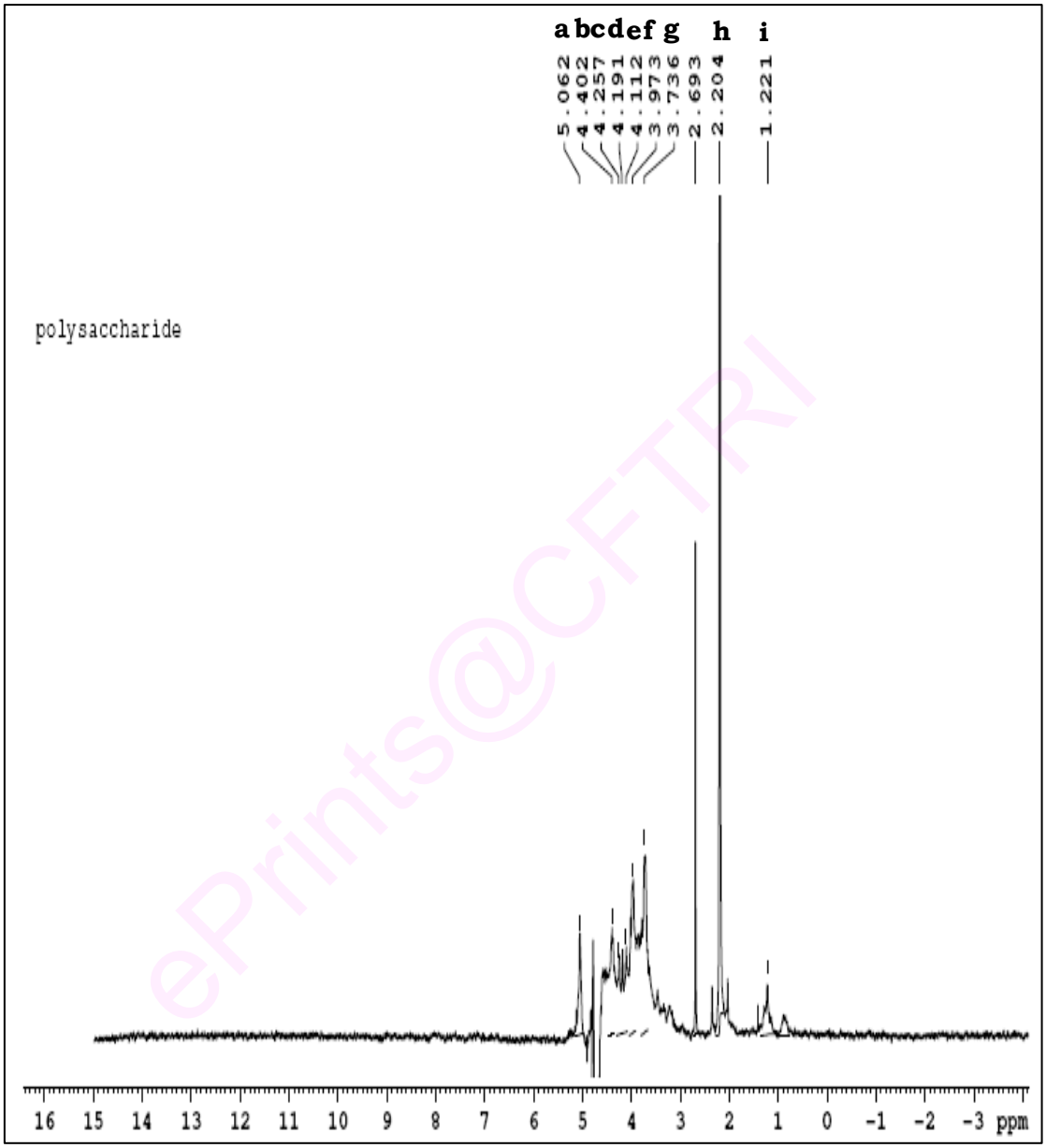


Fig 2.5. ^{1}H-NMR spectrum of BCPP

	^{1}H-NMR chemical shift (signals in ppm)	**Characteristic features**	**Reference**
a	5.06	Anomeric proton of α–D-Gal*p*A	Zhao et al., 2007
b	4.40	Represents H-4 of α–D-Gal*p*A	Zhao et al., 2007
c	4.25	-4)-α–D-Gal*p*A-(1-2)- α–L-Rha*p*-(1- , Represents repeating units of backbone of rhamnogalacturonan type I polymer	Polle et al., 2002
d	4.19	Corresponds to H-4 of -4)-β-D-Gal*p*-(1-	Polle et al., 2002
e	4.11	Corresponds to H-2 of -5)-α–L-Ara*f*-(1- (Arabinan side chain)	Polle et al., 2002
f	3.97	H-4 of β-D-Gal*p*-(1-4	Polle et al., 2002
g	3.73	H-5 of -2)- α–L-Ara*f*-(1-	Polle et al., 2002
h	2.20	Represents O-acetyl group	Zhao et al., 2007
i	1.22	H-6 of -2)-α–L-Rha*p*-(1- represents backbone residue of rhamnogalacturonan type I polymer	Zhao et al., 2007

Table. 2.5. Characteristic features of ^{1}H-NMR spectrum of BCPP

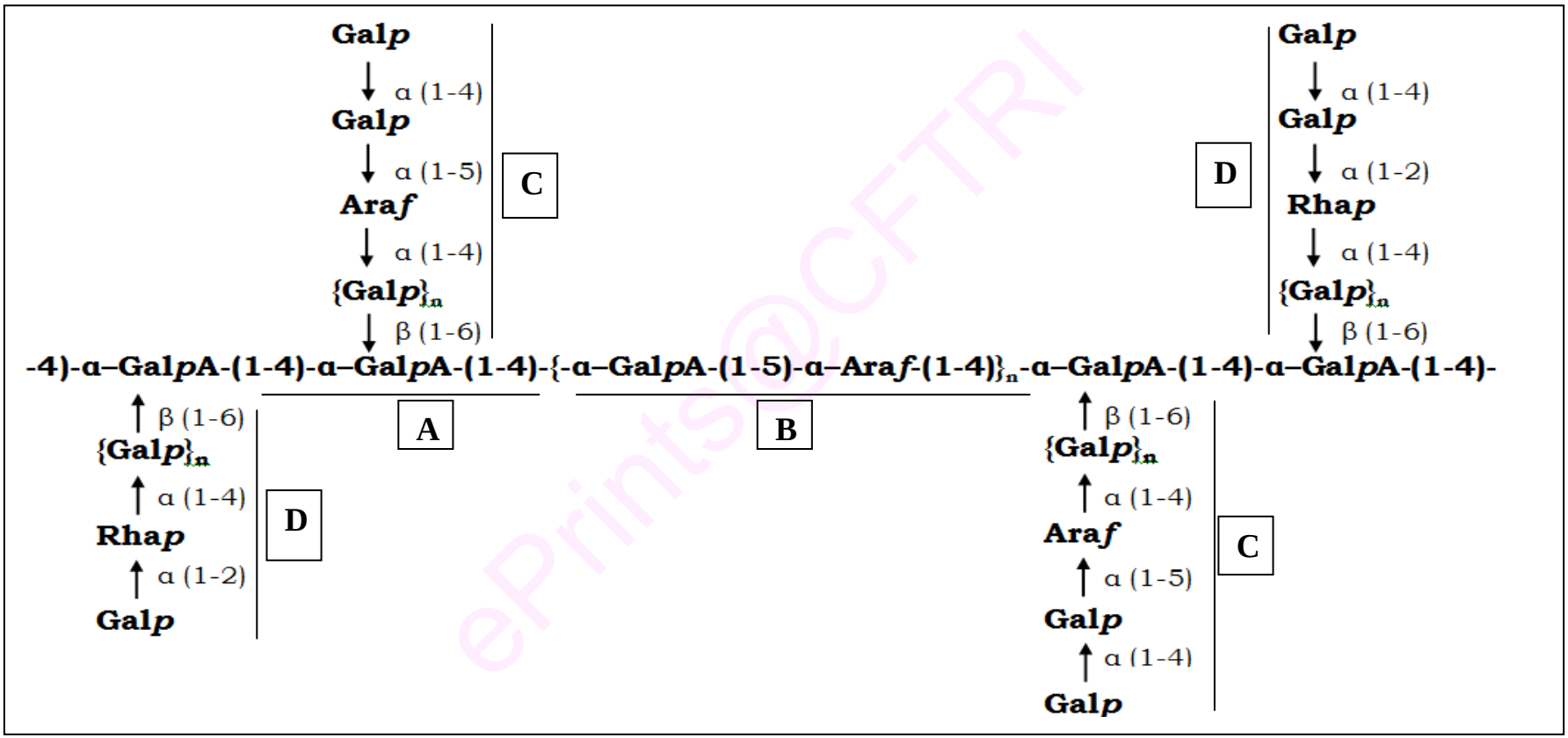


Fig 2.6. Tentative structure of SRPP

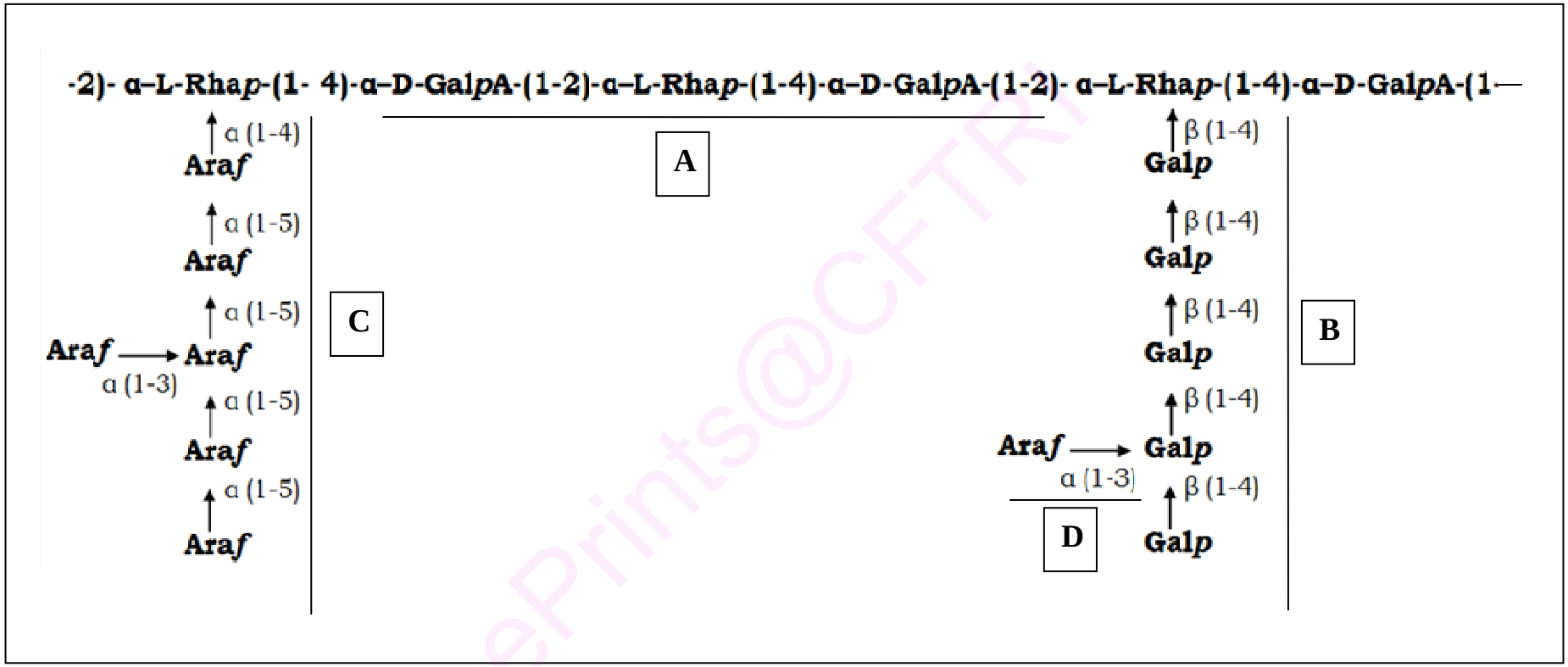


Fig 2.7. Tentative structure of BCPP

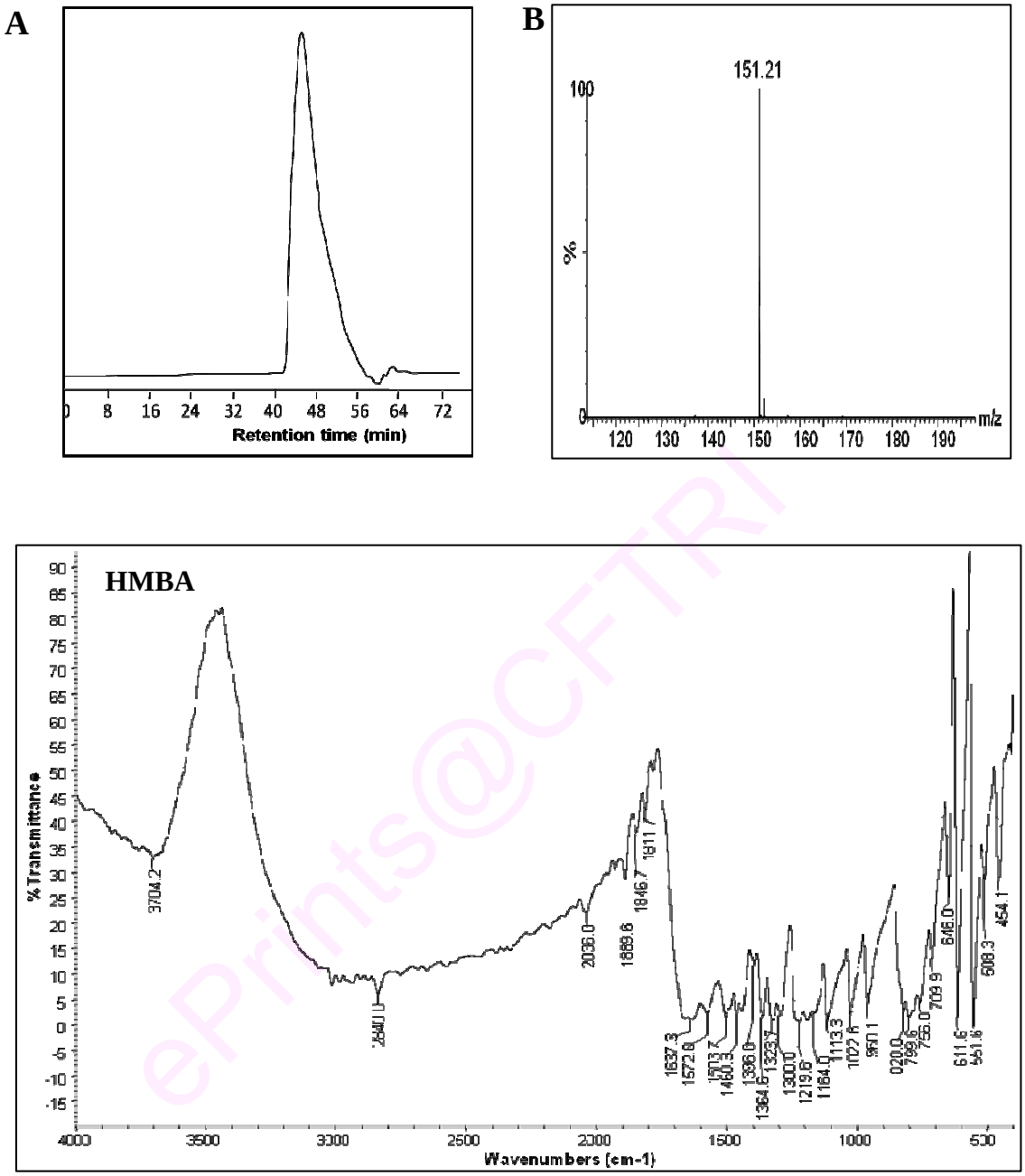


Fig 2.8. Characterization of HMBA: **Fig A** Showing HPLC profile with a single peak at 48.41 min retention time corresponds to HMBA. **Fig B** – ESI-MS profile of HMBA. **Fig C** showing FT-IR spectrum of HMBA with characteristic absorption peaks for C=C stretching of benzene ring (1450 – 1600 cm^{-1}), C-H stretching (2840 cm^{-1}), C=O stretching (1637 cm^{-1}), C-O stretching (1022 cm^{-1}) and C-O-CH_3 stretching (1300 cm^{-1}).

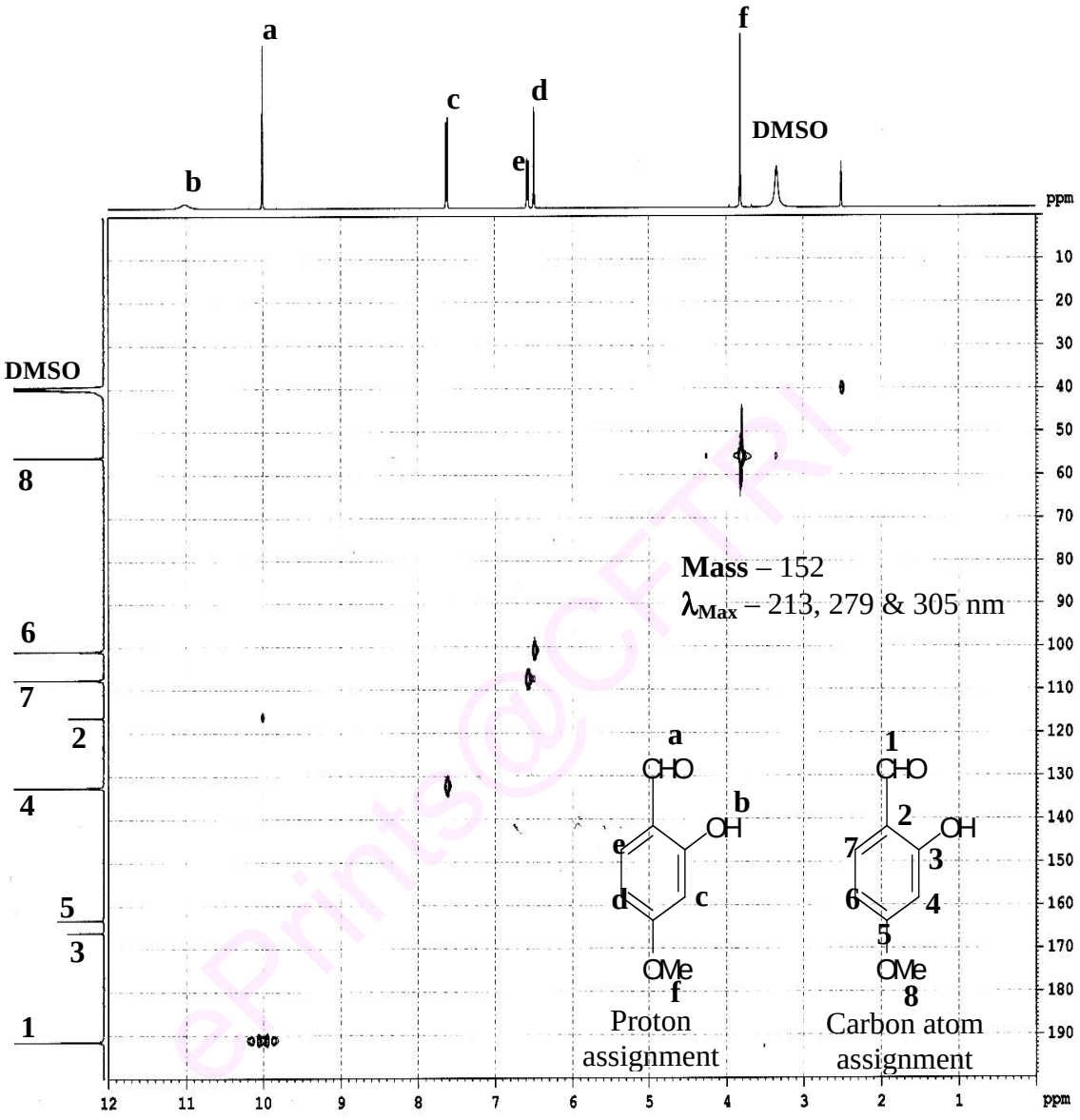


Fig 2.9. Two-dimensional NMR spectrum of 2-hydroxy-4-methoxy benzaldehyde (HMBA).

Activities		**SRPP**	**BCPP**	**GRPP ***	**HMBA**
% Ulcer prevention		80	73	95	64
% Ulcer healing		90	80	Not tested	Not tested
% Mucin recovery	Ulcer prevention	87	85	96	66
	Ulcer healing	99	90	Not tested	Not tested
ATPase (IC_{50} - µg/mL) Lansoprazole 19.3 µg/mL		77	170	27.2	49
Anti-*H. pylori* (MIC - µg/mL) Amoxicillin 26 µg/mL		150	Nil	60	39
Anti-adhesive (MIC - µg/mL)		49.5	90.0	Not tested	Nil
Phenol (mg/g)		120	26	5.76	>95%
DPPH (IC_{50}- µg/mL) Gallic acid 1.1 µg/mL		40	432	205	213
Reducing power units (U/g)		3200	1152	987	Not tested

Table 2.6. Comparison of antiulcer activities of pectic polysaccharides of different sources obtained from *in vitro* and *in vivo* studies (Chapter 1, 3 & 4).

*For the sake of correlation studies, GRPP (Ginger pectic polysaccharide) data was taken from Siddaraju M.N., Ph.D. Thesis, 2008, submitted to University of Mysore, Mysore.

2.6. Discussion

Ulcer is a major disease of concern in recent days due to several changes in the lifestyle. Stress, increased exposure to pollutants/infectious agents, inadequate intake of healthy foods, constant intake of drugs to subside the encountering problems, excess intake of alcohol etc., results in gastric irritation, gastric mucosal damage and ulcers which at times can be fatal (Miller 1987; Langman et al., 1991; Ernst & Gold, 2000).

Attempts are being made to develop drugs that can neutralize the gastric acidity, repair the damaged mucosal layer etc. However, long term use of these synthetic drugs has been shown to cause severe gastric disturbances and gastric acidity and associated problems by itself (Cowan, 1999). As highlighted in Chapter 1, attention is being given to the use of herbal/dietary components as nutraceuticals, to overcome the problems mentioned. Although substantiating amount of work has been put already in this area of exploring the knowledge of traditional medicine and to elucidate the scientific basis for the beneficial effects of such compounds in predicting its efficacy, it was posing problems in popularizing such medicines amongst the growing population who are either susceptible for ulcers or the victims of ulcers. In chapter 2 therefore, we undertook detailed study on identification and characterization of potential antiulcer and ulcer healing compounds from selected dietary sources and the results output clearly provide a greater hopes towards the use of such compounds as safer, effective, non-toxic and beneficial sources to combat the existing, continuing and increasing problem of gastric ulcers. Use of traditional medicine for symptoms control although was not uncommon, predictability and reliability needed to be established at molecular level in order to reemphasize the use of such sources as a simple strategy to cure or prevent complex disease conditions like ulcer.

Studies thus focused towards the isolation and characterization of pectic polysaccharides from Swallow root (SRPP) and Black cumin (BCPP) based on the justification provided in chapter 1. It was predicted that compounds possessing antioxidant, H^+, K^+ -ATPase/*H. pylori* inhibitory activities acted as effective combination to control gastric ulcer. In the current chapter

therefore, pectic polysaccharides from Swallow root (SRPP), Black cumin (BCPP) and one of the major components of Swallow root – 2-hydroxy-4-methoxy benzaldehyde (HMBA), which was not exhibiting antioxidant property but showed potentials to protect against *H. pylori* and ulcers, were isolated, purified and active fractions were subjected to characterization studies to understand their role in preventing or curing ulcers.

SRPP and BCPP showing potential *in vitro* antiulcer properties were subjected to purification and characterization. Both SRPP and BCPP were chromatographed on DEAE cellulose column chromatography and fractions obtained were examined for the activity. Attempts were also made to interpret the structure-function relationship so that the reliability of functioning of these polysaccharides against gastric ulcers could be explored.

Detailed structural analysis revealed that, SRPP is an arabinogalacturonan type pectic polysaccharide with a molecular mass of ~ 700 kDa, while BCPP appeared to be rhamnogalacturonan type-I pectic polysaccharide with molecular mass of ~ 500 kDa . Results are supported by the work of Masato et al., (1994), where they patented a rhamnan homopolysaccharide, rhamnose or a rhamnose oligomer as an active antiulcer component. It is intriguing to observe that rhamnan was also isolated from surface of Bifidobacterium (Masato et al., 1994) and this has been known to offer beneficial properties including ulcer healing properties in the gut. However, the mechanism of action was not clearly elucidated. In this scenario, it is gratifying to observe the presence of similar components from dietary sources which are safer to consume, in this case it is Black cumin. Studies clearly show the effective healing ability of BCPP as stated in chapter 3 and BCPP had the capability to modulate the signaling cascade to trigger mucosal cell proliferation, production of gastric mucin and reorganizing ability of damaged mucosal layer in a desirable manner so that it can function normally and to evade the disease pathogenicity and pathogenicity-associated complications.

SRPP has been deduced as an arabinogalacturonan type of pectic polysaccharide (Fig 2.6), containing galacturonan and arabinogalacturonan

chains in the backbone structure with galactan and arabinogalactan side chains.

Predominant levels of uronic acid (141 mg/g), arabinose (50%) and galactose (32%) in SRPP justifies the structure (Table 2.2). Evidences have been provided from FT-IR and NMR structural studies. SRPP also contained higher levels of phenolics (120 mg/g) suggesting that both phenolics as well as carbohydrates may play a significant role in the bioactivity observed as per Table 2.6. Thus this novel phenol-bound pectic polysaccharide of Swallow root (SRPP) prevented and healed ~80 to 90% of gastric ulcers in rats at 200 mg/kg b.w. concentration. The fact that it showed significant mucosal recovery (~80-99%) revealed that, SRPP by virtue of negative charges may bind to gastric mucin, an enveloping layer found on the gastric epithelium.

BCPP (Fig 2.7 and Table 2.5) on the other hand is a rhamnogalacturonan type-I (RG-I) pectic polysaccharide. The main polysaccharide backbone is a repeating units of α–D-galacturonic acid and α–L-rhamnopyranosyl units. Arabinan and galactan were found as a side chains with few galactose units of galactan side chain substituted with arabinose indicating the presence of arabinogalactan also as a side chain component. The composition analysis of BCPP (Table 2.2) revealed rhamnose (29%), arabinose (42%) and galactose (24%) as major sugar residues with uronic acid content of 30 mg/g and phenolics of 26 mg/g (Table 2.6).

In other words although both SRPP and BCPP differed in their structure, both of them exhibited significant prevention and healing of ulcers (~70 to 90%). Almost similar levels (~80 to 90%) of mucosal protection may suggest that ulcer protection could be by preventing mucosal damage which could be by attack of free radicals. Similar levels of mucoprotective activity, although ~10 folds differences in antioxidant activity between SRPP and BCPP may suggest that mucosal cell protection could be by routes other than antioxidative mechanism also. Alternatively, it is possible that having pectins with polygalacturonic acid in both the cases may exhibit negative charge typical of a pectic polysaccharide and hence may bind strongly to the positively charged mucosal layer (positive charge due to enhanced

aminosugars). Thus carbohydrate-carbohydrate interaction may have provided an enveloping of ingested pectic polysaccharides - SRPP/BCPP in an experimental animal, which may enable the protection to mucosal layer against free radicals or acid induced mucosal damage. Effective healing of ulcers in acetic acid induced ulcer model also substantiates the fact that, more of mucoprotective than antioxidative mechanism may enunciate ulcer healing. Studies substantiate the observation made by Matsumuto et al., (2002) who provided evidence for the first time, the antiulcer effect of a *Bupleurum falcatum L.* pectic polysaccharide. As highlighted in the study, protective activity against a wide variety of experimental gastric mucosal lesions could be due to (a) reinforcement of resistance of the mucosal barrier by a protective coating of polysaccharide; (b) anti-secretory activity on acid or pepsinogen and (c) the prevention or scavenging of free radicals which are generated during initiation of ulcer pathogenicity by an ulcerogen. We have provided evidence for the first time the effective H^+, K^+ -ATPase inhibitory effect both by SRPP and BCPP which substantiates the antisecretory role of SRPP/BCPP. Therefore the combination effect of both reinforcement of resistance of the mucosal barrier and the reduction of aggressive factors might be involved in ulcer prevention as well as during ulcer healing.

It is interesting to observe that despite so many similarities in structure of SRPP/BCPP with *B. falcatum* polysaccharide, notable difference were also found to exist. SRPP and BCPP as depicted in chapter 3 showed potent PGE_2 triggering (Fig 3B.6 and 3B.7 of chapter 3), which in turn has been believed to be the underlying mechanism for cytoprotection and mucosal cell proliferation that is required for repair of mucosal damage. *B. falcatum* however was reported to lack this property. Taking information from the action of sulfated mucopolysaccharide of animal origin, heparin that had the ability to stimulate PGE_2 (Li et al., 1998 & 1999) and no PGE_2 stimulation by *B. falcatum* pectic polysaccharide (Matsumuto et al., 2002) which did not encounter the presence of sulfate group, it may be presumed that sulfated polysaccharide domain may be important for PGE_2 stimulatory activity. Presence of sulfated groups as evidenced by FT-IR signals in both SRPP and BCPP may justify this statement (Fig 2.3, Table 2.3).

Further *H. pylori* being a major ulcerogen it was essential to determine the potentiality of SRPP/BCPP in inhibiting *H. pylori* growth and colonization with respect to their structures. It should be notified here that SRPP although could inhibit the growth of *H. pylori* at 150 µg/mL and as evidenced by scanning electron microscopic data (Fig 1.7 of chapter 1), BCPP did not show growth inhibitory effect. Further, inhibition of *H. pylori* adhesion to gastric mucin, an important interaction required for *H. pylori* invasion into the mucosal layer was inhibited effectively both by SRPP and BCPP (Fig 4.5 of chapter 4), although two folds increased activity was observed in SRPP than BCPP. Data is intriguing and suggests that, there is a definite prerequisite for inhibition of *H. pylori* growth and cell adhesion. Differential interaction between *H. pylori* surface molecule with SRPP or BCPP which could be due to differences in the structure may be responsible for the activity. Also differential activity in two different assays between two sources - SRPP and BCPP may reveal that SRPP with arabinogalacturonan type may interact better than rhamnogalacturonan type-I (RG-1). *H. pylori* inhibition in the growth inhibition assay by SRPP alone could be by binding of SRPP-arabinogalacturonan core or arabinogalactan side chain structure to *H. pylori* thereby not allowing the proliferation. RG-I of BCPP may not bind to *H. pylori* surface molecule that much effectively. Data thus suggest a stringent requirement of the precise and compatible domain to bind to *H. pylori* in order to execute the action of *H. pylori* inhibition by SRPP and BCPP respectively. Data also suggest that SRPP with arabinogalacturonan structure may be atleast 2 folds better than BCPP.

In other words this chapter provides evidence for the first time that a RG type-I structure depicted for antiulcer activity in BCPP may inhibit ulceration via inhibition of gastric H^+, K^+ -ATPase and exhibiting antioxidant activity rather than inhibiting *H. pylori* growth (Table 2.6). It is pertinent to mention here that Bifidobacterium rhamnan offer protection to gastric ulcers (Masato et al., 1994). It is clear however by the current study that rhamnan type although found to be present in Bifidobacterium, may not protect the system against *H. pylori*. Thus the gastroprotective effect could be through a different route.

Antioxidant activity yet another activity exhibited by polysaccharide has been shown to be essential for antiulcer activity. SRPP exhibiting better free radical (~10 folds) and reducing power (~2 folds) ability than BCPP may also add to the increased potency of SRPP. Antioxidant activity was attributed to the phenolics and nature of phenolics which are covalently bound to the pectic polysaccharide. Indeed antioxidant activity of SRPP and BCPP is correlated to the total phenolic content.

In vivo antioxidant effect of SRPP/BCPP and their potential ability to inhibit malondialdehyde - an oxidative stress marker accumulation and normalization of antioxidant and antioxidant enzyme levels that were depicted during ulceration by SRPP and BCPP upon their feeding to rats confirms the antioxidant potencies and the bioavailability of such pectic polysaccharides (Table 3A.5, 3A.6, 3A.7, 3B.1, 3B.2 of chapter 3).

It has been assumed widely that polysaccharides are not absorbed from the digestive tract because of their high molecular weight. However, Sakurai et al., (1996) demonstrated that the antiulcer polysaccharide, bupleuran 2IIc can be detected in the liver by using a specific antibody after oral administration of BR2 to mice, and these results suggested that at least part of the orally administered bupleuran 2IIc was incorporated in to the circulation. The size of the polysaccharides as well as their characteristic monosaccharide composition may seem to modulate biological activity in a differential manner. Paulsen and Barsett (2005) pointed out that most of these molecules presented a typical rhamnogalacturonan type-I backbone. They generally possessed arabinogalactan II side chain, while a few of them were found with arabinogalactan type 1 chains. Then, it can be ascertained that the structure and the variability of rhamnogalacturonan type-I side chains are the most important structural features that could modulate the bioactivity of pectins.

In conclusion, our results suggest that the pectic polysaccharides from Swallow root and Black cumin may be responsible for antiulcer activity and like *B. falcatum* pectic polysaccharide, SRPP and BCPP may function against ulcers both by reinforcement of resistance of the mucosal layer and by the reduction of aggressive factors. Data thus supports the use of herbal

prescriptions containing Swallow root and Black cumin for the treatment of gastric ulcers.

Regarding structure-activity relationship of HMBA, it is intriguing to note that *D. hamiltonii* has come out as one of the best antiulcer sources. Traditional knowledge has indicated that hot water decoction of this root has been used as a remedy for stomach pain, burning and other gastric disturbances. Looking for components in aqueous extracts of Swallow root, highlighted the abundance of HMBA in addition to other phenolics (Chapter 1).

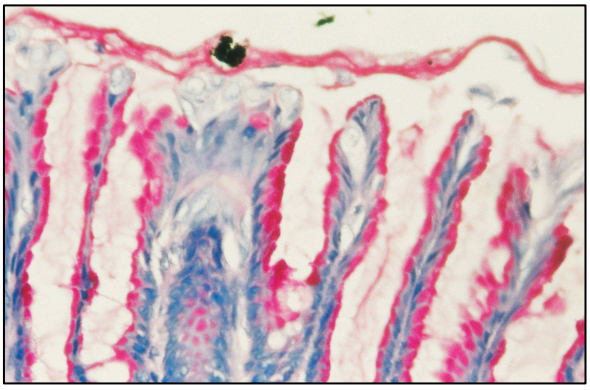
Benzaldehyde moiety with hydroxyl group is responsible for antioxidant property. However poorer antioxidant property has been attributed to the substitution of -OCH3 group instead of hydroxyl group at para position. Further potent H^+, K^+ -ATPase/*H. pylori* inhibitory properties similar to that of cinnamic acid may be attributed to the hydrophobic nature of the compound that can interact well with membrane domains of H^+, K^+ -ATPase enzyme and *H. pylori* as indicated in our earlier paper (Nanjundaiah et al., 2009). Detailed structure-activity relationship of HMBA with various cytotoxic actions of *H. pylori* that could happen during ulcerations is described in chapter 4. It also had shown inhibition of gastric ulcer together with significant mucin protection substantiating the significant antiulcer property of HMBA.

2.7. Summary and conclusions

- Chapter 2 addresses isolation, purification and characterization of potential antiulcer compounds form Swallow root and Black cumin.
- Pectic polysaccharides of Swallow root (SRPP) and Black cumin (BCPP) and a predominant bioactive phenolic component (HMBA) were selected for structure-function studies.
- SRPP and BCPP were fractionated and the active fraction (0.15 M ammonium carbonate) was passed through sepharose CL-4B column; confirmed the homogeneity and determined the molecular weights as 700 kDa and 500 kDa for 0.15 M-SRPP and 0.15 M-BCPP respectively.
- Interpretation of biochemical and biophysical tests indicated that SRPP is an arabinogalacturonan type polysaccharide with galacturonan and arabinogalacturonan chains in the backbone and arabinogalactan unit in the side chain; while BCPP exhibited a typical rhamnogalacturonan type-I structure with rhamnogalacturonan unit in the backbone and arabinan and galactan units in the side chains.
- The structure of major phenolic compound of Swallow root is confirmed as 2-hydroxy-4-methoxy benzaldehyde (HMBA) by HPLC, FT-IR, ESI-MS and NMR studies.
- Structure-activity relationship indicates that, H^+, K^+ -ATPase and *H. pylori* growth inhibitory potency is due to hydrophobic nature of HMBA, unlike SRPP/BCPP although it showed increased potency in inhibiting H^+, K^+ -ATPase (IC_{50} 49 µg/mL) and *H. pylori* growth (MIC 39 µg/mL). However, HMBA could not inhibit *H. pylori* adhesion to mucosal membrane suggesting role of polysaccharides as antiadhesives.
- Together, HMBA, SRPP and BCPP exhibited different antiulcer potential with varied potency and has been attributed to characteristic structures derived. Arabinogalacturonan structure of SRPP appears to be more active than rhamnogalacturonan type-I of BCPP in exhibiting potential antiulcer activity.

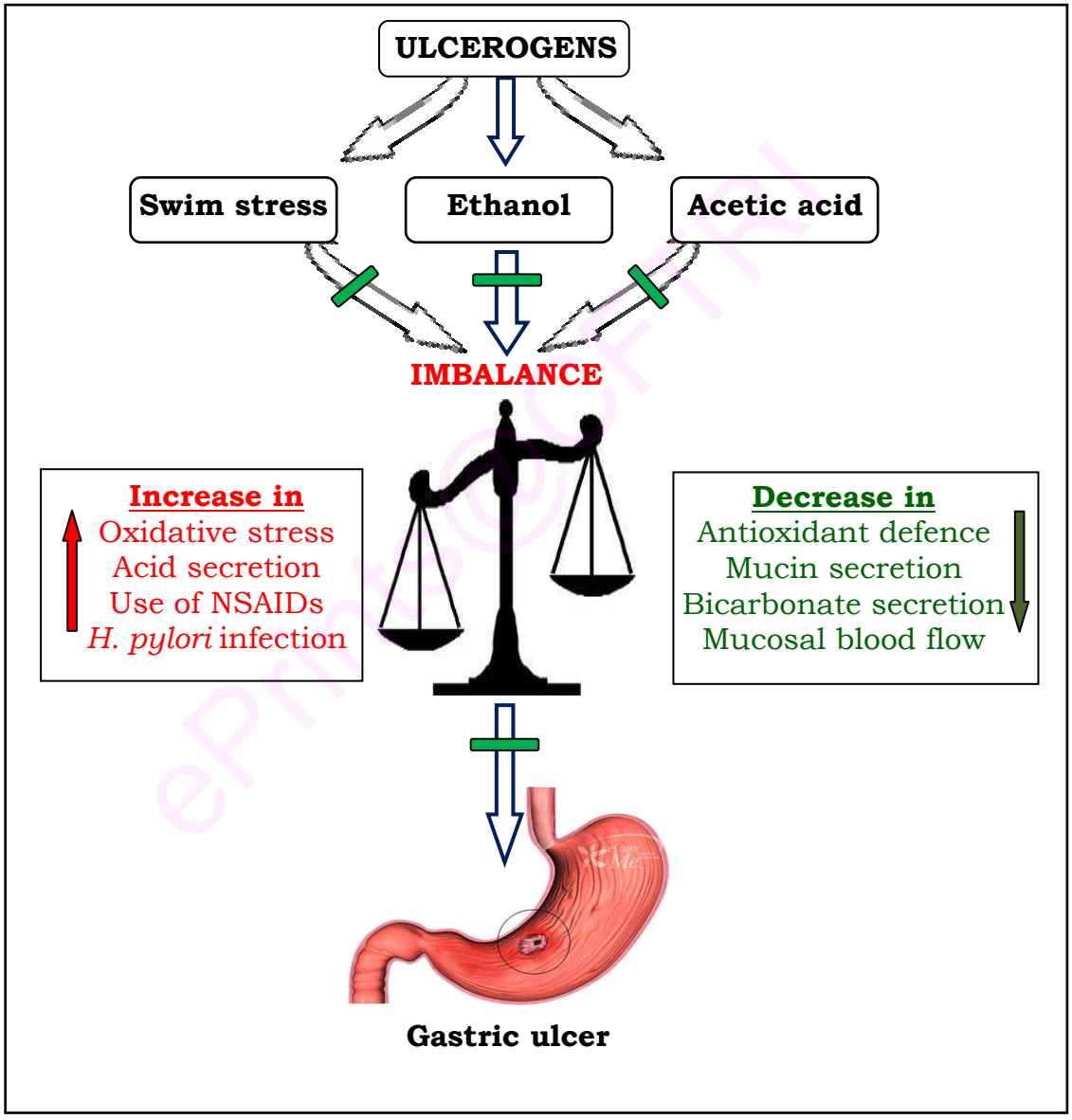
CHAPTER 3

Determination of *in vivo* antiulcer potency and mechanism of action of active antiulcer compounds

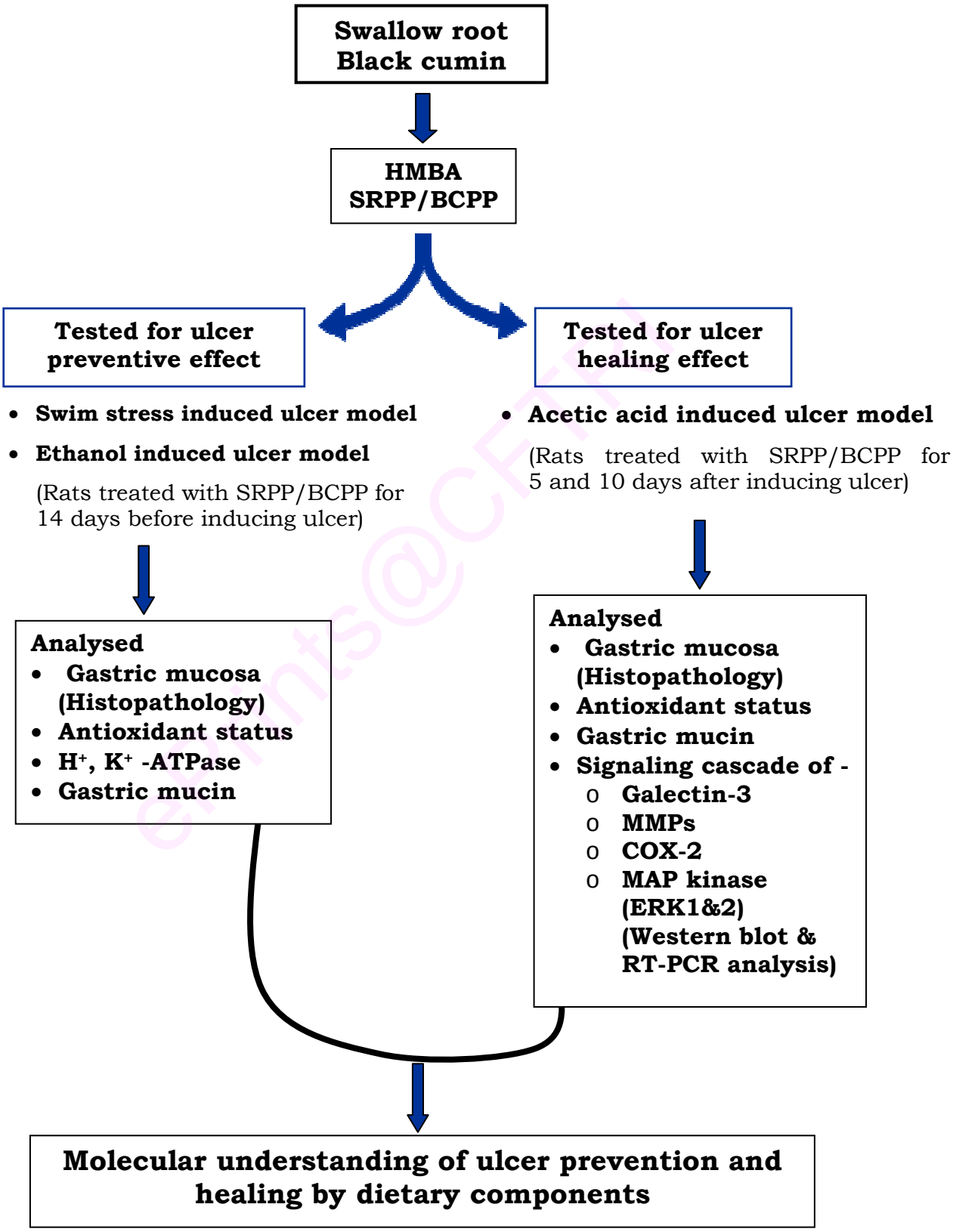


3.1. Hypothesis

Can selected dietary components prevent and heal gastric ulcers ?



3.2. Work plan


Swallow root
Black cumin
HMBA
SRPP/BCPP
Tested for ulcer preventive effect
Tested for ulcer healing effect
Swim stress induced ulcer model
Ethanol induced ulcer model
(Rats treated with SRPP/BCPP for 14 days before inducing ulcer)
Acetic acid induced ulcer model
(Rats treated with SRPP/BCPP for 5 and 10 days after inducing ulcer)
Analysed
Gastric mucosa (Histopathology)
Antioxidant status
H+, K+ -ATPase
Gastric mucin
Analysed
Gastric mucosa (Histopathology)
Antioxidant status
Gastric mucin
Signaling cascade of -
Galectin-3
MMPs
COX-2
MAP kinase (ERK1&2) (Western blot & RT-PCR analysis)
Molecular understanding of ulcer prevention and healing by dietary components

3.3. Introduction

Gastric ulcers develop due to loss of the delicate balance between gastro-protective and aggressive factors. Reduction in gastroprotective factors such as mucus, bicarbonate secretion, prostaglandins and gastric mucosal blood flow and; enhancement of aggressive factors like increase of acid/pepsin secretion and, *Helicobacter pylori* infection results in gastric ulceration (Miller, 1987; Baragi et al., 1997; Adam & Kris, 1999; Ernst & Gold, 2000; Brzozowski et al., 2005). Mucosal damage, an initial step in ulcer development has been known to be due to oxidative stress (OS) by Reactive Oxygen Species (ROS), hypersecretion of HCl through H^+, K^+-ATPase action (Phull et al., 1995), harboring of *H. pylori* on damaged mucin layer (Odenbreit, 2005), and the blockade of cyclooxygenase enzyme system by NSAIDs (Khanna et al., 2006) as depicted in Fig 3A.8.

The ability of the gastric mucosa to resist injury by endogenous secretions and by ingested irritants and infections can therefore be attributed to number of factors that have been collectively referred to as "mucosal defence" (Wallace 1996). Mucus thus play an important role in prevention of mechanical injury to the epithelium and in providing a microenvironment over sites of superficial injury in which rapid repair (restitution) can occur.

Many experimental studies have been carried out to understand the aetiology of ulcers; however ulcer healing being an active and complicated process of reconstruction of mucosal architecture with the involvement of inflammation, cell proliferation, re-epithelialization, formation of granulation tissue, interaction between various cells, matrix and tissue remodelling (Tarnawski, 2005), the probable mechanism of action of the selected potential antiulcer/ulcer healing compounds are yet to be elucidated. Reconstruction or rejuvenation of mucosal architecture, needed for ulcer healing is dependent on elicitation of signalling molecules that can modulate the complete healing process including modulation of specific markers. Prostaglandins appear to play a key role in ulcer healing process as they are involved in upregulation of gastric mucin synthesis, triggering of mucosal cell proliferation, promotion of angiogenesis and several other functions to restore mucosal integrity and hence ulcer healing (Brzozowski et al., 2005).

The discovery of "adaptive cytoprotection" in gastric mucosa mediated through prostaglandins (Robert et al., 1979) contributed significantly towards gastroprotection. The role of cyclooxygenases and their products – prostaglandins have been the focus in recent days for better understanding of ulcer healing mechanisms (Brzozowska et al., 2002; Brzozowski et al., 2005). The quality of ulcer repair remains crucial for the stability of the injured tissue and for preventing recurrence. Remodeling of extracellular matrix is yet another important process requiring interactions between extracellular matrix proteins including collagens, matrix metalloproteinases, cytokines, and growth factors on one side and the granulation tissue supplying the connective tissue cells to restore the lamina propria and the endothelial cells necessary for angiogenesis on the other side together responsible for ulcer healing (Robbins, 1984). Recent studies also showed differential role of MMP-9 and MMP-2 during aggravation or healing of gastric ulcers (Baragi et al., 1997; Swarnakar et al., 2005; Shahin et al., 2001).

The role of signaling molecules such as galectin-3 which are known to regulate inflammatory processes, via a cascade of matrix metalloproteinases (MMPs) and its hitherto association with the elicitation of prostaglandins, a marker of inflammatory reaction needs to be determined for the complete understanding of the healing process. In addition, potential molecules participating in various steps of ulceration and healing such as oxidants, antioxidants in addition to galectin-3, MMPs, PGE_2, COX-2 and MAP kinases (ERK1&2) needs to be addressed to understand the mode of action of antiulcer polysaccharides.

In the context that ulcer is a multi-step disease, although antiulcer drugs are available in the market such as proton pump blockers, histamine receptor blockers, mucosal protectants and antimicrobials, they have the limitations to exert multi-step antiulcer effect as a single entity. Also they have been shown to pose adverse side effects (Waldum et al., 2005). Although suppressors of acid secretion have been a mainstay for promotion of ulcer healing from past three decades, there is an increased interest in recent years in the mechanisms through which ulcers heal to ensure the speed and quality of healing to avoid recurrences (Wallace, 2005). Mere

blockade of induction of damage can offer effective prevention, however, unless the molecule has inherent capacity to activate or deactivate defence or ulcer pathogenicity routes respectively and stimulation of cells to undergo appropriate mechanisms to selectively activate the cascade of wound healing process, the compound may not be an effective ulcer healing agent.

The modest approach to control ulceration hence is via stimulation of gastric mucin synthesis, enhancement of antioxidant levels in the stomach, scavenging of ROS, inhibition of H^+, K^+-ATPase and *H. pylori* growth (Bandyopadhyaya et al., 2002). Although the antisecretory drugs such as H^+, K^+-ATPase pump inhibitors - omeprazole, lansoprazole; histamine H_2-receptor blockers - ranitidine, famotidine are being used to control acid secretion and acid related disorders, they are not the drugs of choice since they pose adverse effects on human health and not advisable particularly during pregnancy.

Despite the elaborative studies on antiulcer potentials on some of the natural pectic polysaccharides, how these pectic polysaccharides either protect or enhance the synthesis of gastric mucin was poorly understood. In other words an understanding of ability of the polysaccharide in interacting with the multi-step complex ulcer pathogenicity and their precise role in triggering the signaling cascade is yet to be elucidated.

In the light of the above facts, it is pertinent to address natural products from food/plant for potential antiulcer compounds. Due to lack of side effects compared to synthetic drugs, approximately 60% of the world population relies entirely on such natural medications. In Indian traditional medicines, several plants have been employed to treat gastrointestinal disorders, including gastric ulcers (Sathyavathi et al., 1987). Antiulcer properties have been attributed generally to phenolics (Sung-Sook et al., 2005; Reyes-Chilpa et al., 2006) and occasionally to polysaccharides (Matsumoto et al., 2002; Ye et al., 2003; Gao et al., 2004) of plant extracts.

According to previous chapter (Chapter 1) Swallow root and Black cumin were considered as better antiulcer sources since they exhibited highest antiulcer indices of 81% and 68% respectively (Table 1.5 of chapter 1). Further pectic polysaccharide fractions (SRPP and BCPP) of these two

sources were identified as active fractions as evaluated by *in vitro* assays (Fig 1.7, 1.8 & Table 1.8 of chapter 1).

Apart from SRPP and BCPP, the phenolic compound – HMBA was also considered as potent antiulcer source (Discussed in chapter 4), since it exhibited potent inhibitory effect on both H^+, K^+ ATPase with IC_{50} of 49 µg/mL and anti-*H. pylori* activity with MIC of 39 µg/mL. Therefore the current chapter aimed to determine gastroprotective potential of SRPP, BCPP and HMBA using different gastric ulcer models in Wistar albino rats. Since ulcer prevention and healing processes involve different mechanisms, we studied these two process using appropriate gastric ulcer models.

Accordingly the chapter was subdivided into **"Part A"** and **"Part B"**.

In "**Part A**" the ulcer preventive effect of SRPP, BCPP and HMBA were studied using swim stress and ethanol induced gastric ulcer model in Wistar albino rats. Work envisaged the multi-potent role of phenolic-bound polysaccharides - upregulation of mucin, antioxidant levels, modulation of oxidative status, inhibition of H^+, K^+ -ATPase activity against swim and ethanol stress induced ulcers in experimental animal models, and also reveals the multi-step action of phenolic polysaccharide in preventing gastric ulceration.

Further, in "**Part B**" ulcer healing mechanism of SRPP and BCPP were studied using acetic acid induced gastric ulcer model. Since acetic acid induced gastric ulcers are similar to human gastric ulcers in their pathophysiology and healing process, we elaborated the study to understand the mode of action of pectic polysaccharides – SRPP and BCPP. The study also envisages the role of these pectic polysaccharides in modulation of signaling cascades involved in either ulcer pathogenesis or healing process. Since HMBA showed a moderate effect on gastroprotection as evidenced by ulcer preventive studies, it was not included during studies on mode of action during ulcer healing in acetic acid induced ulcer model. However, since it acts as a potent inhibitor of *H. pylori* it was studied exclusively for anti-*H. pylori* effect including its mode of action on *H. pylori* in chapter 4.

Part A

Ulcer preventive effect of SRPP, BCPP and HMBA in swim stress and ethanol induced ulcer models

3A.4. Materials and methods

Monoclonal anti-gastric mucin antibody, alcian blue, Glutathione, Tetramethoxy propane and Bovine serum albumin were procured from Sigma Chemicals (St. Louis, MO, USA), Alkaline phosphatase conjugated-rabbit anti-mouse IgG secondary antibody and p-nitrophenyl phosphate from Genei (Bangalore, India). All other reagents were of analytical grade purchased from Qualigens fine chemicals (Mumbai, India).

3A.4.1. Preparation of pectic polysaccharides – SRPP/BCPP and HMBA

SRPP and BCPP are pectic polysaccharides isolated from Swallow root and Black cumin respectively using a classical procedure as described in section-1.4.4 of chapter 1. The SRPP and BCPP were dissolved in distilled water at required concentrations and used for animal experiments. HMBA is a phenolic compound isolated from Swallow root as described in section 2.4.3.1 of chapter 2. HMBA at required concentration was initially dissolved in ethanol and diluted with distilled water (final ethanol concentration of 5%, v/v) and used for animal experiments. Appropriate vehicle controls were also included in the experiment.

3A.4.2. Animals and experimental design

Ulcer preventive ability of pectic polysaccharides – SRPP & BCPP and phenolic component HMBA was tested using Wistar albino rats. Wistar albino rats weighing about 180–220 g maintained under standard conditions of temperature, humidity and light were provided with standard rodent pellet diet (Amruth feeds, Bangalore, India) and water *ad libitum*. The study was approved by the institutional ethical committee, which follows the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. 49,1999), Government of India, New Delhi, India.

The ulcer preventive activity of SRPP and BCPP were tested at concentrations of 100 mg/kg b.w. and 200 mg/kg b.w. in both swim stress and ethanol induced ulcer models in two different experimental setup. While the preventive effect of HMBA against gastric ulcer could be tested only in ethanol induced ulcer model at 10 and 20 mg/kg b.w. concentrations in Wistar albino rats. Ranitidine (50 mg/kg b.w.) and omeprazole (20 mg/kg b.w.) were used as standard antiulcer drugs. The animals were categorized into different groups of 6 rats each as shown in Table 3A.1.

Control groups	
1	Healthy
2	Swim stress induced ulcer (SS)
3	Ethanol stress induced ulcer (ES)
4	SRPP control (200 mg/kg b.w.)
5	BCPP control (200 mg/kg b.w.)
6	HMBA control (20 mg/kg b.w.)
Set 1 (SRPP)	
7	SRPP 100 mg/kg b.w. + SS
8	SRPP 200 mg/kg b.w. + SS
9	SRPP 100 mg/kg b.w. + ES
10	SRPP 200 mg/kg b.w. + ES
11	Ranitidine 50 mg/kg b.w. + SS
12	Ranitidine 50 mg/kg b.w. + ES
Set 2 (BCPP)	
13	BCPP 100 mg/kg b.w. + SS
14	BCPP 200 mg/kg b.w. + SS
15	BCPP 100 mg/kg b.w. + ES
16	BCPP 200 mg/kg b.w. + ES
17	Omeprazole 20 mg/kg b.w. + SS
18	Omeprazole 20 mg/kg b.w. + ES
Set 3 (HMBA)	
19	HMBA 10 mg/kg b.w. + ES
20	HMBA 20 mg/kg b.w. + ES

Table 3A.1: Experimental groups with sample concentration and ulcer induction models. SS and ES indicate swim stress and ethanol stress respectively.

The sample was administered orally at indicated doses for 14 days to respective groups. Healthy, swim stress and ethanol stress control groups were administered with saline. Animals were fasted for 18 h with free access to water before inducing ulcers. On 14th day ulcer was induced by either swim stress or ethanol treatment to respective groups as follows. Sample control animals fed with only samples without inducing ulcers were also setup in order to evaluate toxicity if any, encountered by samples.

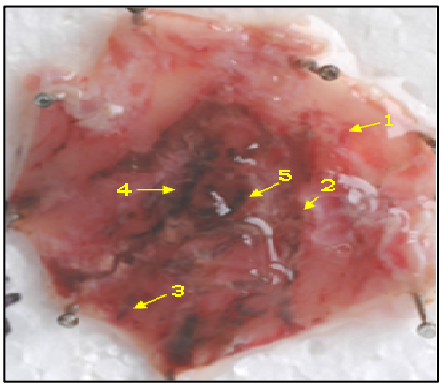
In swim stress induced ulcer model, on day 14, after 30 min of sample treatment, all the animals except healthy and sample control groups, were subjected to forced swim stress individually, by making them to swim in a jar of 30 cm height and 15 cm diameter containing water up to 15 cm height and maintained at room temperature for 3 h (Brady et al, 1979).

In the ethanol stress induced ulcer model, on day 14, after 30 min of sample treatment to respective rats, the gastric ulcers were induced to all the rats except healthy and sample control groups by administering absolute ethanol at a dose of 5 mL/kg b.w. for 1 h (Jainu & Devi, 2006).

After ulcer induction animals were sacrificed under deep ether anesthesia and stomach was removed, cut opened along the curvature to count ulcer index (Kulkarni & Goel, 1996). Stomach, liver and serum samples were collected and used for analysis of biochemical parameters.

3A.4.3. Determination of Ulcer Index (UI)

Stomachs of animals subjected to ulcer induction and those pretreated with test samples were cut opened along the curvature, washed with saline and ulcers were scored according to the protocol (Kulkarni & Goel, 1996). Lower to higher (0.5 to 3.0) grading was provided to milder to severe symptoms respectively. Ulcer score was recorded as follows, 0.5-ulcerous red coloration, 1.0-spot ulcers, 1.5-hemorrhagic streaks of <3 mm, 2.0-hemorrhagic streaks of 3 to 5 mm, 3.0- hemorrhagic streaks of more than 5 mm. Mean ulcer scores for each experimental group were calculated and expressed as the ulcer index (UI) (Kulkarni & Goel, 1996) as depicted below.



1. Ulcerous red coloration
2. Spot ulcers
3. Hemorrhagic streaks of <3 mm
4. Hemorrhagic streaks of 3-5 mm
5. Hemorrhagic streaks of >5 mm

No. lesions x ulcer score = Ulcer incidence (Ui)

Ui1 + Ui2 + Ui3 + Ui4 + Ui5 = **Ulcer Index** (UI)

3A.4.4. Histological and immunohistological evaluation of stomach tissue

Histological and immunohistological evaluation was done as described previously (Morise et al., 1998). The formalin (10%) fixed gastric tissue samples were embedded in paraffin, sectioned (5 µm) and stained with haematoxylin and eosin reagents. Another set of slides were immunostained with 1:500 (v/v) diluted monoclonal anti-human gastric mucin antibody (MAb-GM) followed by goat anti-mouse IgG peroxidase conjugate (GENEI, Bangalore, India) at 1:1000 (v/v) dilution followed by peroxidase substrate TMB/H_2O_2. Slides were mounted with DPX and observed under the microscope for pathological changes like, damaged mucosal epithelium, glandular structure, inflammatory exudates, proliferated fibroblasts, mixed leukocyte infiltrate and cellular debris to evaluate the degree of ulceration and gastroprotection. In case of BCPP (set 2), the stomach sections were stained with H & E and PAS reagents.

3A.4.5. Quantitative estimation of gastric mucin

Adhered mucus content of the fundic part of the stomach was determined by alcian blue dye binding method as described by Corne et al (1974). Briefly, equal weights of stomach tissue of healthy control, ulcer induced and sample treated rats were incubated for 2 h with 1% of mucus binding-alcian blue in 50 mM sodium acetate buffer pH 5.8 containing 0.16 M sucrose. After incubation, suspension was centrifuged at 3000 *g* for 10 min and the

supernatant containing unbound alcian blue was measured at 598 nm. The results were expressed as amount of alcian blue binding per gram of stomach tissue. The quantity of alcian blue binding is directly proportional to the mucin content.

3A.4.6. Assessment of H^+, K^+-ATPase

Equal weight of gastric tissue from animals of each group was homogenized using Tris-HCl buffer pH 7.4. The gastric membrane vesicles enriched in H^+, K^+-ATPase were prepared and the H^+, K^+-ATPase activity was assessed in equal amount of protein as described in chapter 1, Section 1.4.8.

3A.4.7. Evaluation of antioxidant status

The antioxidant status of the rats of different groups were determined by estimating the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, antioxidant molecule - glutathione and lipid peroxidation levels (as Thiobarbituric acid reactive species) in serum and homogenates of stomach and liver tissues as follows.

3A.4.7.1. Preparation of serum and tissue homogenates

After dissection, blood was collected in tubes and kept in a slanting position at room temperature for 20 min followed by storage at 4 ^{0}C for 20 min to allow the separation of serum. Serum was centrifuged at 500 *g* for 10 min and the serum was stored at –20 °C for further studies. Stomach and liver tissue were removed and five percent homogenates were prepared using cold 0.15 M phosphate buffer and centrifuged at 5000 *g* for 20 min at 4 °C. The supernatants obtained were collected and analyzed for biochemical parameters as described below and total protein was estimated as described earlier (Lowry et al., 1951).

3A.4.7.2. Estimation of superoxide dismutase (SOD, EC 1.15.1.1)

The activity of SOD was assayed using nitroblue tetrazolium (NBT) as the substrate (Flohe and Otting, 1984). Briefly 0.1 mL of tissue homogenate or serum was taken in Beckman quartz cuvette of 1 cm path length. To this, a mixture containing 1 mL of sodium carbonate (50 mM), 0.4 mL of NBT (24 µM) and 0.2 mL of EDTA (0.1 mM) was added and the zero min reading was taken at 560 nm. The reaction was initiated by the addition of 0.4 mL of 1

mM hydroxylamine hydrochloride. The reaction mixture was then incubated at 25 °C for 5 min and the reduction of NBT was read at 560 nm. A parallel control without homogenate or serum was also run and was considered as 100% auto oxidation. The enzyme activity was expressed as unit/mg protein.

Superoxide dismutase activity was also measured spectrophotometrically at 425 nm as decrease in auto oxidation of epinephrine in presence of this enzyme and the activity was expressed as units/mg/min (Misra & Fridovich, 1972). One unit is defined as the amount of protein (enzyme) required for inhibiting 50% of auto oxidation of epinephrine.

3A.4.7.3. Estimation of Catalase (CAT, EC 1.11.1.6)

The activity of catalase was assayed according to the method described previously (Aebi, 1983). Briefly, 0.1 mL of tissue homogenate or serum was added to 1.9 mL of phosphate buffer, pH 7.0 and absorbance was measured at 240 nm. To this 1 mL of hydrogen peroxide was added and the absorbance was measured for 3 min at 240 nm using phosphate buffer as blank solution. The activity of catalase was expressed as amount of H_2O_2 utilized/mg protein/min.

3A.4.7.4. Glutathione peroxidase (POX, EC.1.11.1.9)

The activity of glutathione peroxidase was determined according to the method described previously (Flohe and Gunzler, 1984). The mixture containing 0.1 mL of tissue homogenate or serum, 0.1 mL of 10 mM glutathione reductase (0.24 U) and 0.1 mL of 10 mM GSH was preincubated for 10 min at 37 °C and, thereafter 0.1 mL of NADPH solution was added. The hydroperoxide independent consumption of NADPH was monitored for 3 min. Overall reaction was started by adding 0.1 mL of prewarmed hydroperoxide solution and the decrease in absorption at 340 nm was monitored for 3 min and the activity was expressed as nanomoles of NADPH oxidized/min/mg protein.

3A.4.7.5. Measurement of glutathione

Glutathione (GSH) is a tripeptide. It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. Glutathione, an antioxidant, helps protect cells from reactive oxygen species such as free radicals and peroxides (Pompella *et al.,*

2003). Glutathione is nucleophilic at sulfur and attacks poisonous electrophilic conjugate acceptors.

Thiol groups are kept in a reduced state in animal cells. In effect, glutathione reduces any disulfide bond formed within cytoplasmic proteins to cysteines by acting as an electron donor. In this process, glutathione is converted to its oxidized form glutathione disulfide (GSSG). Glutathione is found almost exclusively in its reduced form, since the enzyme that reverts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced glutathione to oxidized glutathione within cells is often used scientifically as a measure of cellular toxicity (Pastore, 2003).

GSH in tissue homogenate and serum was measured as described previously (Sedlak & Lindsay, 1968). An aliquot of 1.0 mL of homogenate or serum was precipitated with 10% trichloroacetic acid and centrifuged. The supernatant (1.0 mL) was added to 2.0 mL of 0.8 M Tris-HCl, pH 9.0, containing 20 mM EDTA and mixed with 0.1 mL of 10 mM 5,5-dithiobis-2-nitrobenzoic acid. The intense yellow colour of nitromercaptobenzoate was read at 412 nm. For calibration, a standard curve was prepared treating varied concentrations of reduced glutathione with 5,5-dithiobis-2-nitrobenzoic acid under similar conditions.

3A.4.7.6. Measurement of lipid peroxidation

Malondialdehyde (MDA), one of the well known secondary products of lipid peroxidation formed after exposure to reactive oxygen species and free radicals was used as indicator for cell membrane injury, since this is commonly encountered in disease conditions. Thiobarbituric acid (TBA) test is one of the most frequently used tests for measuring the peroxidation of lipids. Thiobarbituric acid reacts with MDA forms a pink colour chromogen, which can be detected spectrophotometrically at 532 nm.

Lipid peroxide content as thiobarbituric acid reactive species (TBARS) in serum and tissue homogenate was measured as described previously (Ohkawa et al., 1979). Briefly, 1 mL of the membrane fraction was allowed to react with 2 mL of 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.25 N HCl reagent, heated in a boiling water bath for 15 min, cooled, and

centrifuged at 1000 *g* for 10 min at RT. The absorbance of the supernatant was measured at 535 nm, and the amount of malondialdehyde (MDA) produced was quantified using a standard curve of 1,1,3,3 tetramethoxypropane.

3A.4.8. Toxicity studies

To evaluate the toxic effects of selected components SRPP, BCPP and HMBA, serum of sample control groups and the healthy control groups were analyzed for total protein and liver function enzymes like SGPT (serum glutamate pyruvate transaminase), SGOT (serum glutamate oxaloacetate transaminase) and alkaline phosphatase (ALP) using kit methods following manufacturer's protocol (Agappe). A Thiobarbituric acid reactive substance (TBARS) was also measured as a marker of oxidative stress condition.

3A.4.9. Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed using one way ANOVA followed by Duncan's multiple range test (DMRT) to test the significant differences between treated and control groups. Data was computed for statistical analysis by using SPSS statistical software.

3A.5. Results

3A.5.1. Macroscopic assessment of gastroprotection by SRPP, BCPP and HMBA

As indicated under materials and methods, ulcer preventive ability of SRPP and BCPP was studied employing both swim stress and ethanol induced ulcer models; while gastroprotective effect of HMBA could be studied only in ethanol stress induced ulcer model. Results are therefore presented accordingly as described below.

The observation of mucosal surface of stomachs of healthy, ulcer induced and SRPP or BCPP treated groups (Fig 3A.1 & 3A.2) showed that, the healthy stomach looks like normal without any visible changes like reddening, bleeding or lesions in the stomach surface mucosa. Rats treated with only SRPP or BCPP (sample control groups) without ulcer induction also showed no lesions, and was similar to that of healthy controls. However rats treated with forced swim stress for 3 h or ethanol stress for 1 h showed damage in the gastric wall with a hemorrhagic form of lesions and intraluminal bleeding. Oral treatment of SRPP at 100 and 200 mg/kg b.w. for 14 days gave a dose dependent protection. SRPP at 200 mg/kg b.w. showed maximum protection of 85% against swim stress and 80% against ethanol induced ulcers (Fig 3A.3).

BCPP also showed a gastroprotective effect against both swim stress and ethanol induced gastric ulcers as indicated by reduction in lesions and bleeding in BCPP treated groups (Fig 3A.2). At 200 mg/kg b.w. concentration BCPP showed protection of 77% against swim stress and 73% against ethanol induced ulcers (Fig 3A.4). The known antiulcer drugs ranitidine and omeprazole showed a good protection of more than 80% against both the ulcer induction models. Quantitative reduction in the ulcer index by SRPP and BCPP treatment are depicted in Fig 3A.3 and Fig 3A.4 respectively.

Gastroprotective effect of HMBA was tested in ethanol induced gastric ulcer model in Wistar albino rats. HMBA was orally fed at 10 mg and 20 mg/kg b.w. concentrations respectively. The visible changes observed in the cut opened stomachs of HMBA pretreated and ulcer induced rats showed a moderate protection of gastric mucosa by HMBA against ethanol induced ulceration (Fig 3A.5). This was evidenced by higher ulcer index of 80 in ulcer

control which was reduced to 40 and 30 upon HMBA treatment, indicating 51% and 64% protection by HMBA at 10 mg and 20 mg/kg b.w. respectively (Fig 3A.5E).

3A.5.2. Effect of SRPP, BCPP and HMBA on H^+, K^+ -ATPase activity

H^+, K^+ -ATPase is a proton pump present in parietal cells of gastric wall that causes increase in gastric acidity and gastric ulcers. Approximately 3 to 4 folds increase in H^+, K^+-ATPase activity in ulcer-induced stomach tissue as shown in Table 3A.2 and 3A.3, was inhibited in a dose dependent manner by both SRPP (Table 3A.2) and BCPP (Table 3A.3). SRPP at 200 mg/kg b.w. reduced ~44% and 61% (1.5 folds) of H^+, K^+-ATPase activity in ethanol and swim stress-induced ulcer models respectively when compared to that of ulcer controls (Table 3A.2). BCPP however showed 30 to 46% reduction in H^+, K^+-ATPase activity at 200 mg/kg b.w. concentration in swim and ethanol stress induced ulcer models as opposed to 90% normalization by omeprazole at 20 mg/kg b.w.

HMBA exhibited a good inhibitory effect on H^+, K^+ -ATPase activity in ethanol induced ulcer condition as indicated in Table 3A.4. Results showed 2 folds increase in H^+, K^+ -ATPase level in ulcerous condition, whereas HMBA pretreatment showed dose dependent inhibition of H^+, K^+ -ATPase level; at 20 mg/kg b.w. the H^+, K^+ -ATPase levels were normalized similar to that of healthy rats.

The results were also supported by *in vitro* quantitative estimation of H^+, K^+ -ATPase inhibitory activity of SRPP, BCPP and HMBA. The *in vitro* results indicated that SRPP inhibited sheep stomach H^+, K^+ -ATPase activity with an IC_{50} of 77 µg/mL and BCPP inhibited the activity at IC_{50} of 170 µg/mL as opposed to that of standard proton pump inhibitor lansoprazole (26 µg/mL) (Table 1.8 of chapter 1). HMBA however inhibited H^+, K^+ -ATPase activity with an IC_{50} of 49 µg/mL indicating that HMBA is potent inhibitor of H^+, K^+ -ATPase activity than SRPP and BCPP.

3A.5.3. Gastric mucin protection by SRPP, BCPP and HMBA

Gastric wall mucus is damaged during ulcer development and becomes the first target of stress-induced reactive oxygen species. Mucin oxidation or degradation takes place and subsequently looses the protective effect. In the

current study, we evaluated the effect of *in vivo* ingestion of SRPP and BCPP on protection of gastric wall mucus during ulceration induced by swim/ethanol stress. Since alcian blue binds to carboxylated mucopolysaccharides as well as sulfated and carboxylated glycoproteins, any disruption in gastric mucin results in reduction in the dye binding, which can be quantitated. The gastric mucin of stomach tissue was decreased to less than 20 mg/g in swim stress and ethanol induced ulcerous rats, when compared to that of healthy controls that contained more than 40 mg/g (Table 3A.2 & 3A.3). Rats pretreated with either SRPP or BCPP showed 80 to 87% recovery in gastric mucin content at 200 mg/kg b.w., whereas HMBA (Table 3A.4) showed 60 to 66% recovery in mucin content at 10 and 20 mg/kg b.w. respectively. It should be noted here that the results of HMBA and SRPP/BCPP cannot be compared since HMBA used is a pure phenolic compound, while SRPP and BCPP are pectic polysaccharides.

The gastric mucin protective effect of SRPP and BCPP were further substantiated by histological observation of stomach tissues stained with either gastric mucin antibody (Fig 3A.6) or periodic acid Schiff's (PAS) reagent (Fig 3A.7) as indicated in figures.

3A.5.4. Histopathological assessment of gastroprotection by SRPP and BCPP

Histological changes of gastric mucosa were observed in H & E stained section of stomach tissue. Fig 3A.6 and 3A.7 shows an intact mucosal layer, well organized, elongated glandular structures in healthy groups. Swim stress and ethanol induced ulcer groups resulted in damaged mucosal epithelium, disorganized glandular structures, inflammatory exudates deposits, infiltration of leucocytes and cellular debris. SRPP (Fig 3A.6) and BCPP (Fig 3A.7) pretreated groups showed reduction in these pathological changes.

Results also showed a significant difference in gastric mucin layer among different groups. The mucin staining by gastric mucin antibody (Fig 3A.6) and PAS reagent (Fig 3A.7) showed a continuous PAS stained layer representing gastric mucin in healthy group. Reduction in intensity and area of stained region as observed in ulcerous stomach is an indication of loss of gastric mucin and reduction in mucin production. Since ethanol directly

affects mucin layer and mucin producing cells by dehydration, cell death and mucosal exfoliation, we could find very less mucin content in ethanol induced ulcer group of animals (Fig 3A.7). Whereas SRPP (Fig 3A.6) and BCPP (Fig 3A.7) treated groups showed increased mucin content with continuous layer of mucin overlaying mucosal epithelium, similar to that of healthy stomach indicating the mucin protective ability of SRPP and BCPP. The standard drugs ranitidine (Fig 3A.6) and omeprazole (Fig 3A.7) also showed a similar kind of protection to gastric mucin.

Since HMBA showed a less potent gastroprotective effect than that of SRPP and BCPP, it is expected that substantiating histopathological changes in stomach tissue of HMBA treated rats may be observed.

3A.5.5. Effect of SRPP, BCPP and HMBA on oxidant and antioxidant status during ulcer prevention

Swim stress and ethanol induced ulcerations have been known to affect the antioxidant status of the animal. Therefore we have tested the antioxidant status by estimating level of lipid peroxidation, reduced glutathione content and antioxidant enzymes – SOD and CAT. Tables 3A.5 and 3A.6 indicate the effect of SRPP on antioxidant, antioxidant enzymes and TBARS levels in stomach/liver homogenate and the serum of swim/ethanol stress induced models. Similar changes observed with BCPP treated groups have been depicted in Tables 3A.7. The results indicate ~ 2 folds increased level of SOD and glutathione peroxidase and ~1.8 folds decrease in CAT and GSH during ulcerous conditions. These changes were normalized in rats which were pretreated with either SRPP or BCPP. Approximately 4 folds increase in TBARS levels depicts lipid peroxidation or damage of stomach tissue in ulcerous animals and SRPP and BCPP treatment showed significant (2-3 folds) reduction in TBARS levels, suggesting the ability of SRPP and BCPP to modulate oxidative stress level *in vivo.*

Table 3A.8 showed the effect of HMBA on antioxidant status during ulcer induction. Approximately 2 folds increased level of TBARS and ~2 folds decreased GSH levels were observed in ethanol induced ulcerous rats. The depleted level of antioxidant molecule - GSH and increased TBARs levels were recovered to normal level in HMBA pretreated rats at 20 mg/kg b.w.

3A.5.6. Evaluation of toxicity of SRPP, BCPP and HMBA

Toxicity studies with SRPP, BCPP and HMBA were carried out in rats for safety evaluation. These studies indicated no lethal effect of SRPP, BCPP and HMBA when orally fed for 14 d. There were no significant differences in total protein, TBARS levels, SGPT, SGOT and ALP between normal and SRPP or BCPP or HMBA treated rats (Table 3A.9), indicating no adverse effect on the major organs. After the above treatment schedules, animals remained as healthy as control animals with normal food and water intake, body weight gain and behavior, further substantiating the non-toxic nature of the samples.

3A.5.7. Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed using one way ANOVA followed by Duncan's multiple comparison test. Data was computed for statistical analysis by using SPSS statistical software. A *P* value of < 0.05 was considered to be statistically significant.

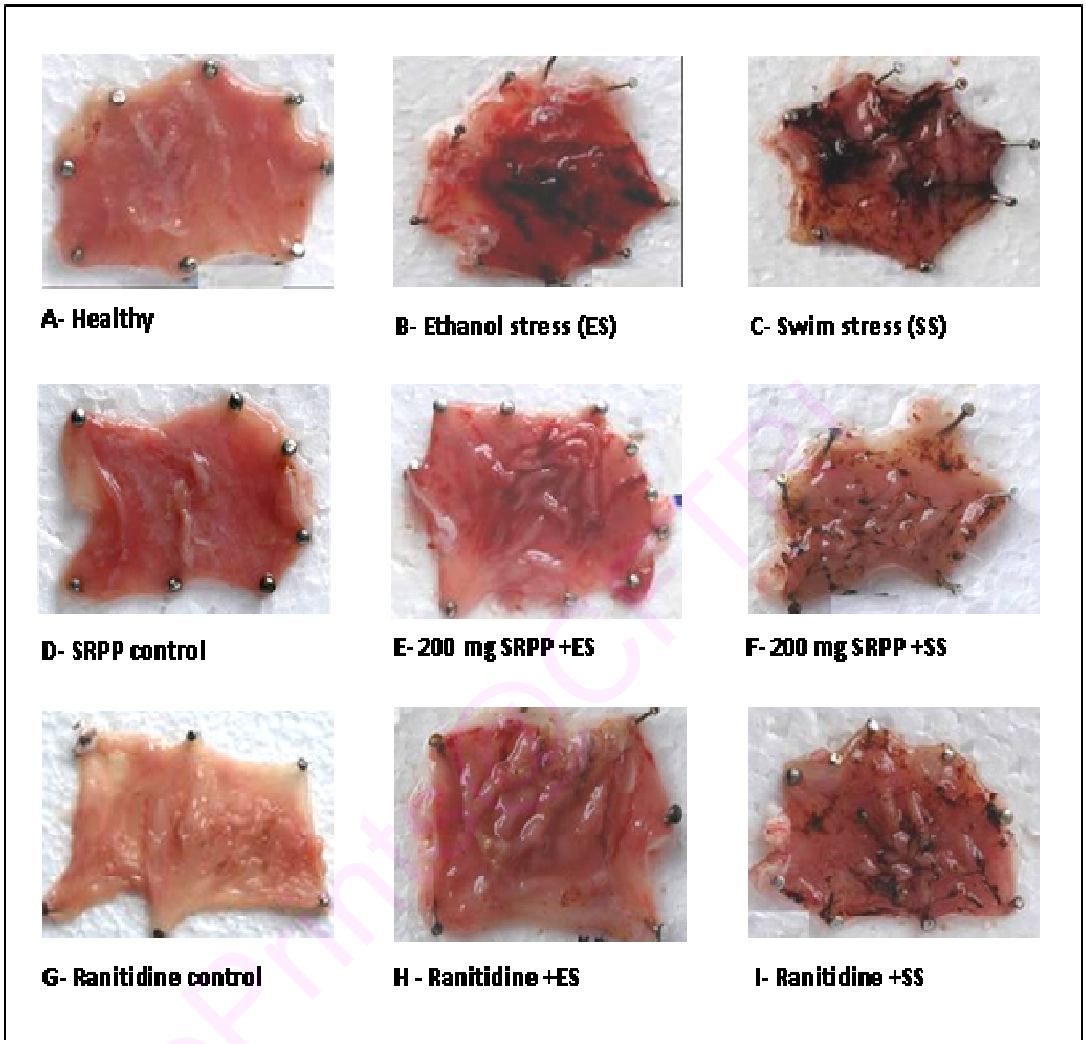


Fig 3A.1. Gastroprotective effect of SRPP in swim stress/ethanol induced ulcer models;
Macroscopic observation of ulcers in ulcer induced/protected stomachs. Ulcer was induced in animals by either swim stress (SS) or ethanol stress (ES) in group of pretreated/untreated animals at indicated concentrations. In healthy (**A**), SRPP control (**D**) and Ranitidine control (**G**)-no ulcer lesions or damage in the stomach tissue were observed. In ethanol stress (**B**) and swim stress (**C**) induced animals ulcers score were very high. SRPP (**E** and **F**) and ranitidine (**H** and **I**) treated animals showed reduced stomach lesions.

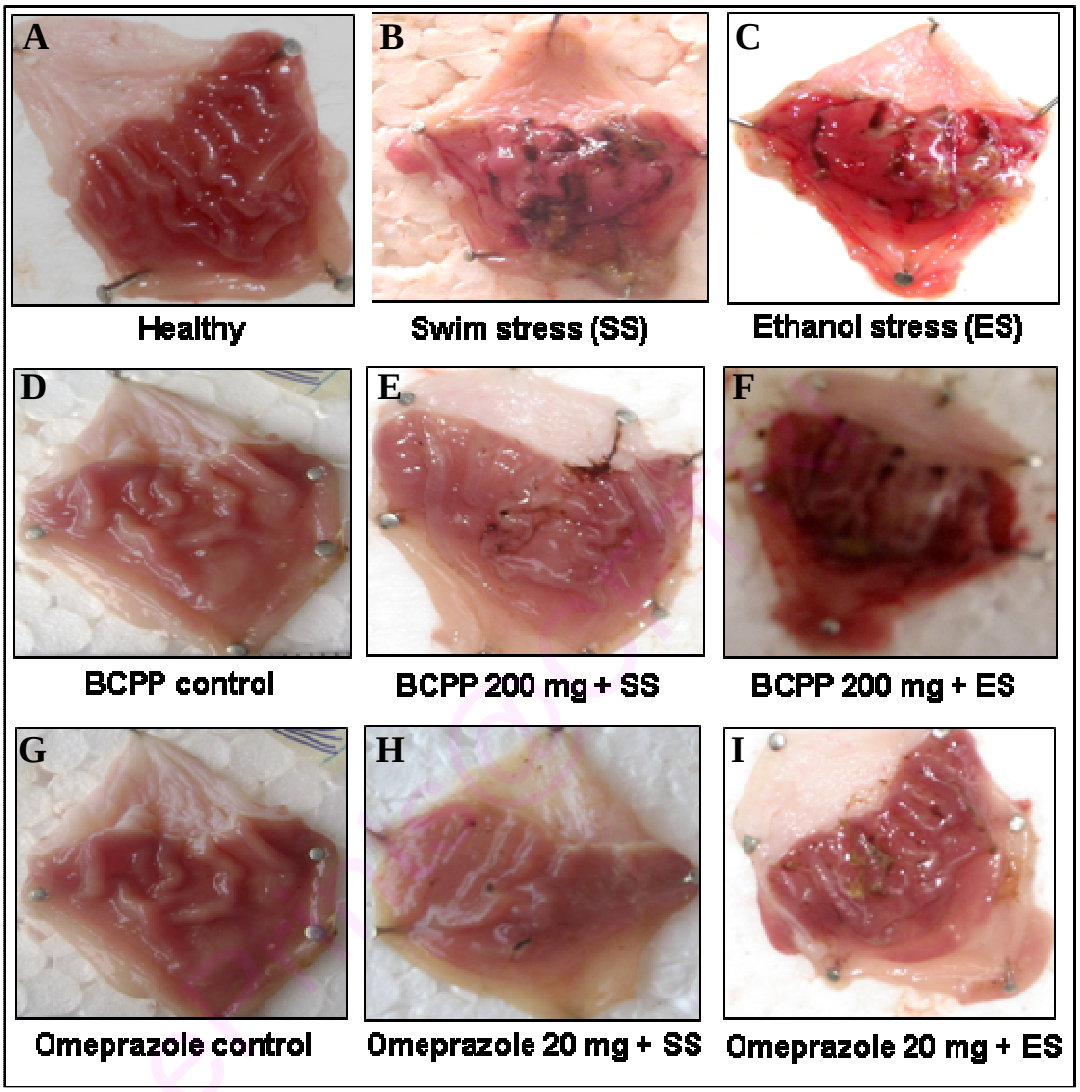
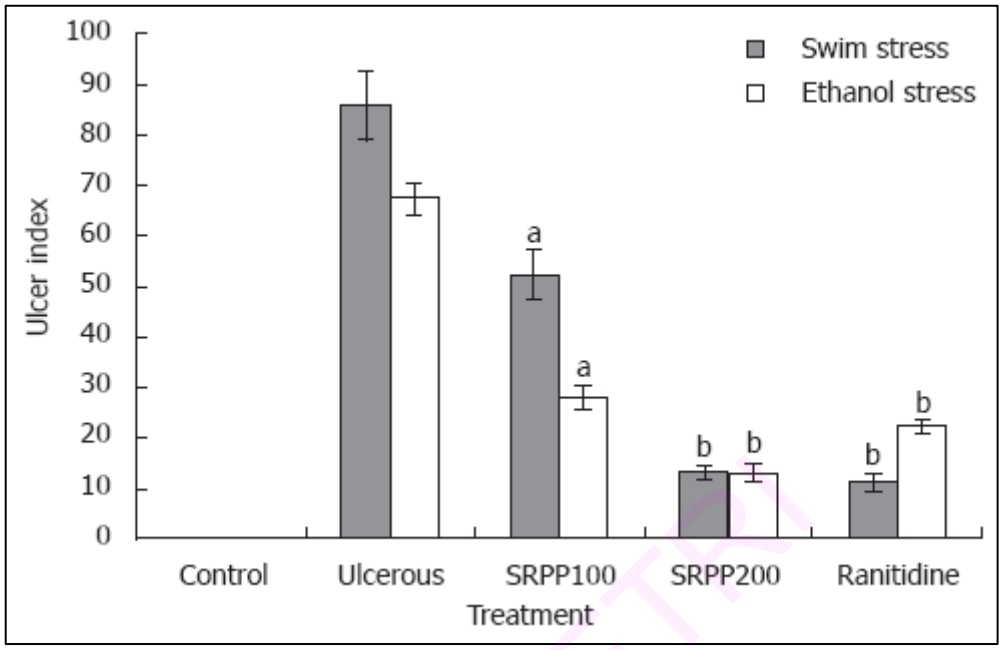
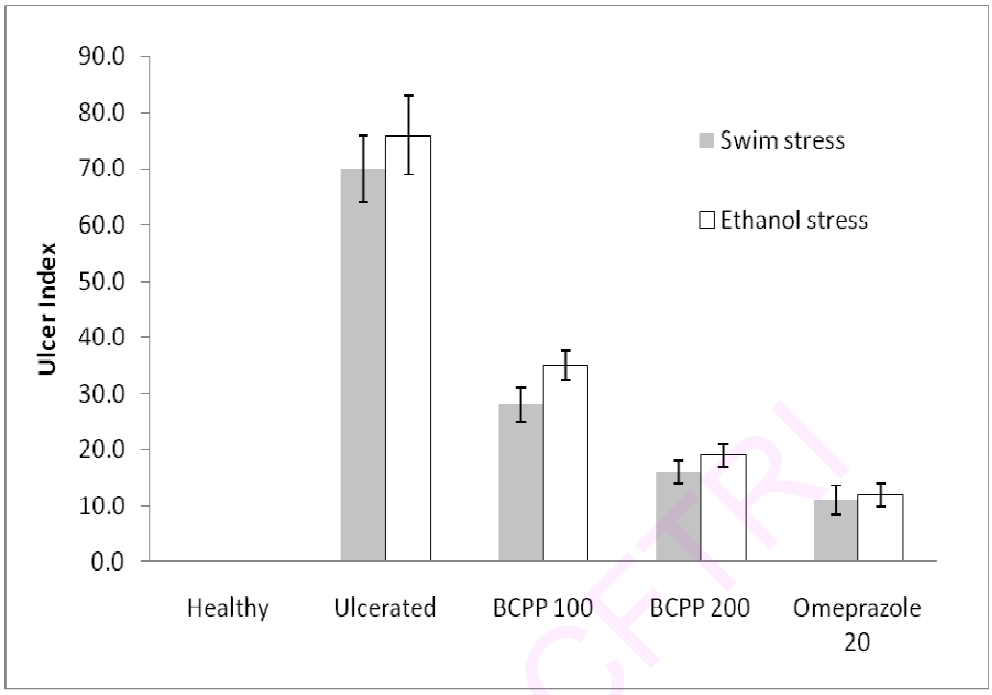


Fig 3A.2. Gastroprotective effect of BCPP in swim stress/ethanol induced ulcer models;
Cut opened stomach pictures showing ulcerated regions with blood streaks in both swim (B) and ethanol (C) induced ulcer groups. BCPP treatment shows reduction in ulcerated regions (E,F) and blood streaks. Omeprazole also provided protection against both swim and ethanol induced ulcers (H,I).



Groups of animals (n=6)	Control	Ulcer induced	SRPP 100 mg kg^{-1} b.w.	SRPP 200 mg kg^{-1} b.w	Ranitidine 50 mg kg^{-1} b.w
Swim stress Mean Ulcer Index ± SEM	0	86.0 ±6.8	52.2^{a} ±5.1	13.3^{b} ±1.4	11.2^{b} ±1.5
% Protection	-	0	40	85	87
Ethanol stress Mean Ulcer Index ± SEM	0	67.4 ± 3.2	28.2^{a} ± 2.5	13.4^{b} ± 1.8	22.4^{b} ± 1.2
% Protection	-	0	58	80	66

Fig 3A.3. Effect of SRPP on gastric lesions in swim/ethanol stress induced ulcer models; Ulcers were scored as described under materials and methods and expressed as ulcer index. Maximum ulcer index observed during stress induction was reduced in a concentration dependent manner. A- $P < 0.05$ and b- $P < 0.01$ between ulcerated and treated groups.



Groups of animals (n=6)	Control	Ulcer induced	BCPP 100 mg kg^{-1} b.w.	BCPP 200 mg kg^{-1} b.w	Omeprazole 50 mg kg^{-1} b.w
Swim stress Mean Ulcer Index ± SD	0	70.0 ± 5.94	28.0^{a} ±3.16	16.6^{b} ± 2.07	11.2^{b} ±1.5
% Protection	-	0	60	77	84
Ethanol stress Mean Ulcer Index ± SD	0	76.0 ± 7.02	35.0^{a} ± 2.63	21.0^{b} ± 2.06	12.3^{c} ± 2.08
% Protection	-	0	53	73	84

Fig 3A.4. Effect of BCPP on gastric lesions in swim/ethanol stress induced ulcer models; Ulcers were scored as described under materials and methods and expressed as ulcer index. Maximum ulcer index observed during stress induction was reduced in a concentration dependent manner. Significant difference between groups were tested by DMRT at *p<0.05.*

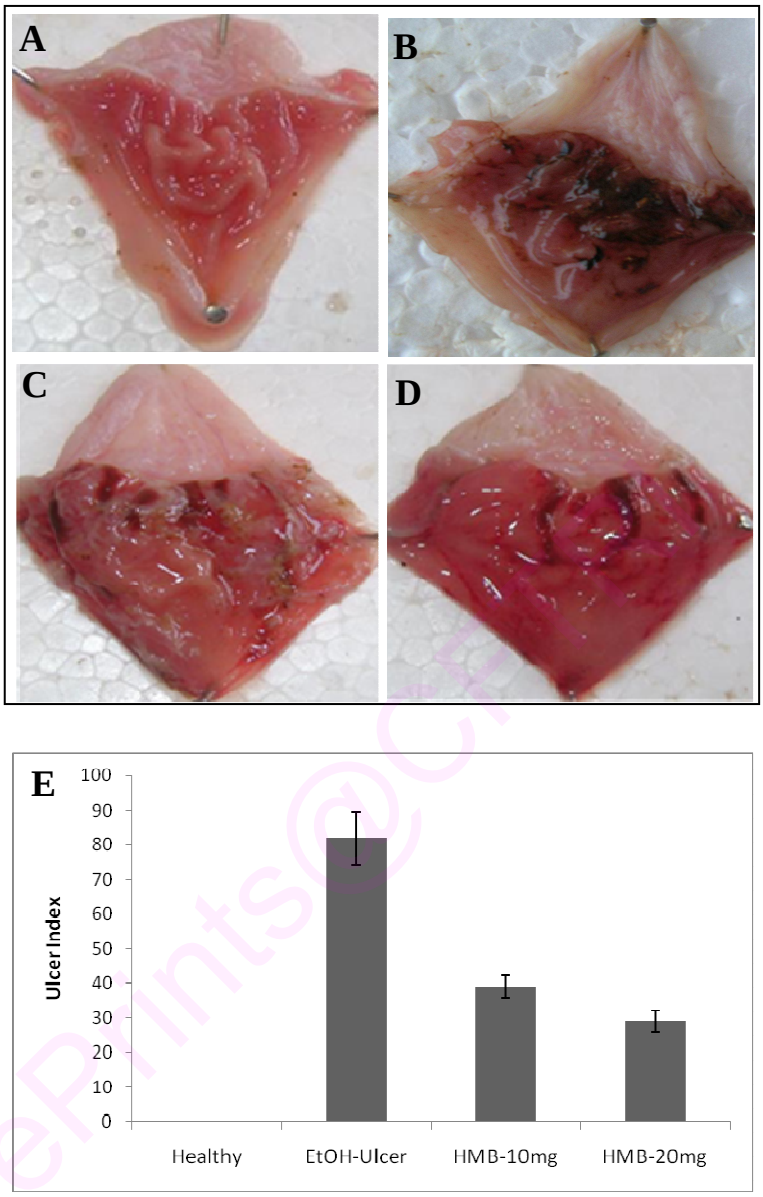


Fig 3A.5. Gastroprotective effect of HMBA in ethanol induced ulcer model:
Stomach pictures showing normal appearance in healthy (A), ulcerated regions with blood streaks in ethanol induced ulcer groups (B). HMBA pretreatment at 10 mg (C) and 20 mg/kg b.w. (D) showing decreased gastric lesions when compared to ulcer control group. Fig E shows ulcer index. HMBA pretreatment showed 51% and 64% protection at 10 and 20 mg/kg b.w. concentrations respectively.

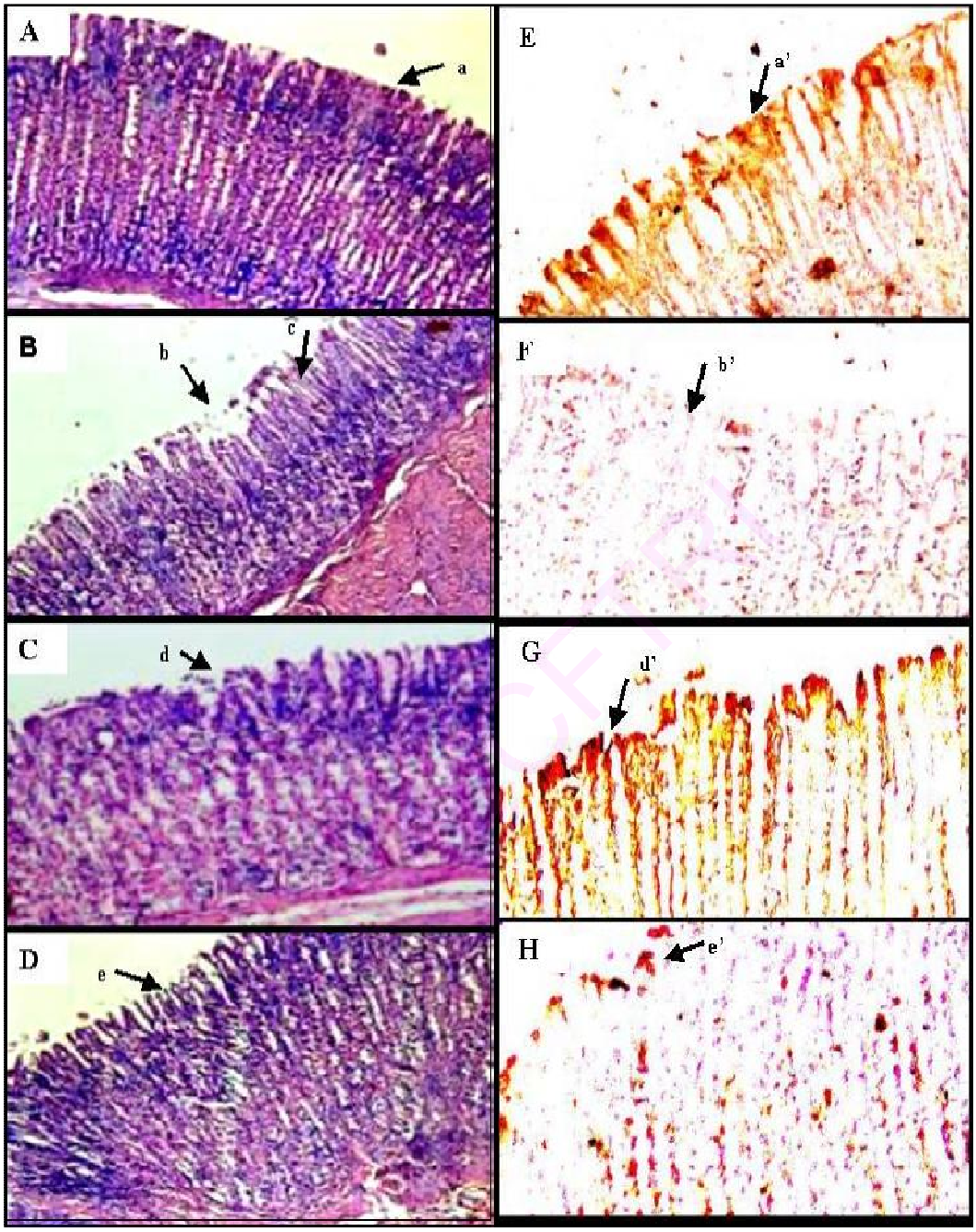


Fig 3A.6. Histopathologic/Immunohistopathologic observation of stomach from ulcer induced/SRPP and Ranitidine treated animals:
A-**D** indicates H&E staining sections (× 40), while **E**-**H** reveal immunostained sections with anti-gastric mucin antibody (× 40), and magnified the selected portion in computer photoshop). Control (**A**, **E**) shows intact mucosal epithelium with organized glandular structure (a) and intense brown staining for gastric mucin by antibody (a'). Ulcer induction (**B**, **F**) showed damaged mucosal epithelium (b) and disrupted glandular structure (c), loss of brown staining (b') in figure **F** indicate the loss of gastric mucin. Complete recovery of damaged mucosa (d and d' of **C**, **G**) by SRPP and partial recovery by ranitidine (e and e' of **D**, **H**) treatments were observed.

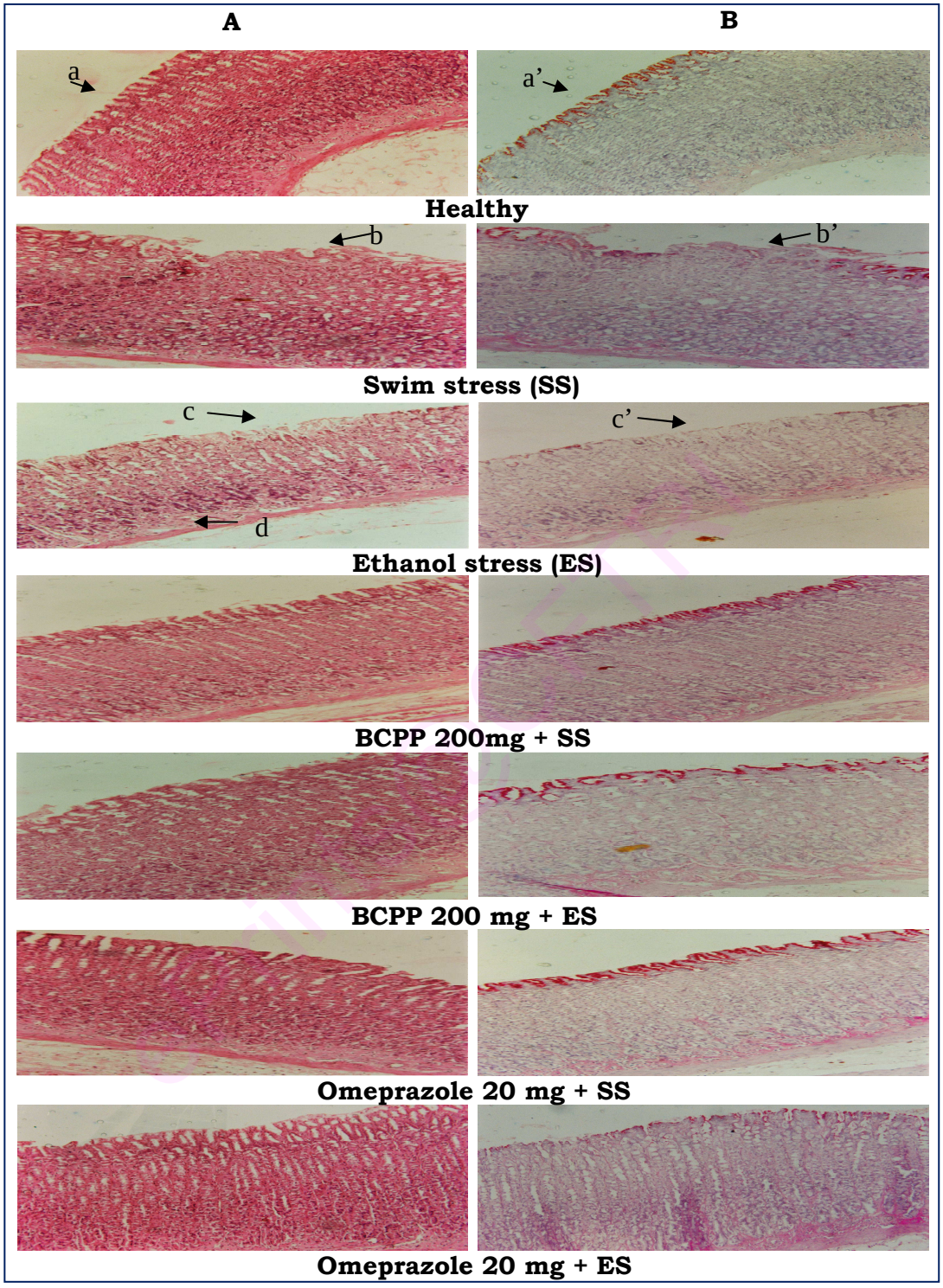


Fig 3A.7. Histopathological changes during ulcer prevention by BCPP: Sections of stomach tissues stained with H & E (Plate A) and PAS (Plate B), Figure shows well organized glandular structure with intact mucin covering epithelial cells in healthy stomach (a,a'). Whereas swim (b,b') and ethanol stress (c,c') erodes mucosal layer as indicated by discontinuous and decreased PAS stained regions. Stress induction also showed inflammatory exudates (d). BCPP and omeprazole treatment prevented these changes, showing continuous mucosal layer with increased mucin content.

Group, n=6	**Mucin content (mg AB binding/g tissue)**	**H^+, K^+-ATPase (µ moles Pi released/mg/h)**
Healthy	45.04^{d} ±4.128	0.807^{a}± 0.072
Swim stress induced ulcer model		
Swim stress induced	17.78^{a} ±2.557	2.209^{c} ± 0.152
SRPP 100 mg kg^{-1} b.w.	27.13^{b} ±4.082	1.771^{b} ± 0.081
SRPP 200 mg kg^{-1} b.w.	35.35^{c} ± 3.221	1.601^{b} ± 0.091
Ranitidine 50 mg kg^{-1} b.w.	31.42^{bc} ±2.327	1.621^{b} ± 0.092
Ethanol stress induced ulcer model		
Ethanol stress induced	16.32^{a} ± 3.821	2.621^{d} ± 0.211
SRPP 100 mg kg^{-1} b.w.	32.13^{b} ±3.457	2.123^{c} ± 0.241
SRPP 200 mg kg^{-1} b.w.	39.53^{bc} ±3.082	1.512^{b} ± 0.121
Ranitidine 50 mg kg^{-1} b.w.	37.13^{b} ±1.507	1.485^{b} ± 0.124

Table 3A.2. Effect of SRPP on gastric mucin and H^+, K^+-ATPase levels during swim stress and ethanol induced ulcers in rats.
Values are mean ± SD (n=6). Significant differences between treated and ulcer induced groups was tested by DMRT.

Group, n=6	Mucin content (mg AB binding/g tissue)	H^+, K^+-ATPase (µ moles Pi released/mg/h)
Healthy	$41.2^{c} \pm 4.32$	$0.615^{d} \pm 0.211$
Swim stress induced ulcer model		
Swim stress induced	$21.0^{a} \pm 3.60$	$2.260^{a} \pm 0.251$
BCPP 100 mg kg^{-1} b.w.	$24.8^{a} \pm 3.96$	$1.961^{a} \pm 0.268$
BCPP 200 mg kg^{-1} b.w.	$34.2^{b} \pm 4.60$	$1.780^{b} \pm 0.21$
Omeprazole 20 mg kg^{-1} b.w.	$39.3^{c} \pm 3.05$	$0.813^{c} \pm 0.091$
Ethanol stress induced ulcer model		
Ethanol stress induced	$18.8^{a} \pm 4.76$	$2.331^{a} \pm 0.268$
BCPP 100 mg kg^{-1} b.w.	$22.6^{a} \pm 5.08$	$2.107^{a} \pm 0.276$
BCPP 200 mg kg^{-1} b.w.	$35.2^{b} \pm 6.57$	$1.547^{b} \pm 0.192$
Omeprazole 20 mg kg^{-1} b.w.	$37.3^{b} \pm 8.62$	$1.315^{b} \pm 0.099$

Table 3A.3. Effect of BCPP on gastric mucin and H^+, K^+-ATPase levels during swim stress and ethanol induced ulcers in rats:
Values are mean ± SD (n=6). Results are significantly different among different groups, which is tested by DMRT at $p < 0.05$.

Group, n=6	Mucin content (mg AB binding/g tissue)	H^+, K^+-ATPase (µ moles Pi released/mg/h)
Healthy	$36.6^{c} \pm 4.1$	$0.178^{c} \pm 0.016$
Ethanol stress (ES) induced ulcer	$16.8^{a} \pm 1.9$	$0.338^{a} \pm 0.029$
HMBA-10 mg + ES	$22.2^{a} \pm 2.6$	$0.202^{b} \pm 0.14$
HMBA-20 mg + ES	$24.4^{b} \pm 3.4$	$0.173^{c} \pm 0.19$

Table 3A.4. Effect of HMBA on gastric mucin and H^+, K^+-ATPase levels during ethanol induced ulcers in rats.
Values are mean ± SD (n=6). Significant difference among groups are analysed by DMRT at $P<0.05$.

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (µmol H_2O_2/mg/min)	Glutathione Peroxidase (ηmoles/mg)	GSH (η mol/g tissue)	TBARS (ηmoles)
Stomach						
Healthy	$2.23^{c} \pm 0.21$	$9.86^{a} \pm 1.1$	$829.2^{c} \pm 41.6$	$0.21^{a} \pm 0.009$	$224^{c} \pm 10.0$	$0.31^{a} \pm 0.01$
Ulcerated	$1.95^{a} \pm 0.13$	$19.10^{c} \pm 1.8$	$462.4^{a} \pm 30.2$	$0.49^{d} \pm 0.01$	$121^{a} \pm 18.9$	$1.12^{c} \pm 0.20$
SRPP 100 mg/kg	$1.90^{a} \pm 0.09$	$16.32^{bc} \pm 2.1$	$488.1^{ab} \pm 32.8$	$0.34^{b} \pm 0.02$	$174^{b} \pm 22.1$	$0.94^{c} \pm 0.10$
SRPP 200 mg/kg	$2.10^{b} \pm 0.19$	$13.06^{b} \pm 2.6$	$679.6^{b} \pm 9.9$	$0.22^{a} \pm 0.01$	$208^{c} \pm 16.5$	$0.55^{ab} \pm 0.00$
Ranitidine	$2.16^{b} \pm 0.22$	$15.22^{b} \pm 1.2$	$505.5^{ab} \pm 35.5$	$0.39^{c} \pm 0.01$	$136^{a} \pm 12.1$	$0.92^{b} \pm 0.10$
Serum						
Healthy	$6.62^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9$	$0.221^{a} \pm 0.004$	$23.6^{c} \pm 3.0$	$0.16^{a} \pm 0.01$
Ulcerated	$6.84^{a} \pm 0.53$	$264.6^{d} \pm 32$	$22.90^{a} \pm 3.1$	$0.286^{c} \pm 0.02$	$11.1^{a} \pm 1.8$	$0.32^{d} \pm 0.02$
SRPP 100 mg/kg	$6.35^{a} \pm 0.59$	$201.1^{c} \pm 36$	$28.63^{b} \pm 2.3$	$0.298^{d} \pm 0.03$	$16.5^{b} \pm 2.1$	$0.261^{c} \pm 0.03$
SRPP 200 mg/kg	$6.95^{a} \pm 0.48$	$168.2^{b} \pm 21$	$40.12^{c} \pm 3.8$	$0.268^{b} \pm 0.03$	$19.8^{b} \pm 12.9$	$0.162^{a} \pm 0.01$
Ranitidine	$6.35^{a} \pm 0.63$	$196.3^{bc} \pm 23$	$30.82^{b} \pm 2.9$	$0.226^{a} \pm 0.02$	$12.8^{a} \pm 2.6$	$0.18^{b} \pm 0.01$
Liver						
Healthy	$24.2^{c} \pm 0.31$	$261.5^{b} \pm 41$	$28.42^{d} \pm 3.1$	$0.32^{a} \pm 0.02$	$414^{c} \pm 51$	$0.98^{a} \pm 0.13$
Ulcerated	$21.9^{a} \pm 0.23$	$142.4^{a} \pm 18$	$22.18^{bc} \pm 2.6$	$0.58^{c} \pm 0.05$	$221^{a} \pm 26$	$2.41^{d} \pm 0.23$
SRPP 100 mg/kg	$23.1^{b} \pm 0.28$	$164.2^{a} \pm 13$	$19.63^{bc} \pm 2.4$	$0.36^{ab} \pm 0.03$	$315^{b} \pm 36$	$1.84^{c} \pm 0.16$
SRPP 200 mg/kg	$23.9^{b} \pm 0.28$	$361.5^{d} \pm 39$	$15.54^{a} \pm 2.1$	$0.28^{a} \pm 0.02$	$214^{a} \pm 24$	$1.26^{b} \pm 0.11$
Ranitidine	$23.6^{b} \pm 0.26$	$314.4^{cd} \pm 36$	$17.34^{a} \pm 1.9$	$0.32^{a} \pm 0.02$	$254^{a} \pm 28$	$1.41^{b} \pm 0.12$

Table 3A.5. Effect of SRPP on antioxidant/antioxidant enzymes and TBARS levels in swim stress induced ulcer model: Values are mean ± SD, (n = 6). Range was provided by Duncan multiple range statistical test done at $P < 0.05$. Different letters **a** to **d** in each column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups. SOD-Superoxide dismutase; GSH- Reduced glutathione; TBARS- Thiobarbituric acid reactive substances.

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (µmol H_2O_2/mg/min)	Glutathione Peroxidase (ηmoles/mg)	GSH (ηmol/g tissue)	TBARS (ηmoles)
Stomach						
Healthy	$2.23^{a} \pm 0.21$	$09.86^{a} \pm 1.1$	$829.2^{c} \pm 41.6$	$0.21^{a} \pm 0.009$	$224^{d} \pm 23.2$	$0.31^{a} \pm 0.1$
Ulcerated	$2.32^{a} \pm 0.09$	$17.86^{c} \pm 2.4$	$201.5^{a} \pm 18.9$	$0.30^{c} \pm 0.01$	$102^{a} \pm 12.6$	$1.26^{d} \pm 0.3$
SRPP 100 mg/kg	$2.16^{a} \pm 0.16$	$16.21^{c} \pm 1.0$	$193.3^{a} \pm 62.5$	$0.26^{b} \pm 0.01$	$162^{b} \pm 15.5$	$0.92^{c} \pm 0.1$
SRPP 200 mg/kg	$2.41^{a} \pm 0.20$	$11.09^{b} \pm 1.0$	$540.5^{b} \pm 40.2$	$0.33^{c} \pm 0.02$	$196^{c} \pm 16.4$	$0.54^{b} \pm 0.1$
Ranitidine	$2.42^{a} \pm 0.19$	$12.42^{b} \pm 1.4$	$468.6^{c} \pm 31.6$	$0.22^{a} \pm 0.03$	$152^{b} \pm 16.3$	$0.96^{c} \pm 0.2$
Serum						
Healthy	$6.621^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9^{a}$	$0.221^{a} \pm 0.04$	$23.6^{d} \pm 3.0$	$0.16^{a} \pm 0.01$
Ulcerated	$6.525^{a} \pm 0.69$	$282.3^{d} \pm 26$	$28.36^{a} \pm 3.2^{b}$	$0.315^{c} \pm 0.03$	$09.6^{a} \pm 1.2$	$0.46^{d} \pm 0.03$
SRPP 100 mg/kg	$6.358^{a} \pm 0.70$	$228.4^{c} \pm 32$	$34.25^{ab} \pm 3.3^{b}$	$0.286^{b} \pm 0.03$	$18.6^{c} \pm 2.2$	$0.32^{c} \pm 0.04$
SRPP 200 mg/kg	$6.245^{a} \pm 0.56$	$172.3^{b} \pm 2$	$39.60^{b} \pm 4.51^{a}$	$0.243^{b} \pm 0.02$	$18.2^{c} \pm 1.9$	$0.18^{a} \pm 0.02$
Ranitidine	$6.321^{a} \pm 0.69$	$210.7^{c} \pm 28$	$34.12^{ab} \pm 4.6^{b}$	$0.252^{b} \pm 0.03$	$14.6^{b} \pm 1.6$	$0.21^{ab} \pm 0.02$
Liver						
Healthy	$24.2^{a} \pm 0.31$	$261.5^{b} \pm 1.1$	$28.42^{c} \pm 3.1$	$0.32^{b} \pm 0.02$	$414^{c} \pm 51$	$0.98^{a} \pm 0.13$
Ulcerated	$24.3^{a} \pm 0.31$	$118.1^{a} \pm 16$	$19.64^{b} \pm 2.2$	$0.48^{bc} \pm 0.03$	$392^{bc} \pm 41$	$2.98^{d} \pm 0.31$
SRPP 100 mg/kg	$23.5^{a} \pm 0.21$	$121.8^{a} \pm 15$	$18.32^{b} \pm 1.6$	$0.39^{b} \pm 0.03$	$268^{b} \pm 25$	$2.15^{c} \pm 0.22$
SRPP 200 mg/kg	$26.4^{a} \pm 0.41$	$325.4^{c} \pm 34$	$13.17^{a} \pm 1.6$	$0.29^{a} \pm 0.02$	$241^{ab} \pm 28$	$1.65^{b} \pm 0.14$
Ranitidine	$26.8^{a} \pm 0.29$	$254.5^{b} \pm 26$	$14.24^{a} \pm 1.8$	$0.31^{a} \pm 0.03$	$211^{a} \pm 28$	$1.61^{b} \pm 0.16$

Table 3A.6. Effect of SRPP on antioxidant/antioxidant enzymes and TBARS levels in ethanol induced ulcer model: Values are mean ± SD, (n = 6). Range was provided by Duncan multiple range statistical test done at $P < 0.05$. Different letters **a** to **d** in each column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups. SOD-Superoxide dismutase; GSH- Reduced glutathione; TBARS- Thiobarbituric acid reactive substances.

Groups	SOD (U/mg protein)	Catalase (µmol H_2O_2/mg/ min)	GSH (ηmol/g tissue)	TBARS (ηmol MDA/mg protein)
Healthy	$29.86^{c} \pm 3.7$	$914^{c} \pm 0.47$	$115.0^{b} \pm 12.6$	$0.32^{d} \pm 0.06$
Swim stress (SS)	$41.78^{a} \pm 4.8$	$623^{a} \pm 0.09$	$86.2^{a} \pm 16.4$	$1.09^{a} \pm 0.24$
BCPP 100 mg + SS	$32.58^{b} \pm 2.0$	$676^{a} \pm 0.15$	$111.9^{b} \pm 7.1$	$0.51^{b} \pm 0.09$
BCPP 200 mg + SS	$31.13^{b} \pm 2.0$	$998^{b} \pm 0.38$	$116.6^{b} \pm 9.6$	$0.52^{b} \pm 0.09$
Omeprazole 20 mg + SS	$32.63^{b} \pm 2.2$	$1125^{d} \pm 0.28$	$105.4^{b} \pm 6.0$	$0.60^{c} \pm 0.01$
Ethanol stress (ES)	$34.86^{a} \pm 4.2$	$508^{a} \pm 0.06$	$88.0^{a} \pm 10.5$	$0.78^{a} \pm 0.03$
BCPP 100 mg + ES	$36.14^{a} \pm 3.9$	$942^{b} \pm 0.31$	$100.8^{b} \pm 26$	$0.59^{b} \pm 0.03$
BCPP 200 mg + ES	$29.67^{b} \pm 5.5$	$1013^{d} \pm 0.34$	$106.2^{b} \pm 15$	$0.51^{c} \pm 0.06$
Omeprazole 20 mg + ES	$31.23^{b} \pm 3.7$	$963^{c} \pm 0.32$	$102.7^{b} \pm 35$	$0.59^{b} \pm 0.01$

Table 3A.7. Effect of BCPP on antioxidant status of stomach tissue during swim stress and ethanol induced ulcer models:
SOD-Superoxide dismutase; GSH- Reduced glutathione; TBARS-Thiobarbituric acid reactive substances.
Values are mean ± SD. Values are significantly different when compared between ulcer group with healthy control and BCPP/Omeprazole treated groups. Letters '**a** – **d**' in each column represent increased level of difference between groups, tested by DMRT at *P<0.05.*

Groups	SOD (U/mg protein)	Catalase (µ mol H_2O_2/ mg protein)	GSH (µ mol/mg protein)	TBARS (η mol MDA/ mg protein)
Healthy	$26.40^{b} \pm 0.99$	$170.6^{c} \pm 0.10$	$65.6^{c} \pm 0.08$	$0.562^{b} \pm 0.09$
Ethanol stress induced ulcer	$39.56^{a} \pm 0.81$	$80.3^{a} \pm 0.25$	$36.3^{a} \pm 0.12$	$0.914^{a} \pm 0.18$
HMBA 10mg + ES	$29.10^{b} \pm 0.74$	$110.3^{b} \pm 0.35$	$45.9^{b} \pm 0.04$	$0.648^{b} \pm 0.21$
HMBA 20mg + ES	$27.26^{b} \pm 0.46$	$120.2^{b} \pm 0.31$	$52.3^{b} \pm 0.05$	$0.470^{c} \pm 0.01$

Table 3A.8. Effect of HMBA on antioxidant status of stomach tissue during ethanol induced ulcer condition:
SOD-Superoxide dismutase; GSH- Reduced glutathione; TBARS-Thiobarbituric acid reactive substances; MDA- malondialdehyde.
Values are mean ± SD. Values are significantly different when compared between different group. Letters '**a** – **c**' in each column represent increased level of difference between groups, tested by DMRT at *P<0.05.*

A (SRPP)

Parameters	**Healthy**	**SRPP treated**
Total protein (mg/mL)	34.8[a] ± 3.22	36.18[a]± 2.81
SGOT (U/mg protein)	18.34[a] ± 1.55	16.22[a] ± 1.34
SGPT (U/mg protein)	21.31[a] ± 2.70	23.21[a] ± 2.29
ALP (U/mg protein)	35.52[a] ± 3.87	33.62[a] ± 2.95
TBARS (η moles/mg protein)	0.166[a] ± 0.08	0.186[a] ± 0.11

B (BCPP)

Parameters	**Healthy**	**BCPP treated**
Total protein (mg/mL)	46.37 ± 3.96	42. 53 ± 4.64
SGOT (U/mg protein)	19.60 ± 2.02	16.64 ± 1.42
SGPT (U/mg protein)	30.81 ± 3.52	28.64 ± 3.11
ALP (U/mg protein)	22.51 ± 2.14	23.62 ± 2.42
TBARS (η moles/mg protein)	0.325 ± 0.06	0.365 ± 0.09

C (HMBA)

Parameters	**Healthy**	**HMBA treated**
Total protein (mg/mL)	33.37 ± 3.43	38. 53 ± 2.64
SGOT (U/mg protein)	20.60 ± 2.12	23.64 ± 2.42
SGPT (U/mg protein)	27.81 ± 3.52	31.63 ± 4.21
ALP (U/mg protein)	29.31 ± 3.24	33.62 ± 3.42
TBARS (η moles/mg protein)	0.295 ± 0.03	0.315 ± 0.04

Table 3A.9. Toxicity studies of SRPP (A), BCPP (B) and HMBA (C): SGOT: serum glutamate oxaloacetate transaminase; SGPT : serum glutamate pyruvate transaminase; ALP: alkaline phosphatase; TBARS: thiobarbituric acid reactive substances. All data are the mean ± SD (*n*=6)

	SRPP	**BCPP**	**HMBA**
Carbohydrate content	>90%	>90%	-
Phenolic content	0.12%	0.026%	>90%

Table 3A.10. Percent composition of carbohydrate and phenolics in antiulcer compounds

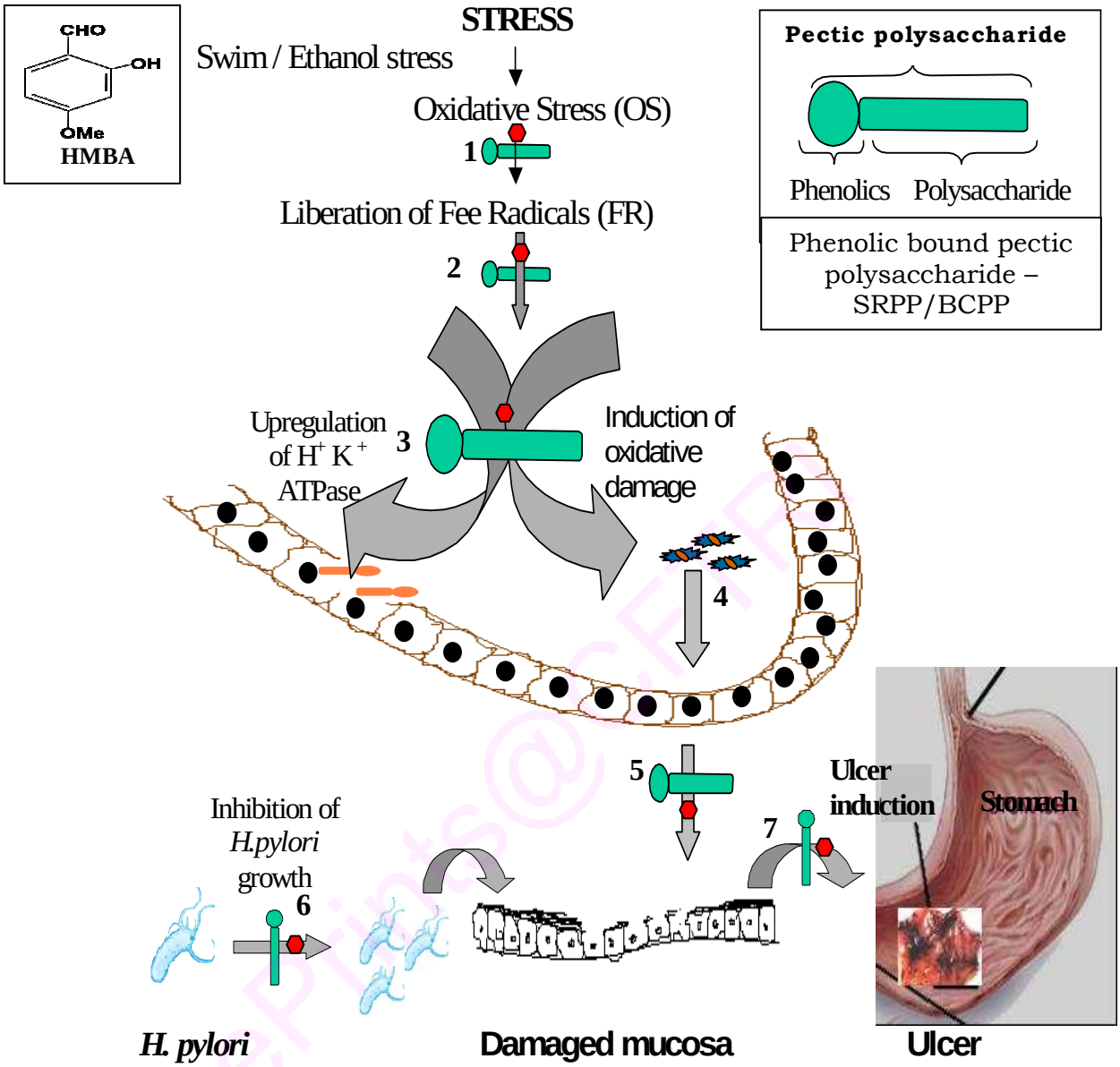


Fig 3A.8. Scheme representing various steps of ulcer pathogenicity and multi-step anti-ulcer action by phenol-bound pectic polysaccharides and HMBA: (●) and (▬) represents phenolic and polysaccharide portions of pectic polysaccharide **(●▬)** respectively and HMBA is represented by (●). Swim/Ethanol stress leading to OS (1) and liberation of FR (2). FR upregulated H^+, K^+-ATPase (3) and induce oxidative damage to mucosa (4) leading to mucosal damage (5). *H pylori* may invade on to damaged mucosa and together may cause ulcers (7). SRPP has the ability to inhibit steps 1-7 including the growth and colonization of *H. pylori* (6). (The data on anti-*H. pylori* activity is provided in chapter 4)

3A.6. Discussion

In recent days phytomedicines from medicinal plants and nutraceuticals from food sources are the attractive sources of new and natural drugs. However, the active ingredients, mode of action and *in vivo* efficacies have been rarely established, which is very crucial to understand the long-term potency of the antiulcer sources. Among the majority of identified sources – frequently, phenolics (Zayachkivaska et al., 2005; Reyes-Chilpa et al., 2006) and occasionally polysaccharides are implicated as antiulcer agents (Matsumoto et al., 2002; Ye et al., 2003; Gao et al., 2004). Higher levels of antioxidant properties in *D. hamiltonii* probably next to green tea with multiple compounds (Harish et al., 2005) may play a critical role in inhibiting oxidative stress induced mucosal damage in ulcers.

Gastric ulcers have multiple aetiopathogenesis. Stress ulcers are due to both physiological and psychological factors, which affect gastrointestinal defence (Gratrix et al., 2007). The increased accumulation of acid due to influx of H^+ into the lumen of the stomach by parietal cell's H^+, K^+-ATPase leading to autodigestion of the gastric mucosa (Goel & Bhattacharya, 1991) and generation of free radicals etc. adds to the complexities in pathogenesis. A study from Xie et al., (2005) showed that bilateral cervical vagotomy prevented the gastric mucosal lesion induced by swim stress. It suggested that swim stress induced gastric mucosal lesion is resulted from parasympathetic over activity and related increase in acid output is the basis of reduced mucosal resistance due to mucosal ischemia and oxidative stress injury.

Ethanol stress on the other hand is known to act on the gastric mucin directly affecting the mucosal defence. Ethanol damages the plasma membrane and leads to intracellular accumulation of sodium and water by increasing the membrane permeability. These changes ultimately cause cell death and gastric mucosal exfoliation (Desai et al., 1997). Ethanol is also known to release the endogenous ulcerogenic mediators. These could participate in mucosal injury either by causing vascular changes like mucosal edema and increased mucosal permeability or by non-vascular effects like mucus depletion and enzyme release in the stomach (Szabo et al., 1985).

Therefore an antiulcer compound should possess either or all of the defence mechanisms such as proton pump inhibition, increasing mucosal resistance by increased mucin synthesis, enhancement of antioxidative defence in addition to cytoprotection.

Nevertheless both swim stress and ethanol induced ulcer models cause ulcerations as depicted (Fig 3A.1, 3A.2, 3A.5) in the current study in addition to observations from other investigators also (Hollander et al., 1985; Miller, 1987). Our earlier studies indicated that phenolic antioxidants were efficient in inhibiting upregulated H^+, K^+-ATPase and in recovering the depleted levels of antioxidant and antioxidant enzymes (Naik et al., 2007).

In the current chapter, we studied the antiulcerogenic potential of a pectic polysaccharide with bound phenolics isolated from Swallow root (SRPP) and Black cumin (BCPP) and phenolic compound - HMBA isolated from Swallow root. In human nutrition, pectic polysaccharides play a key role as low energy foods and break down products have been known to have health beneficial properties.

The effect of SRPP, BCPP and HMBA on gastric ulcers induced by swim and ethanol stress was investigated *in vivo* in rat models. Oral administration of 100 and 200 mg/kg b.w. of SRPP/BCPP and 10 and 20 mg/kg b.w. of HMBA reduced gastric lesions as evidenced from the data (Fig 3A.1-3A.5). Swim and ethanol stress induced effects such as erosion, ulceration or gastric hemorrhages and perforations of the stomach were modulated by the inhibition of H^+, K^+-ATPase and enhancement of downregulated gastric mucin, antioxidants/antioxidant enzyme levels. Histological studies indicated characteristic changes of gastric mucosal damage (Fig 3A.6 and 3A.7) as indicated by damaged mucosal epithelium, disorganized glandular structures, inflammatory exudates, infiltration of leucocytes and cellular debris in swim and ethanol stress induced ulcer groups and reduction of these changes by SRPP (Fig 3A.6) and BCPP (Fig 3A.7) pretreated groups. Current data, together with the results of our previous paper (Naik et al., 2007), indicate clearly that phenolic antioxidants of SRPP may contribute to H^+, K^+-ATPase inhibition, rather than the polysaccharide per se since Swallow root antioxidants inhibited H^+, K^+-ATPase at 36 µg/mL as apposed to that of SRPP (77 µg/mL).

Ethanol induced gastric lesions are thought to arise as a result of direct damage of gastric mucosal cells, resulting in the development of free radicals and oxidation of lipid. Few reports showed that *Solanum nigrum* extract possesses significant antioxidant activity as one of the possible gastroprotective mechanisms against ethanol induced gastric ulceration (Jainu & Devi, 2006). Phenolic-bound pectic polysaccharides – SRPP and BCPP may also act similarly in reducing ulcerations, but with varied efficiency depending on their phenolic content.

As indicated in Table 3A.10 SRPP and BCPP contained exclusively carbohydrates along with lower levels of phenolics. HMBA on the other hand is exclusively phenolic component without any carbohydrates. It is intriguing to observe that despite significant differences in the composition, the net result of ulcer preventive results were good, although vary between SRPP/BCPP and HMBA to some extent.

SRPP and BCPP could show >80%, while HMBA could show up to ~63% gastroprotection (Fig 3A.3, 3A.4, 3A.5). Since both SRPP and BCPP contained covalently bound phenolics at the concentration of 12% (w/w) and 2.6% (w/w) respectively (Table 3A.10), it is imperative to understand the level of phenolics and carbohydrates under the conditions of the experiment. It is estimated that SRPP and BCPP contained 24 and 5.2 mg GAE phenolics/kg b.w. as opposed to 20 mg/kg b.w. of HMBA in experimental conditions. Despite ~4 folds lower concentrations of phenolics in BCPP than that of HMBA assuming similar rate of absorbability, ulcer preventive effect was greater in case of animals fed with polysaccharides (SRPP and BCPP) suggesting that both carbohydrate and phenolics may play a significant role in ulcer preventive activity. Carbohydrate potentiates the ulcer preventive ability. In addition, SRPP, BCPP and HMBA are safer sources since toxicity studies indicated no lethal effect on oral feeding for 14 d (Table 3A.9). To understand the potential role of SRPP and BCPP in mucosal protection, it is important to know that mucin is an insoluble adherent mucus gel, which is quite stable and has significant buffering capacity for neutralization of luminal acid in the presence of bicarbonate. Upregulation of gastric mucin by SRPP/BCPP and HMBA indicate the stabilization of mucosal layer (Table 3A.2-3A.4).

Very good recovery of gastric mucin (>80%) in SRPP and BCPP treated groups as opposed to ~66% in HMBA treated group (Fig 3A.6 & 3A.7 Table 3A.2 - 3A.4) suggest that polysaccharide may have a significant role in gastric mucin synthesis and may suggest that enhancement of mucin may be due to prevention or protection against mucosal injury during ulceration or it may be due to direct increase in the synthesis and this has been discussed and highlighted in Part B of chapter 3.

Further SRPP, BCPP and HMBA possessed H^+, K^+ -ATPase inhibitory and *H. pylori* inhibitory activities. Approximately 2 folds higher levels of H^+, K^+ -ATPase and *H. pylori* inhibitory activity in HMBA (49 and 39 µg/mL) as opposed to that of SRPP (77 and 150 µg/mL) and BCPP (170 µg/mL and no inhibition of *H. pylori*) may suggest the contribution of phenolics in preventing or modulating H^+, K^+ -ATPase/*H. pylori* activity than polysaccharides.

This opens up an understanding on the appropriate mechanism of phenolic-bound polysaccharides and phenolics per se. Data substantiate the previous report that a poorer antioxidant cinnamic acid in case of ginger similar to that of HMBA showed very good H^+, K^+ -ATPase and *H. pylori* inhibitory activity (Nanjundaiah et al., 2009). This could be due to better interaction of the compound with H^+, K^+ -ATPase and *H. pylori* membrane domains similar to that of cinnamic acid probably due to their hydrophobic nature. Polysaccharides due to their exclusive hydrophilic nature may not envisage such interactions and hence may exhibit poorer H^+, K^+ -ATPase and *H. pylori* inhibitory properties.

Although some phenolic components of SRPP and BCPP, i.e. gallic acid is a potent antioxidant, a poorer antioxidant activity of SRPP/BCPP could be due to the presence of higher levels of a poorer antioxidant - HMBA (28% of total phenolics with IC_{50} of 213 µg/mL) in SRPP and presence of insignificant levels of phenolics (26 mg/g) in BCPP. Data together may suggest that SRPP/BCPP and HMBA may exhibit potential ulcer preventive ability via two crucial mechanisms – amplification of mucoprotection and regression of aggressive factors (acid secretion, free radicals), which is important for ulcer preventive or gastroprotective properties of the two components phenolics and polysaccharides. Both may be responsible for ulcer preventive ability.

Polysaccharide may contribute significantly to mucoprotection by augmenting gastric mucin synthesis and; down regulation of aggressive factors (H^+, K^+ -ATPase and *H. pylori*) may be due to bound-phenolics of the polysaccharide. Potential inhibition of H^+, K^+ -ATPase and potent antioxidant activity (although poorer) of HMBA may also result in subsequent prevention of further damages to gastric mucosa and ulceration. Precise molecular mechanisms of antiulcer property of pectic polysaccharide and HMBA have been addressed in detail in part B of chapter 3 and chapter 4 respectively.

There were several schools of thoughts indicating that ulcer preventive/healing component must induce proliferation, amplify cell migration, and enhance angiogenesis etc., in order to enhance re-epithelialization and repair damaged mucosa. However, antiulcer compounds with proliferative ability, and the ability to enhance angiogenesis may be carcinogenic also. This statement is also substantiated by observation of induction of cancer upon the usage of antiulcer drugs on the long-term basis (Waldum et al., 2005). Antiulcer compounds thus should possess cell proliferation ability but regulated. In this context SRPP and BCPP although found to be antiulcerogenic had been shown to be anticancerous (Sathisha et al, 2007). Further chapter 3B provides evidence for regulated proliferation by SRPP/BCPP. Hence the treatment of ulcer by SRPP and BCPP even for longer period of time may not pose side effects.

Literature showed the importance of antioxidant nature of polysaccharide in providing gastroprotection by both phenolics and sulfate group containing polysaccharides (Zhang et al., 2004; Rees, 1991; Qi et al., 2005). In contrast to this observation, BCPP containing relatively less phenolic content - 26 mg GAE/g, when compared to 120 mg GAE/g of SRPP exhibited poorer antioxidant properties *in vitro* (Table 1.8 of Chapter 1); but the *in vivo* study showed protection to antioxidative system as potent as that of SRPP which is evidenced by estimations of GSH, TBARS, SOD and CAT in *in vivo* samples (Table 3A.7). The results may suggest that, BCPP may not directly be involved in scavenging of free radicals, but may be preventing the generation of stress itself by routes other than antioxidative mechanism also which in turn may prevent free radical formation.

Further it is also possible that pectic polysaccharides by virtue of their anionic nature may bind effectively to positively charged amino acid residues of gastric mucin as that of sucralfate and other polysaccharides (Rees, 1991). This binding may avoid gastric mucin damage and hence prevention of further ulceration.

Since the number of polysaccharide components isolated from different sources exhibited different level of gastroprotection against different ulcer induction models (Kiyohara et al., 1994; Gao et al., 2002; Yang et al., 2005; Cipriani et al., 2006; Nergard et al., 2006; Srikanta et al., 2007), a precise study on structure-function analysis was warranted to understand the mode of action of polysaccharide mediated changes during ulcer prevention. SRPP and BCPP are structurally different pectic polysaccharides. SRPP is having arabinogalacturonan backbone with arabinogalactan side chains; while BCPP a rhamnogalacturonan type-I pectic polysaccharide having arabinan and galactan side chains and different uronic acid content (SRPP 141 mg/g and BCPP 30 mg/g) showed equipotency as for as prevention of ulcers considered. The structure-function relationship of these polysaccharides was therefore addressed with prior importance in chapter 2.

The overall protection offered by SRPP and BCPP in terms of reduction in ulcer index, recovery in mucin content, reduction in H^+, K^+-ATPase activity and antioxidant status during ulcer prevention indicates that pectic polysaccharides from dietary sources may be better components for gastroprotection. In addition SRPP and BCPP are safer and promising multi-step ulcer blocking ability as shown in Fig 3A.8.

The outcome of this chapter may be represented schematically as in Fig 3A.8. Both phenolics and polysaccharides have the potential to inhibit multiple steps of ulcer pathogenicity as highlighted in the Fig 3A.8, although to varied degree. Inhibition of oxidative stress/generation of free radicals, inhibition of upregulated H^+, K^+-ATPase and damage of mucosal layer due to increased acidity and free radicals; potential inhibition of *H. pylori* growth and invasion into damaged mucosal layer (Data shown in Chapter 4) may be envisaged to prevent gastric ulcers. Further, inhibitory effect of ulcer initiation steps leading to further blockade of ulcer pathogenicity from the components mentioned – SRPP/BCPP and HMBA may not be ruled out.

3A.7. Summary and conclusions

- Chapter 3A addresses *in vivo* ulcer preventive effect of SRPP, BCPP and HMBA.
- Results indicated that pectic polysaccharides - SRPP & BCPP showed a good gastroprotection at 100 mg/kg b.w. and 200 mg/kg b.w. in a dose dependent manner in both swim stress and ethanol induced ulcer models. Whereas phenolic compound –HMBA showed moderate (~64%) gastroprotection in ethanol induced ulcer model.
- SRPP and BCPP showed reduction of ulcer index up to 80-85% and 73-77% respectively.
- Mucosal protection is evidenced by 78-87% recovery of mucin by SRPP and 83-85% recovery by BCPP pretreatment. Histopathological studies of gastric tissue confirm the gastro/muco protective ability of SRPP & BCPP.
- Both SRPP and BCPP inhibited ~40 to 60% of H^+, K^+-ATPase activity in a dose dependent manner, which was upregulated in ulcerated animals.
- Antioxidant nature due to bound phenolics of SRPP and BCPP is supported by normalization of antioxidant status of animals that were altered during swim stress and ethanol induced ulceration in rats.
- Overall data suggest that SRPP and BCPP may exhibit potential ulcer preventive ability via two crucial mechanisms such as amplification of mucoprotection and regression of aggressive factors (acid secretion, free radicals), which are important for ulcer prevention and gastroprotection. HMBA however exhibited gastroprotection mainly by inhibiting H^+, K^+-ATPase activity.

Part B

Ulcer healing mechanism of SRPP and BCPP in acetic acid induced ulcer model

3B.4. Materials and Methods

3B.4.1. Chemicals

COX-2 monoclonal antibody was obtained from Cayman chemical, USA, Purified mouse anti-human galectin-3 antibody was from BD Pharmingen, USA, MMP-2 and MMP-9 monoclonal antibodies were from Santacruise, USA. Horse radish peroxidase conjugated – rabbit anti-mouse IgG secondary antibody and TMB/H_2O_2 were procured from GENEI, Bangalore, India. GenElute™ Mammalian Total RNA Miniprep Kit, galectin-3 gene specific primers, Taq polymerase, dNTP mix and other PCR reagents, Anti-mitogen activated protein kinase (ERK-1 & ERK-2) antibodies, Prostaglandin E_2, alcian blue, 2-thiobarbituric acid (TBA), reduced glutathione (GSH), 1,1,3,3-tetramethoxypropane (TMP), bovine serum albumin (BSA), Tris–HCl, Triton X-100, periodic acid and gelatin were purchased from Sigma Chemical Co. USA (St. Louis, MO). RevertAid™ First strand cDNA synthesis kit was purchased from Fermentas, Genetix Biotech Asia Private Limited, India. Tween-20, other solvents and chemicals used were of the analytical grade purchased from Sisco Research Laboratories, Mumbai, India.

3B.4.2. Animals and experimental design

Wistar albino rats weighing about 180-220 g maintained under standard conditions of temperature, humidity and light were provided with standard rodent pellet diet (Amruth feeds, Bangalore, India) and water *ad libitum*. The study was approved by the institutional ethical committee, which follows the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. 49, 1999), Government of India, New Delhi, India.

Two sets of experiments were designed individually; one set each for SRPP and BCPP. And each set contains different groups as follows:

In each set, animals were categorized into 5 groups of 6 animals each - namely healthy control, ulcer 5 days, ulcer 10 days controls (ulcer induced with acetic acid, allowed an auto-healing for 5 d and 10 d respectively), ulcer + pectic polysaccharide 5 d and ulcer + pectic polysaccharide 10 d (ulcer induced with acetic acid; treated for 5 d and 10 d respectively with pectic polysaccharides - SRPP and BCPP in two different sets). Gastric ulcers were induced by acetic acid in all the animals except healthy control group. One day after ulcer induction, SRPP and BCPP at 200 mg/kg b.w. were administered orally to respective groups for 5 d and 10 d. Concentration of 200 mg/kg b.w. was selected based on our earlier experiments.

3B.4.3. Induction of gastric ulcers by acetic acid injection

Acetic acid induced ulcer model was employed for ulcer healing study, since it resembles human ulcers in its pathogenesis and healing mechanisms. Gastric ulcers were induced by luminal application of acetic acid as described by Tsukimi and Okabe (1994). Briefly, rats were starved for 18 h before inducing ulcers and laparotomy was done under ether anesthesia via midline gastric incision. The stomachs were clamped with round forceps of 10 mm internal diameter. 120 µL of acetic acid solution (60% v/v in water) was injected into the clamped region and withdrawn into the syringe after 50 sec. The abdomens were then closed and the rats were allowed to recover with free access to food and water. One day after ulcer induction, ulcer + SRPP 5 d and ulcer + SRPP 10 d groups were orally administered with SRPP at 200 mg/kg b.w. for 5 d and 10 d respectively. Similarly ulcer + BCPP 5 d and ulcer + BCPP 10 d groups were orally administered with BCPP at 200 mg/kg b.w. for 5 d and 10 d respectively. Rats of healthy control and ulcer control (autohealing) groups were treated with normal saline. At the end of 5 and 10 d treatments, rats were sacrificed; stomachs were removed and ulcer area was measured. Gastric tissues were fixed in 10% buffered formalin for histological analysis.

3B.4.4. Histological analysis

After measuring the ulcer size, the fundic stomach was sectioned for histological studies. The tissue samples were fixed in 10% formalin and embedded in paraffin. The sections (5 µm) were cut using microtome. One set of sections were stained with haematoxylin and eosin (Morise et al.,

1998), and another set was stained with periodic acid-Schiff's reagent (McManus, 1946), and assessed under Olympus microscope for the integrity of tissue layers & pathological changes and photographed for documentation purpose.

3B.4.5. Analysis of alterations in gastric mucin during ulceration and healing process

Gastric mucin, a protective layer of gastric epithelium has been known to be affected during ulcer pathogenesis. Histological and biochemical alterations in gastric mucin were therefore performed to ensure ulcer healing. Gastric mucin was analyzed by alcian blue binding, electrophoresis and histological methods as follows.

3B.4.5.1. Quantitative estimation of gastric mucin

Adhered mucus content of the fundic part of the stomach was determined by alcian blue dye binding method as described in section 3A.4.5 of chapter 3A.

3B.4.5.2. Analysis of gastric mucin damage by polyacrylamide gel electrophoresis (PAGE)

The gastric mucin was extracted from the stomach as described by Mall et al. (2002). Briefly, mucus was gently scraped from the stomach of healthy control, ulcer induced and pectic polysaccharide treated groups, homogenized with a buffer containing 20 mmol/L sodium phosphate, 5 mmol/L EDTA, 5 mmol/L N-ethylmaleimide and 6 mmol/L guanidinium chloride, at pH 6.5 and centrifuged at 10,000 *g*. The resultant supernatant was analyzed for mucin. Equal concentrations of samples were resolved on SDS-PAGE with a buffer containing 0.2% sodium dodecyl sulphate using a 3.5% (w/v) stacking gel and 7.5% separating gel. The gel was stained for mucin with the periodic acid Schiff's (PAS) reagent (mantle & Allen, 1978) as follows.

After electrophoresis, the gel was removed and fixed in 12.5% trichloroacetic acid solution for 30 min and transferred to 1% periodic acid solution for 50 min at RT in dark condition. After incubation, the gel was washed repeatedly to remove the traces of periodic acid and immersed the gel in Schiff's reagent (0.8% potassium metabisulphite, 10.5 mL of HCl and 0.4% of basic fuschin in 1 L of water) for 60 min at 4 ^{0}C. Finally the gel was immersed in 7%

glacial acetic acid to remove the excess stain and observed the pink colour glycoprotein bands and photographed.

3B.4.6. Evaluation of antioxidant status

Since induction of gastric ulcer by acetic acid generates free radicals and oxidative stress in the stomach, the antioxidant status of stomach tissue was determined by estimating the level of glutathione (antioxidant molecule), Thiobarbituric acid reactive substances - TBARS (Lipid peroxidation products) and protein carbonyls (protein oxidation).

Glutathione and TBARS in the stomach tissue were measured as described previously in section 3A.4.7.5. and 3A.4.7.6. of chapter 3A.

3B.4.6.1. Measurement of protein carbonyl content

Protein oxidation was measured as carbonyl content in the low speed supernatant of the fundic stomach homogenate (Levine et al., 1990). The fundic stomach from healthy control, ulcerated, and pectic polysaccharide treated groups were homogenized in 50 mM sodium phosphate buffer, pH 7.4, in a Potter-Elvehjem glass homogenizer for 2 min to get 20% homogenate. After centrifugation at 600 *g* for 10 min, the proteins from 1.0 mL of the supernatant were precipitated with 10% trichloroacetic acid and allowed to react with 0.5 mL of 10 mM 2,4-dinitrophenylhydrazine for 1 h. After precipitation with 20% trichloroacetic acid, the protein was washed thrice with a mixture of ethanol-ethyl acetate (1:1, v/v), dissolved in 1.0 mL of a solution containing 6 M guanidine HCl in 20 mM potassium phosphate adjusted to pH 2.3 with trifluoroacetic acid, and centrifuged at 3000 *g*. The supernatant was read for carbonyl content at 362 nm and calculated the amount of protein carbonyl content using molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

3B.4.7. Estimation of prostaglandin E_2

The prostaglandin E_2 was extracted from the stomach of all the groups as described by Rajakrishnan et al. (2000). Briefly, stomach tissue was homogenized with ice-cold mixture of chloroform/methanol (1:1, v/v), centrifuged at 6000 *g* for 10 min at 4 ^{0}C and the supernatant was evaporated using nitrogen gas. Ethanol (1.5 mL) and water (10 mL) were then added and acidified with 1 N HCl, and passed through sep-pak

cartridges (waters C_{18} column 10 x 10 mm). The column was eluted successively with ethanol/water (15:85, v/v), water, petroleum ether and ethyl acetate. The ethyl acetate fraction was evaporated under nitrogen, redissolved in mobile phase and analyzed by HPLC system (model LC-10A, Shimadzu) consisting of Sphereclone 5µ ODS2 column (4.6 x 150 mm, Shimadzu) using a diode array UV-detector (operating at λmax 200 nm). A solvent system consisting of acetonitrile : 0.0174 M orthophosphoric acid (40 : 60, v/v), pH 3 was used as mobile phase. Retention time of the peak was compared with that of standard prostaglandin E_2.

3B.4.8. Analysis of matrix metalloproteinases by gelatin zymography

The fundic part of the gastric mucosa was suspended in phosphate-buffered saline minced, and incubated for 10 min at 4 ^{0}C. After centrifugation at 12,000 *g* for 15 min the supernatant was discarded. The pellet was extracted in the lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton-X-100) and centrifuged at 12,000 *g* for 15 min. Tissue extracts were preserved at -70 ^{0}C and used for future studies.

For the assay, mucosal extracts were electrophoresed in SDS-polyacrylamide gel containing 1 mg/mL gelatin under nonreducing conditions. The gels were washed in 2.5% Triton X-100 and incubated in a buffer containing 40 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$ for 18 h at 37 ^{0}C and stained with 0.1% Coomassie Blue followed by destaining (Baragi et al., 1997). The zones of gelatinolytic activity were observed as unstained regions.

3B.4.9. Quantitative estimation of galectin-3

Galectin-3 was estimated in stomach homogenate by ELISA method. Monoclonal anti-human galectin-3 antibody (BD Pharmingen, USA) was employed at 1:1000 (v/v) dilution as primary antibody. Alkaline phosphatase conjugated rabbit anti-mouse IgG (GENEI, Bangalore, India) at 1:5000 (v/v) dilution followed by paranitrophenylphosphate (p-NPP) were used as secondary antibody and substrate respectively for ELISA. The values were expressed as mg of galectin-3 per mg protein (Sathisha et al., 2007).

3B.4.10. RT-PCR analysis of galectin-3

Total RNA from rat stomach tissue was extracted using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and RNA concentration

and purity were determined by measuring absorbance at 260 and 280 nm. The first strand cDNA was generated with 5 µg total RNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's protocol.

PCR was performed using galectin-3 specific primer, with the primer sequences as follows; forward: 5' CATATGGCAGACAATTTTTCGCTC 3' and reverse: 5' GGATCCTTATATCA TGGTATATGAAGCAC 3'. The PCR mixture (25 µL) contained 2 µL of cDNA prepared, 1X PCR buffer, 2.5 mM of dNTPs and 1 unit of Taq DNA polymerase and 25 pmoles of each primer. PCR for galectin-3 gene was performed at initial denaturation at 94 ^{0}C for 3 min followed by 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 55 ^{0}C for 1 min and extension at 72 ^{0}C for 1.30 min with a final extension of 72 ^{0}C for 10 min (Zhuang *et al.*, 2008). The thermal cycler used was PeQLab Primus96 advanced Gradient PCR system (PEQLAB Biotechnologie GmbH, Deutschland).

A 10 µL aliquot of the PCR product was analyzed by 1.5% agarose gel electrophoresis. The size of the galectin-3 amplicon was verified by comparing with a 100bp ladder which was used as a molecular size marker. Bands were stained with ethidium bromide and observed under UV light transilluminator (Hero-Lab Gmbh. Germany) and documented.

3B.4.11. Western blot analysis: COX-2, MAP kinase (ERK-1 & 2), MMP-2, MMP-9 and galectin-3.

Western blot analysis was performed following the method described previously (Pai et al.,1998). Tissue lysates containing equal amounts of proteins (75 µg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane in transfer buffer (25 mM tris, 125 mM glycine and 20% methanol in water). Blots were stained with Ponceau S stain to ensure equal loading and complete transfer of proteins. The membrane containing the transferred proteins was incubated with blocking buffer (3% gelatin in tris buffer saline), subsequently washed, and incubated with specific primary antibodies for, COX-2 (1:1000, v/v), MAP kinase - ERK-1 & 2 (1:20,000, v/v), MMP-2 (1:1000, v/v), MMP-9 (1:1000, v/v) and galectin-3 (1:1000, v/v) overnight at 4 ^{0}C. Blots were then washed with tris buffer saline containing 0.1% tween-20. Blots were then incubated with specific

peroxidase-conjugated secondary antibodies IgG for 2 h at RT. After washing with wash buffer, bound antibody was visualized by providing substrate TMB/H_2O_2. The density of the protein bands were analyzed using ImageJ software.

3B.4.12. Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed using one way ANOVA followed by Duncan's multiple comparison test. Data was computed for statistical analysis by using SPSS statistical software. A *P* value of < 0.05 was considered to be statistically significant.

3B.5. Results

3B.5.1. Ulcer healing effect of SRPP and BCPP

Acetic acid could induce prominent gastric ulcers in ulcer control (or autohealing) groups, where no pectic polysaccharide treatment was provided. Area of the ulcer was more than 70 mm and 50 mm after 5 d and 10 d of acetic acid injection respectively (Fig 3B.1b,c & Fig 3B.2B,C). Results show that, there was only a little spontaneous healing in ulcer control group even after 10 d of ulcer induction. The group of rats treated with SRPP for 5 d (Fig 3B.1d) and 10 d (Fig 3B.1e) after ulcer induction showed ~70% and nearly complete (~90%) healing of acetic acid induced ulcers, when compared to their respective ulcer control groups as depicted in Fig 3B.1B. In case of BCPP, it also showed increased healing of 63% and 80% after 5 d and 10 d treatment when compared to ulcer control groups as shown in Fig 3B.2F.

3B.5.2. Histological analysis of gastric mucosa

Both H & E and PAS stained sections of stomach tissues were analyzed to understand the histological changes induced by acetic acid and restoration of normal structures by pectic polysaccharide (PP) treatment during ulceration and healing. Fig 3B.3 and 3B.4 shows an uninterrupted mucosal layer with regular glandular arrangements, dense and uniform mucin producing cells in healthy rats. Acetic acid injection showed clear ulcerations and completely damaged gastric mucosa in both 5 d and 10 d ulcer controls. The 5 d ulcer control showed an ulcer margin with granulation tissue at the base of the ulcer and there was no healing process. In case of 10 d ulcer control, the tissue architecture showed initiation of healing zone at ulcer margin as indicated by migrating epithelial cells towards granulation tissue, proliferating cells and formation of small glandular structures, essential processes for the regeneration of gastric mucosa. The rate of healing in ulcer control groups was very slow when compared to either SRPP or BCPP treated groups. PP treatment showed fast healing as indicated by regeneration of mucosal tissue in the ulcerated region, by inducing cell migration, re-epithelialization, glandular development and reduced inflammatory exudates. SRPP showed a rapid healing with well formed glandular structures (Fig 3B.3), whereas BCPP (Fig 3B.4) showed more than 80%

recovery of mucosal structure after 10 d BCPP treatment, but there was still no clear and compact glandular arrangement when compared to that of SRPP treated group.

3B.5.3. Analysis of changes in gastric mucin

Quantitative evaluation of gastric mucin in healthy, ulcer control and PP treated rat stomachs was done by alcian blue dye binding assay. Since alcian blue is a dye which binds to intact gastric mucin, any damage or reduction in gastric mucin results in reduction of dye binding to stomach tissue. More than 70% reduction in dye binding to ulcer control stomachs when compared to that of healthy control (Fig. 3B.5 & 3B.7) was observed. Both SRPP (3B.5A) and BCPP (3B.7A) treated groups showed increase in dye binding which indicates more than 90% recovery in mucin content revealing the mucin protective effect of pectic polysaccharides.

Further, changes in gastric mucin due to acetic acid and effect of SRPP on gastric mucin damage was analyzed by SDS-PAGE electrophoresis followed by staining with PAS reagent (Fig. 3B.5B). An intact large molecular weight glycoprotein band at the start of the running gel in healthy control and SRPP 10 d treated samples; while a lowered level large molecular weight (~180 kDa) glycoprotein band was observed in ulcer control and 5 d SRPP treated groups of rats. In addition to this band a small molecular weight of ~55 - 65 kDa was observed in only ulcer control groups but not in either healthy control or SRPP treated groups.

3B.5.4. Effect of SRPP and BCPP on PGE_2 synthesis

Since PGE_2 is mainly involved in synthesis of gastric mucin and ulcer healing process, level of PGE_2 was estimated in stomach tissue in rats of all groups. PGE_2 was significantly decreased in the beginning (5 d), but after 10 d of ulcer induction there was an insignificant increase in the level of PGE_2 in ulcer controls (or autohealing) groups when compared to that of healthy controls. In case of SRPP and BCPP treated groups, significantly increased PGE_2 concentration was observed during the specified time course (5 and 10 d) of ulcer healing process (Fig. 3B.6A & 3B.7B). The increase in PGE_2 concentration in SRPP and BCPP treatment was 2 to 5 folds when compared to those of healthy rats.

3B.5.5. Modulation of MMPs by SRPP and BCPP

Matrix metalloproteinase plays a major role during ulceration and ulcer healing, particularly in the remodeling and reassembly process. MMP activity was determined by the method of gelatin zymography. In the second set of experiments (BCPP set) in addition to zymography, western blot analysis of MMP-2 and MMP-9 was performed. The zymography result (Fig 3B.6B & 3B.7C) showed an increased level of both pro and active forms of MMP-2 in ulcer control groups in addition to pro-MMP-9 in ulcer 5 d group. SRPP treatment (Fig 3B.6B) for 5 and 10 d after ulcer induction significantly reduced these MMPs. Zymography results of BCPP also showed similar results (Fig 3B.7C). Fig 3B.8B showed increased level of MMP-2 in ulcer controls, which is further increased in 5 d BCPP treated group and again downregulated after 10 d of BCPP treatment. In case MMP-9 (Fig 3B.8C) ~2 folds increase in MMP-9 protein in ulcer control group, down regulated to normal level in a time dependent manner was observed. Thus the results suggest the differential role of MMP-2 & 9 during ulcer pathogenesis and healing process and also the effect of SRPP and BCPP on these processes.

3B.5.6. Modulation of galectin-3 level by SRPP and BCPP

Galectin-3 involved in inflammatory reactions has been believed to play a role during ulceration; and it also acts as a substrate for MMPs. Therefore expression of galectin-3 was estimated by ELISA in the first set and western blot and mRNA expression in the second set of experiments. In the first set (Fig 3B.6C), the study showed up to ~2 folds increase in galectin-3 level as measured by ELISA method, during ulcer condition suggesting no significant protection due to galectin-3 mediated inflammatory reactions during auto-healing process, even during auto-healing condition of up to 10 d. SRPP treatment however significantly reduced galectin-3 level (Fig 3B.6C), indicating the role of SRPP as a blocker of galectin-3 mediated inflammation.

In the second set, we have analyzed the expression of galectin-3 at protein and mRNA levels by western blot and RT-PCR methods. The results presented in Fig 3B.9, show a slight increase in protein and significant increase in mRNA of galectin-3 in ulcer control groups, which is downregulated both at protein and mRNA levels in BCPP treated groups indicating the galectin-3 inhibitory role of BCPP similar to that of SRPP.

3B.5.7. Modulation of COX-2

Current studies attempted to determine the COX-2 and MAP kinase (ERK-1 & 2) levels which are involved in the cascade of PGE_2 production and ulcer healing process. Western blot analysis of COX-2 (Fig 3B.8A) showed that, there is a slight increase of COX-2 protein in ulcer control groups as an indication of autohealing mechanism; this was however further upregulated depending on duration of BCPP treatment in 5 d and 10 d groups when compared to that of healthy group. This result supports the upregulation of PGE_2 and gastric mucin in sample treated groups.

3B.5.8. Modulation of MAP kinase (ERK-1 & 2)

Since COX-2 expression during ulcer healing is MAP kinase (ERK-2) mediated (Jones et al., 1999) phenomenon, the expression pattern of MAP kinase (ERK-1 & 2) in various groups of animals were studied. Results (Fig 3B.8D,E) showed marginal changes of MAP kinase in 5 d ulcer control; while significant increase in 10 d ulcer control ($p < 0.01$) and sample treated groups ($p < 0.01$) when compared to healthy rats indicating the involvement of ERK-1 & 2 in pectic polysaccharide (BCPP) mediated healing of acetic acid induced gastric ulcers.

The western blot analysis of COX-2, MMP-2 & 9 and MAP kinase (ERK-1 & 2) in SRPP treated samples were not analyzed due to non-availability of specific antibodies during experimentation on SRPP.

3B.5.9. Modulation of oxidative status during ulcer healing

Since protein oxidation and lipid peroxidation occurs during acetic acid induced gastric ulcers which delays the healing process, protein carbonyls level was determined as an indication of protein oxidation and; TBARS as an indication of lipid peroxidation. Antioxidant molecule GSH in stomach tissues of all the rats was also measured. Results in Table 3B.1 & 3B.2 showed decreased antioxidant status in ulcer control groups as evidenced by significantly decreased level of reduced glutathione, ~2-3 folds increase in TBARS level and ~7-9 folds increase in protein carbonyl content, when compared to that of healthy control indicating severe oxidative stress condition. Treatment with SRPP (Table 3B.1) or BCPP, (Table 3B.2) showed dose dependent normalization of antioxidant status when compared to ulcer control groups.

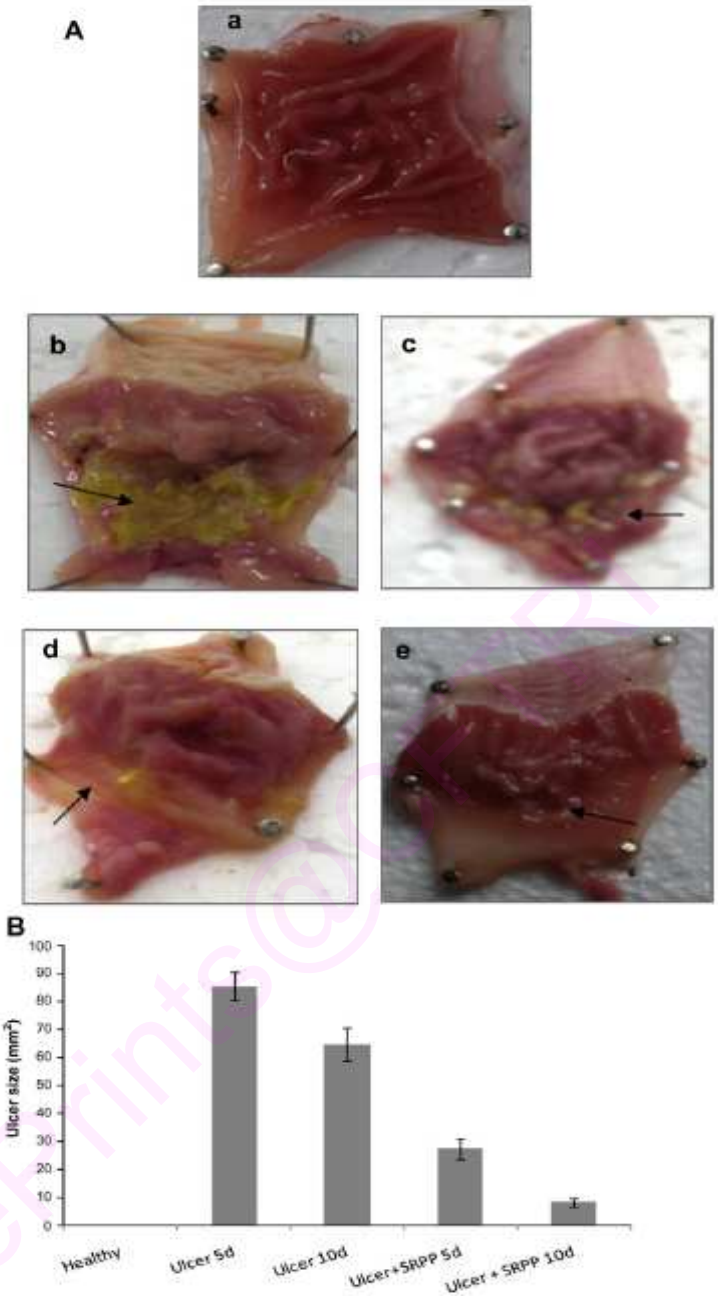


Fig 3B.1. Ulcer healing effect of SRPP in acetic acid induced ulcer model: Macroscopic pictures of stomach tissue (A) and ulcer size (B). Macroscopic observation of ulcer lesions in ulcer induced and protected stomachs. Ulcer was induced in animals by acetic acid solution as described under materials and methods. Healthy control without any treatment showing no ulcer lesion (Aa). A prominent ulcer area was observed after 5 d of acetic acid injection (Ab), and insignificant reduction in ulcer area even after 10 d of auto-healing (Ac). Oral feeding of SRPP at 200 mg/kg b.w. for 5 d and 10 d to acetic acid induced ulcerous rats showed significant reduction in ulcer area (Ad & Ae respectively). Ulcerated area was indicated by arrows. **Fig B** shows graphical representation of ulcer area of ulcerated and protected stomach. Ulcer area was measured according to the procedure explained under materials and methods. Values are mean ± SD (n=6).

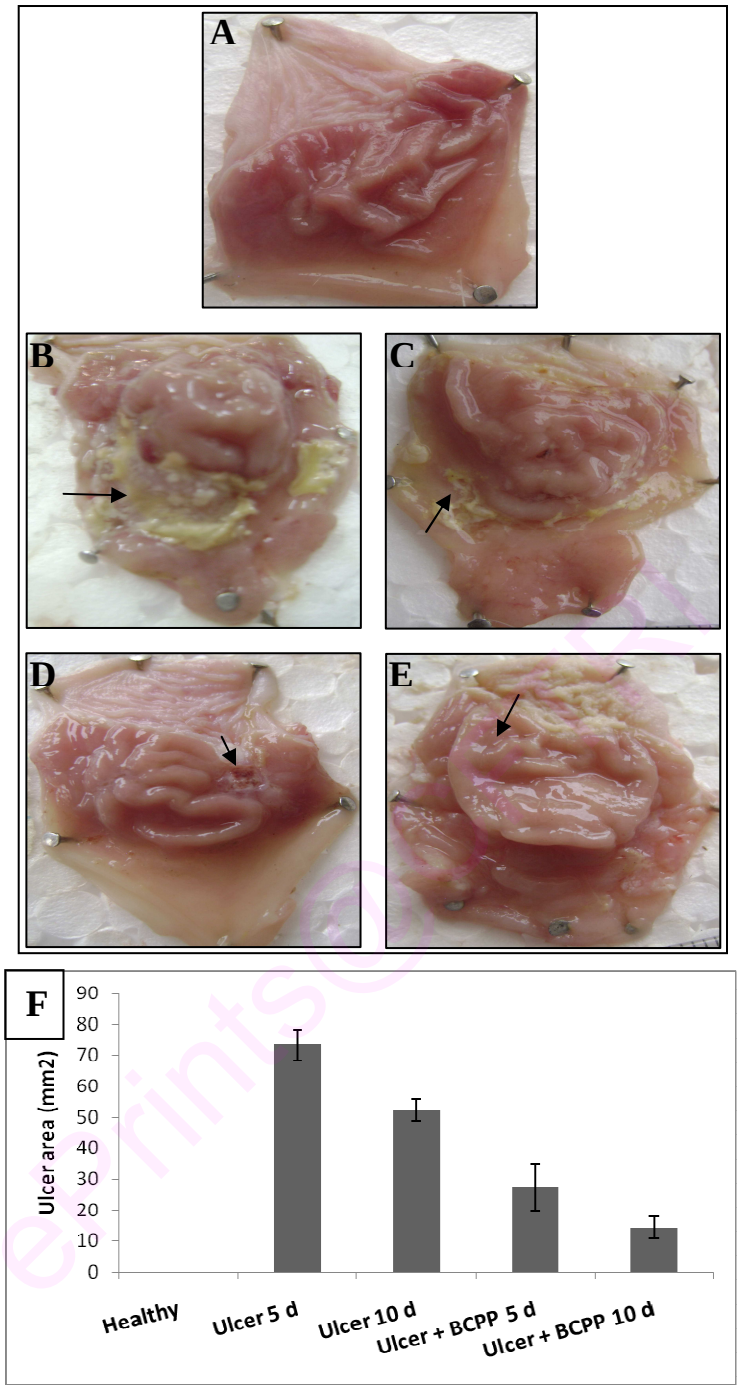


Fig 3B.2. Macroscopic pictures of stomach tissue showing ulcer healing effect of BCPP in acetic acid induced ulcer model: Ulcer was induced in animals by acetic acid solution. Healthy control without any treatment showing no ulcer lesion (A). A prominent ulcer area was observed after 5 d of acetic acid injection (B), and insignificant reduction in ulcer area even after 10 d of auto-healing (C). Oral feeding of BCPP at 200 mg/kg b.w. for 5 d and 10 d to acetic acid induced ulcerous rats showed significant reduction in ulcer area (D & E respectively). Ulcerated area was indicated by arrows. **Fig F** shows graphical representation of ulcer area of ulcerated and protected stomach. Values are mean ± SD (n=6).

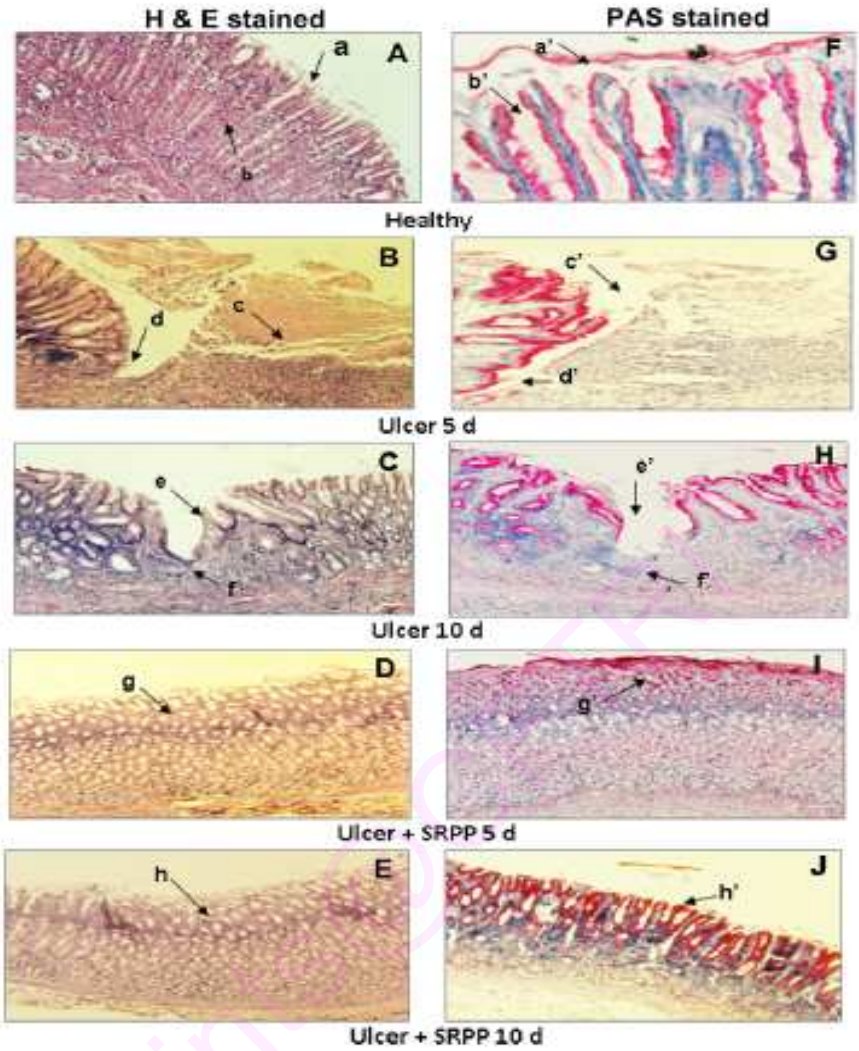


Fig 3B.3. Histopathology of stomach tissue showing ulcer healing effect of SRPP in acetic acid induced ulcer model:. Figure **A-E** showing haematoxylin and eosin stained and **F-J** showing periodic acid-Schiff's reagent stained sections of stomach tissues. Healthy control **(A & F)** shows intact mucosal layer (a), well-organized glandular structures (b) with continuous mucin layer (a') and mucin producing cells (b') along the glands. Stomach shows eroded mucosal layer (c & c') with ulcer lesion penetrating deep into muscularis layer (d) and loss of mucin synthesizing cells (d') was observed after 5 d of acetic acid injection (B & G). Stomach of auto-healing group after 10 d of acetic acid injection (C & H) shows slightly reduced size of ulcer lesion (e & e') with the formation of ulcer base and granulation tissue (f & f'). SRPP treatment for 5 d to ulcer induced rats (D & I) showing completely healed ulcer lesion (g) with mucin synthesizing cells (g'), but there is no glandular organization. Increased duration of SRPP treatment for 10 d to ulcer induced rats (E & J) showing complete recovery with well organized glandular structure (h & h').

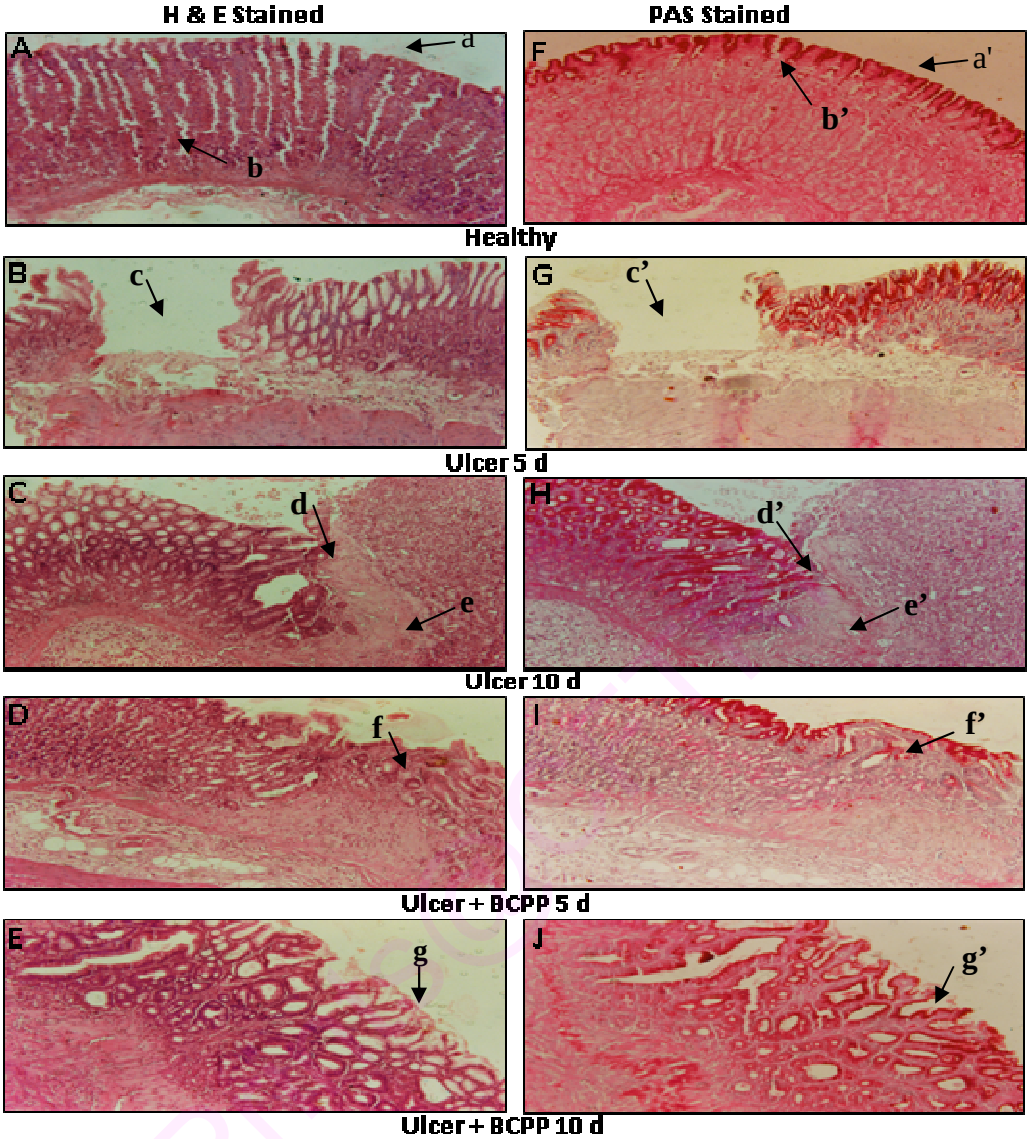


Fig 3B.4. Histopathology of stomach tissue showing ulcer healing effect of BCPP in acetic acid induced ulcer model: Figure **A-E** showing haematoxylin and eosin stained and **F-J** showing periodic acid-Schiff's reagent stained sections of stomach tissues. Healthy control **(A & F)** shows intact mucosal layer (a), well-organized glandular structures (b) with continuous mucin layer (a') and mucin producing cells (b') along the glands. Stomach shows eroded mucosal layer (c & c') with ulcer lesion penetrating deep into muscularis layer and loss of mucin synthesizing cells was observed after 5 d of acetic acid injection (B & G). Stomach of auto-healing group after 10 d of acetic acid injection (C & H) shows slightly reduced size of ulcer lesion (d & d') with the formation of ulcer base and granulation tissue (e & e'). BCPP treatment for 5 d to ulcer induced rats (D & I) showing rapidly healed ulcer lesion (f) with increased mucin synthesizing cells (f'), but there is no glandular organization. Increased duration of BCPP treatment for 10 d to ulcer induced rats (E & J) showing upto 80% healing with formation of new glandular structure (g & g').

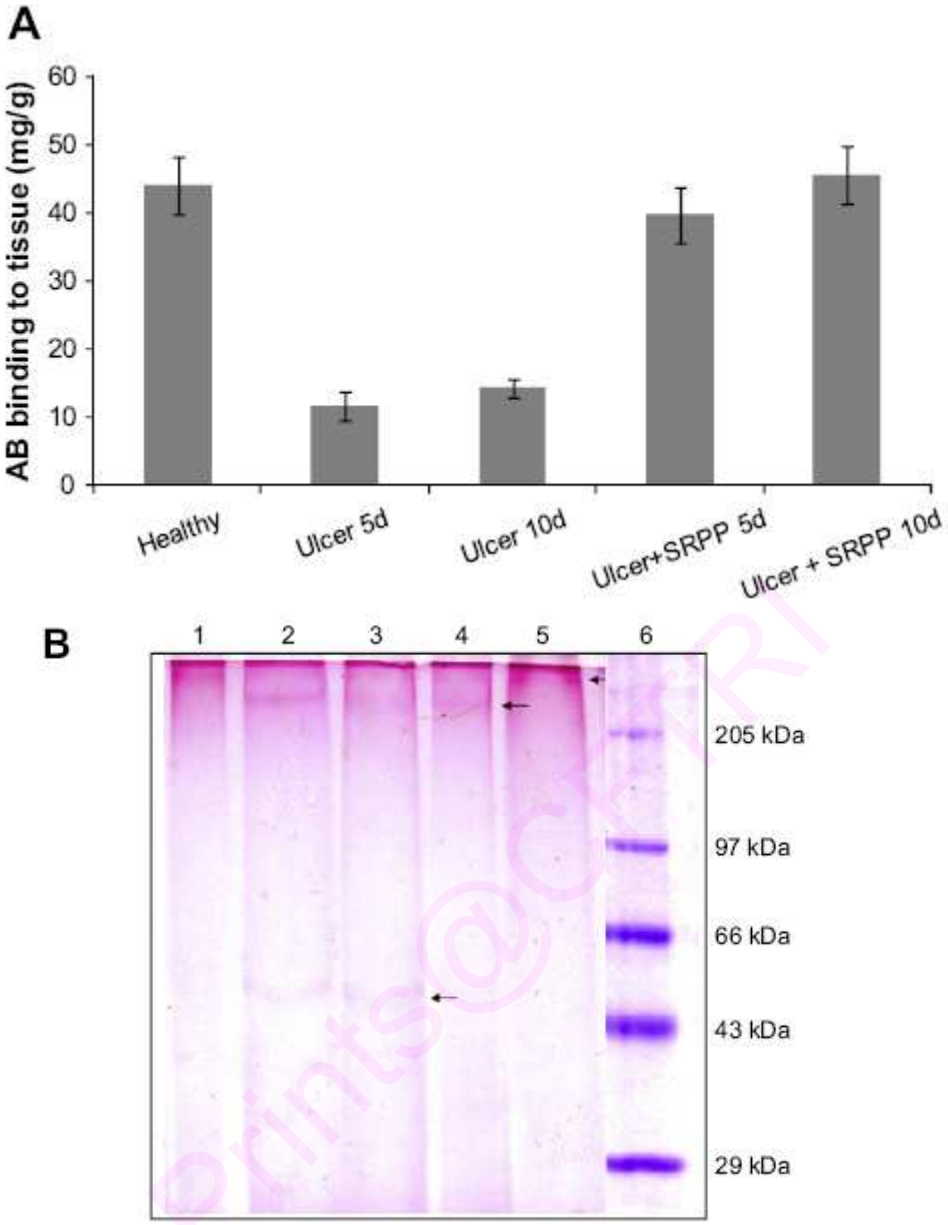


Fig 3B.5. Effect of SRPP on gastric mucin during ulcer healing: Quantitative estimation of gastric mucin by Alcian blue dye binding method(A) and SDS-PAGE analysis of gastric mucin (B): Gastric mucin content was estimated by Alcian blue dye binding method (A) The results were expressed as milligram of Alcian blue binding per gram of stomach tissue , decrease in Alcian blue content indicates decreased mucin content. Values are mean± SD (n=6). **Figure B** showing SDS-polyacrylamide gel electrophoresis of gastric mucin isolated from healthy, ulcer induced and SRPP treated rat stomachs. The gel was stained with periodic acid-Schiff's reagent which specifically stains glycoprotein. Lane 1: healthy control, 2: 5 d ulcer control, 3: 10 d ulcer control, 4: 5 d SRPP treatment after ulcer induction, 5: 10 d SRPP treatment after ulcer induction, 6: higher range molecular weight marker (stained with coomassie brilliant blue). Small molecular weight glycoprotein (~55 kDa) was observed in ulcer control groups as indicated by arrow, which was absent in healthy control and SRPP treated groups. The intensity of large molecular weight (>200 kDa) glycoprotein (indicated by arrow) is reduced in ulcer controls and it is regained in SRPP treated groups.

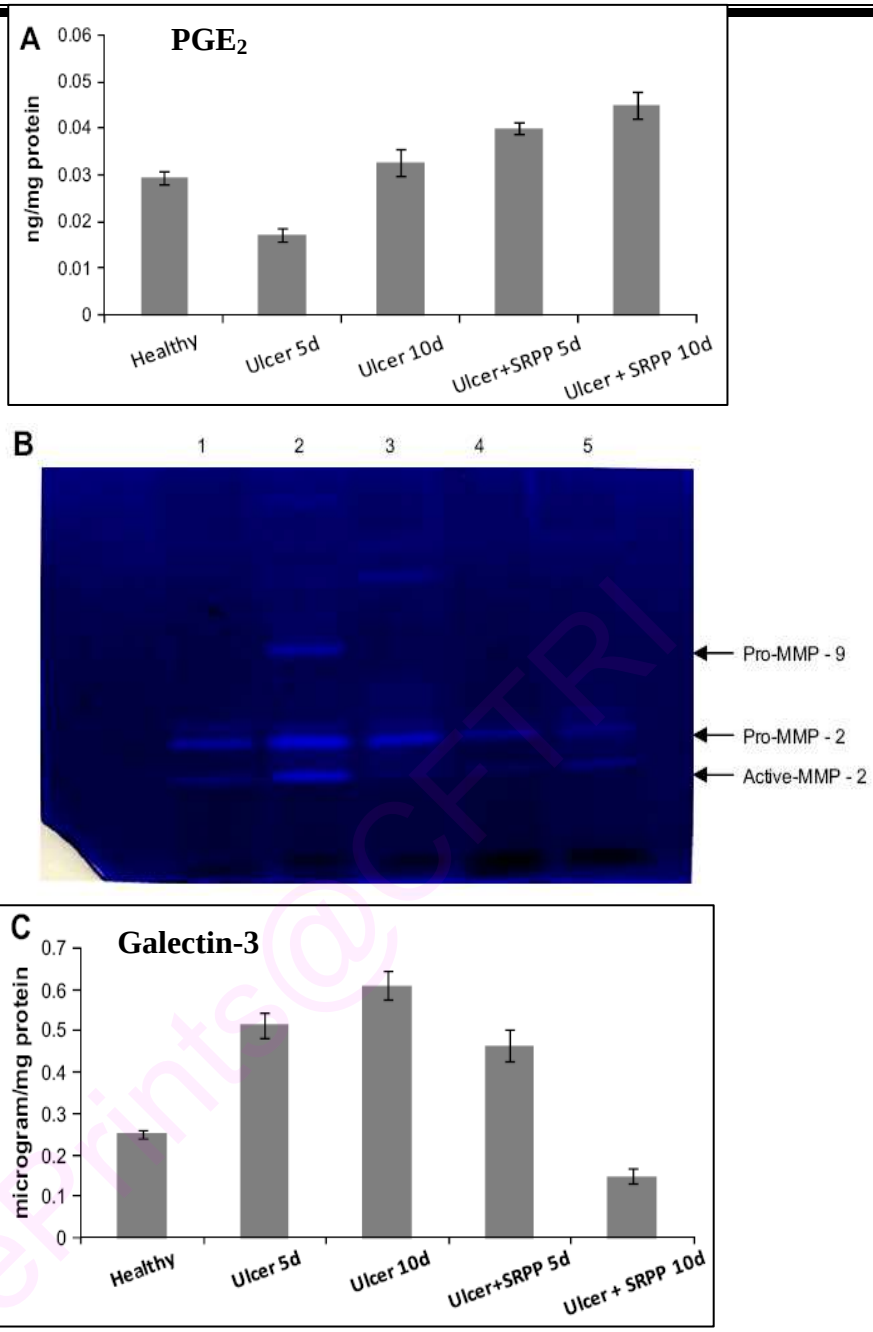


Fig 3B.6. Modulation of PGE_2, MMPs and galectin-3 by SRPP in stomach tissue during healing of acetic acid induced ulcers: PGE_2 level (A), Matrix metalloproteinases (B) and Galectin-3 (C):

A: PGE_2 expressed as ηg/mg protein of stomach tissue. B: Matrix metalloproteinases analyzed by gelatin zymographic method. Lane 1: healthy control, 2: 5 d ulcer control, 3: 10 d ulcer control, 4: 5 d SRPP treatment after ulcer induction, 6: 10 d SRPP treatment after ulcer induction. Both pro and active forms of MMP-2 were increased in ulcer control groups, where as the SRPP treatment showed reduction in band intensity of MMP-2, similar to that of healthy control. The pro-MMP-9 was observed in 5 d ulcer control and it is absent in remaining groups. C: Galectin-3 level in stomach tissue during ulcer healing was determined by ELISA method using monoclonal anti-human galactin-3 antibody. Results were expressed in ELISA absorbance units per milligram protein. Values are mean ± SD (n=6).

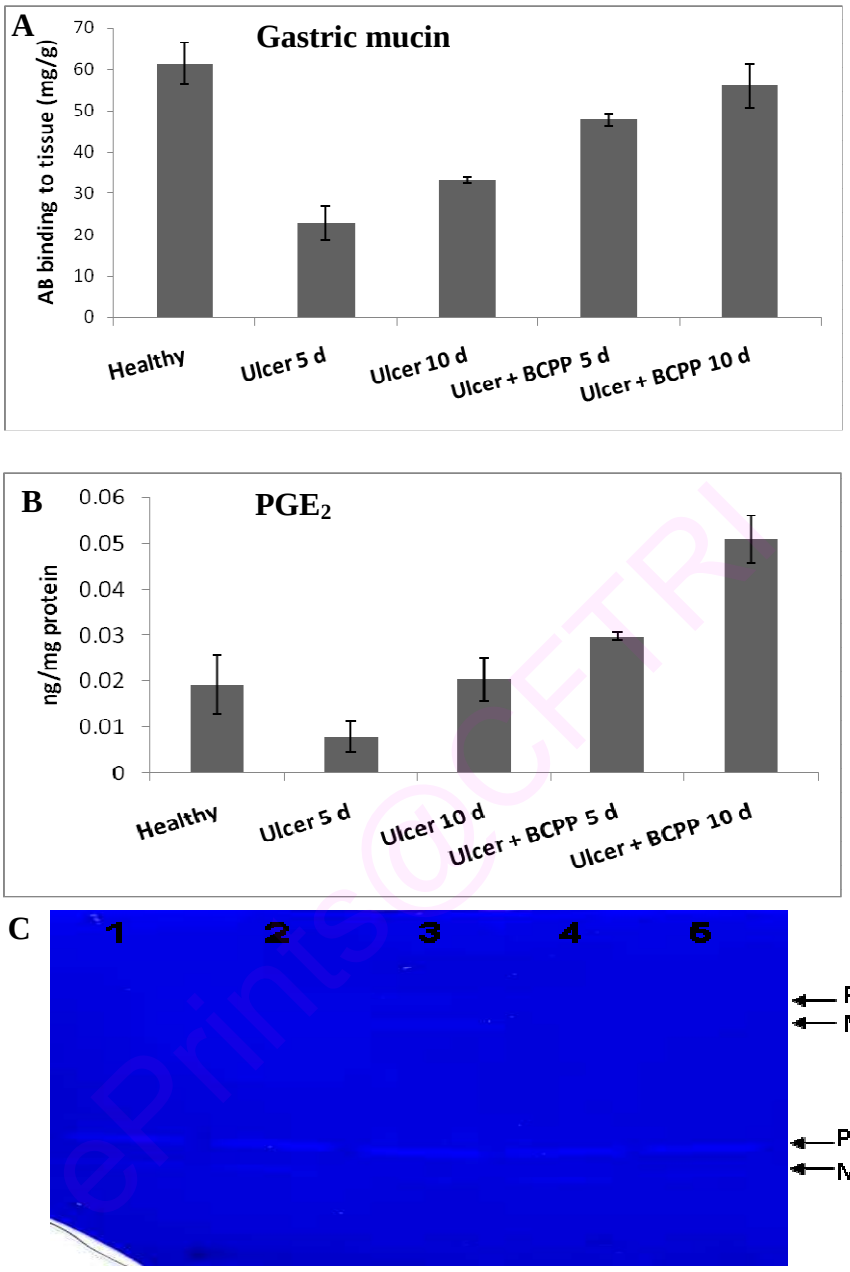


Fig 3B.7. Modulation of gastric mucin (A), PGE_2 (B) and MMP activity levels by BCPP during healing of acetic acid induced ulcers: Gastric mucin content was estimated by Alcian blue dye binding method (A) The results were expressed as milligram of Alcian blue binding per gram of stomach tissue , decrease in Alcian blue content indicates decreased mucin content. Values are mean± SD (n=6). **Fig B** showing PGE_2 expressed as ηg/mg protein of stomach tissue. **Fig C** showing matrix metalloproteinases analyzed by gelatin zymographic method. Lane 1: healthy control, 2: 5 d ulcer control, 3: 10 d ulcer control, 4: 5 d BCPP treatment after ulcer induction, 6: 10 d BCPP treatment after ulcer induction.

Groups (n=6)	GSH (η mol/g tissue)	TBARS (η mol/mg protein)	Protein carbonyls (η mol/mg protein)
Healthy	$155.53^{a} \pm 8.96$	$0.382^{a} \pm 0.036$	$1.150^{a} \pm 0.204$
Ulcer 5d	$101.73^{c} \pm 10.8$	$1.235^{c} \pm 0.161$	$10.54^{d} \pm 0.577$
Ulcer 10d	$115.16^{b} \pm 1.28$	$0.604^{b} \pm 0.030$	$7.741^{c} \pm 0.692$
Ulcer + SRPP 5d	$144.76^{a} \pm 5.39$	$0.607^{b} \pm 0.095$	$3.810^{b} \pm 0.476$
Ulcer + SRPP 10d	$151.66^{a} \pm 5.12$	$0.271^{a} \pm 0.077$	$2.845^{ab} \pm 0.261$

Table 3B.1. Levels of GSH, TBARS and protein carbonyls during ulcer healing by SRPP in acetic acid induced ulcer model: The level of reduced glutathione (GSH), lipid peroxidation products as thiobarbituric acid reactive substances (TBARS) and protein oxidation as protein carbonyls were estimated in the stomach tissue homogenate as described under materials and methods Values are mean ± SD (n=6) The level of significance was tested by Duncan multiple range test at $P < 0.05$. The different letters 'a to d' in the same column indicates the increase in significant difference when compared to healthy group.

Groups	GSH (η mol/g tissue)	TBARS (η mol/mg protein)	Protein carbonyls (η mol/mg protein)
Healthy	$329.00^{d} \pm 20$	$0.232^{c} \pm 0.03$	$4.29^{d} \pm 0.82$
Ulcer 5 d	$130.38^{a} \pm 28$	$0.732^{a} \pm 0.05$	$11.80^{a} \pm 0.19$
Ulcer 10 d	$194.94^{b} \pm 6.5$	$0.586^{b} \pm 0.03$	$8.57^{b} \pm 0.31$
Ulcer + BCPP 5 d	$175.44^{b} \pm 23$	$0.524^{b} \pm 0.01$	$7.14^{c} \pm 0.78$
Ulcer + BCPP 10 d	$252.08^{c} \pm 15$	$0.284^{c} \pm 0.01$	$4.78^{d} \pm 0.11$

Table 3B.2. Levels of GSH, TBARS and protein carbonyls in the stomach tissue during ulcer healing by BCPP in acetic acid induced ulcer model: The level of reduced glutathione (GSH), lipid peroxidation products as thiobarbituric acid reactive substances (TBARS) and protein oxidation as protein carbonyls were estimated in the stomach tissue homogenate as described under materials and methods Values are mean ± SD (n=6) The level of significance was tested by Duncan multiple range test at $P < 0.05$. The different letters 'a to d' in the same column indicates the increase in significant difference when compared to healthy group.

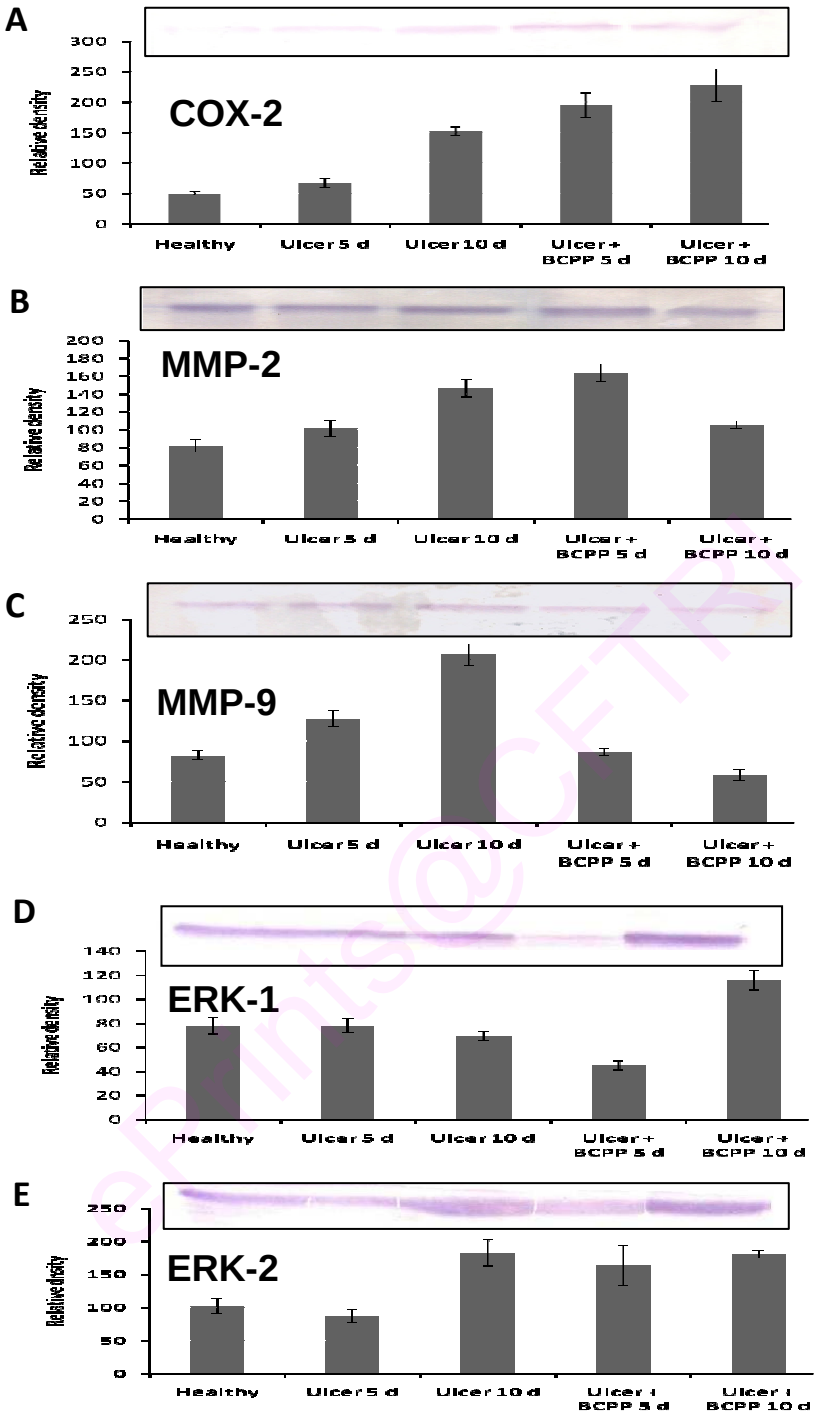


Fig 3B.8. Western blot analysis showing modulation of molecular events by BCPP during healing of acetic acid induced ulcers;
Western blot analysis of COX-2 (A), MMP-2 (B), MMP-9 (C), ERK-1 (D) and ERK-2 (E) in stomach tissue homogenate of healthy, ulcerous and BCPP treated rats using specific monoclonal antibodies

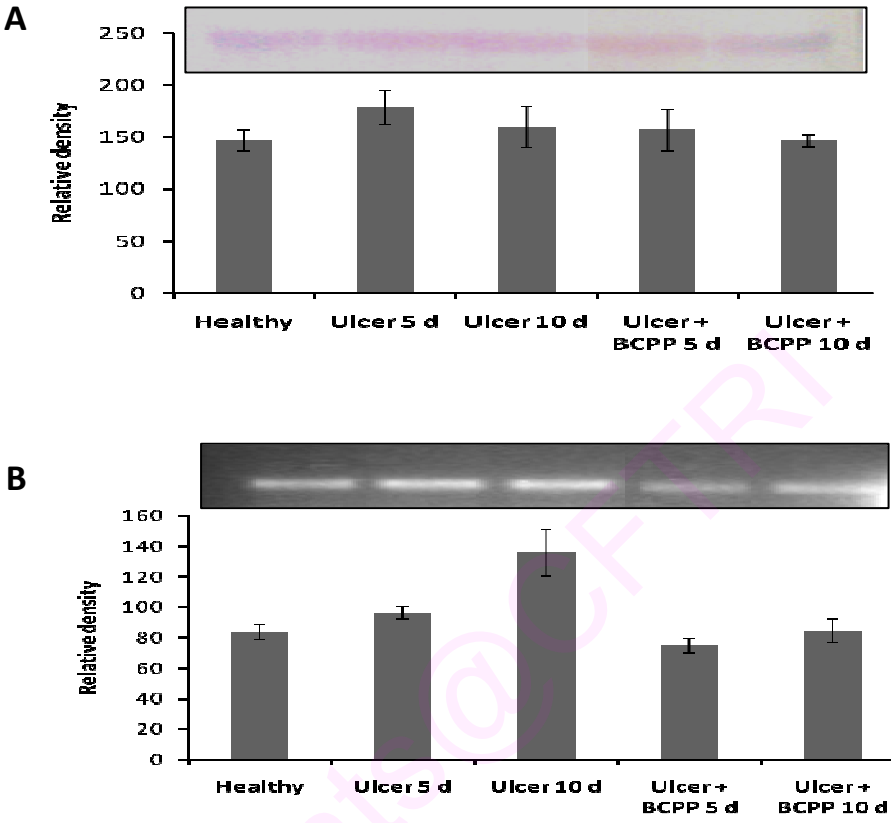


Fig 3B.9. Downregulation of galectin-3 by BCPP during healing of acetic acid induced ulcers:
Western blot analysis (A) and mRNA expression of galectin-3 in stomach tissue homogenate of healthy, ulcerous and BCPP treated.

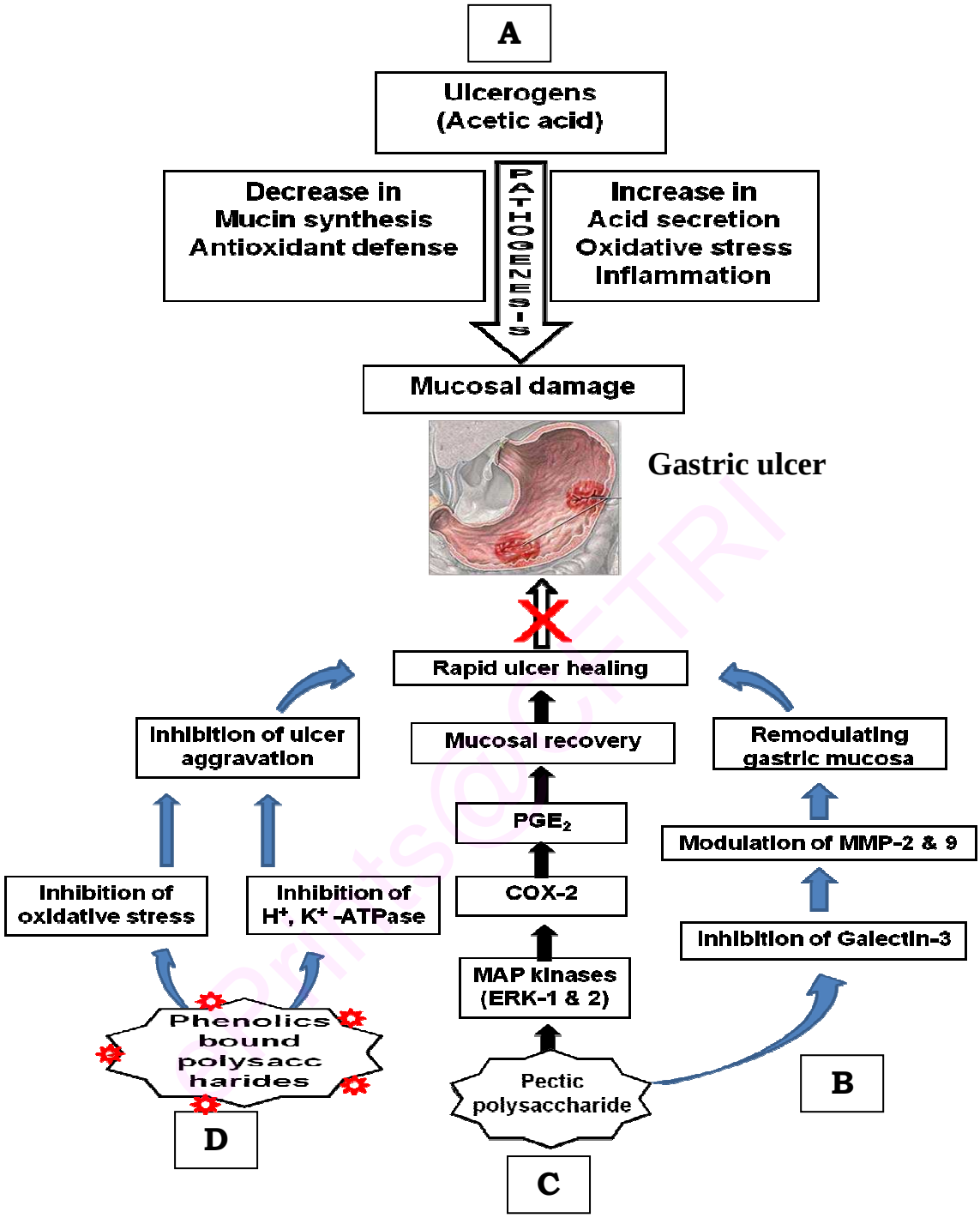


Fig 3B.10. Mode of action of pectic polysaccharides (SRPP/BCPP) during healing of acetic acid induced ulcers: Aggravation of gastric ulcer due to increased acid secretion, oxidative stress, inflammation and; decreased mucin synthesis and antioxidant defence (**A**). SRPP/BCPP inhibits inflammatory reaction leading to remodulation of gastric mucosa (**B**) and also they can directly trigger mucin synthesis through MAP-kinase pathway (**C**). The bound phenolics of SRPP/BCPP also play a role by inhibiting oxidative stress and H^+, K^+ -ATPase activity thus prevents further aggravation of gastric ulcer.

3B.6. Discussion

Previous chapter (Chapter 3A) has indicated ulcer preventive role of some of the selected components from selected dietary sources – Swallow root and Black cumin. Although in swim stress and ethanol induced ulcer models, inhibition of H^+, K^+ -ATPase activity which reduces acid secretion and; levels of antioxidant and antioxidant enzymes that envisages antioxidative protection was demonstrated, the precise mechanism of stimulation of mucosal cell proliferation by PGE_2 & production of mucin for mucosal protection and; the role of anti-inflammatory signaling molecule – galectin-3 was yet to be elucidated.

Ulceration being a complex process of multi-step ulcer pathogenicity, ulcer healing molecule must also have been believed to exhibit multi-step protection for it to be an effective antiulcer agent. Although our previous studies have indicated several dietary components that can heal ulcers effectively (Nanjundaiah et al., 2009), comprehensive action of these compounds on the molecular – molecular interaction and on the signaling cascade was not clearly understood.

The acetic acid induced ulcer model was employed to understand the mechanism of gastric ulcer healing process, since the validity of this model for ulcer healing studies were established and the model resembles human ulcers in terms of both pathological features and healing mechanisms. Acetic acid solution consistently induces penetrating ulcers which begins with vascular injury and subsequently ischemic necrosis leading to ulceration. In addition to this, multi-mechanistic destructive routes like damaging of gastric wall barrier, generation of free radicals, cell apoptosis etc., were also contributed in acetic acid induced ulcer development.

It is important to know that mucin is an insoluble adherent mucus gel; moreover, one of the essential criteria to determine the status of mucus barrier is the state of mucus secretion. This mucus consists of mucin type glycoproteins, which can be determined by ratio of total carbohydrates to protein in gastric juice. These high molecular weight glycoproteins are mainly responsible for viscous and gel forming characteristic of the mucus. Increased mucus secretion by the gastric mucosal cells can prevent gastric

ulceration by several mechanisms, including lessening of stomach wall friction during peristalsis and gastric contractions, improving the buffering of acid gastric juice and by acting as an effective barrier to back diffusion of H^+ ions (Venable, 1986). Results of this chapter (Chapter 3, part B) revealed that gastric mucin damage due to acetic acid treatment was proportionate with increased ulcerations. It was evidenced by the presence of degraded ~55 kDa glycoprotein fragments in ulcerated groups (Fig 3B.5B). This band was generated due to either proteolysis of secreted mucin or due to a failure of mucin synthesizing cells to incorporate these small molecular weight fragments into a segment of gastric mucin polymer during ulcer conditions, particularly due to damaged cells. Disappearance of small molecular weight fragment of mucin after 5 d and 10 d of sample treatment when compared to those of respective ulcer control (or autohealing) rats suggested that polysaccharides (SRPP) may be participating in remodeling process either by inhibiting indirectly the action of MMPs or remodulating the mucin producing cells of gastric wall barrier. Similar results were also observed in BCPP treated groups (Data not shown).

Preliminary studies carried out in our laboratory suggested that SRPP has insignificant role in direct inhibition of MMP activity suggesting that inhibition of MMP could be due to blockade of MMP activation process which occur during ulceration rather than direct interaction of SRPP/BCPP with MMP. Data further suggested that in acetic acid induced ulcer model, there is a significant activation of MMP-2 than MMP-9.

Also results of the zymographic analysis (Fig 3B.6B, 3B.7C) substantiated by western blot analysis (Fig 3B.8) reveal that MMP-9, both pro and active form is expressed in detectable range only during 10 d autohealing (ulcer control) group, which is totally inhibited by SRPP/BCPP. However, relatively increased expression of MMP-9 in 10 d ulcer control than 5 d ulcer control group indicate that, MMP-9 is not directly involved in ulcer pathogenicity. It may be triggered at later stages to participate in the healing or reassembling process. Further, inhibition by SRPP/BCPP suggests that there may be a downregulation of MMP-9 which could be due to less requirement for MMP-9 in SRPP/BCPP treatment, since the healing has already occurred as evidenced by reduction in ulcer area (Fig 3B.1, 3B.2), increased mucin synthesis by histopathological (Fig 3B.3, 3B.4) and biochemical analysis (Fig

3B.5, 3B.6). MMP-2, another member of MMPs has been shown to be involved during the healing process (Swarnakar et al., 2005). Differential regulation of MMP-2 where upregulation of both pro and active MMP-2 in ulcer control groups with relatively less intensity, while ~2 folds higher level of expression in 5 d SRPP/BCPP treated group than 10 d SRPP/BCPP treated group is intriguing because the data substantiates the view that MMP-2 is required for ulcer healing (Fig 3B.6, 3B.7, 3B.8). Increase in MMP-2 in ulcer control groups while downregulation of the same in 10 d SRPP/BCPP treated groups (Fig 3B.8) provide evidence for the rapid healing in SRPP/BCPP treated group via augmenting the proliferation and migration of mucosal cells and tissue protectivity by replacing the damaged mucin layer. The downregulation of MMP-2 by SRPP/BCPP could be due to rapid completion of ulcer healing process (Srikanta et al., 2010). In other words, still overexpression of MMP-2 in 5 d and 10 d BCPP treated group as opposed to downregulation in SRPP treated group suggested that SRPP is more potent and rapidly heals ulcer than BCPP, although BCPP is also an effective ulcer healing agent. Results can be further evidenced by histopathological analysis where completely defined mucin producing mucosal glandular structure in SRPP treatment (Fig 3B.3) while complete formation of mucosal layer with still rearchitecturing activity in BCPP treated group (Fig 3B.4). Together data highlights the fact that MMP-9 may be triggered first during initiation of mucosal damage to reorganize the mucosal layer for functioning; while MMP-2 may enforce its action later during reassembling of newly formed mucosal layer to bring to original architecture of mucosal layer. Results were enumerated both by a zymographic as well as western blot analysis as depicted in Fig 3B.6, 3B.7, 3B.8.

It is pertinent to discuss and unravel the differential roles of MMP-9 and MMP-2 during ulcer pathogenicity or healing process. Earlier investigators (Swarnakar et al., 2005) have also observed the early onset of MMP-9 during ulcer condition than MMP-2 and implicated that MMP-9 is involved in aggravation of ulcers, while MMP-2 has been believed to participate during healing. Based on the observation made in our laboratory and considering the insights of MMPs activity, it is imperative to delineate the roles of MMP-9 and MMP-2. As we have suggested, MMP-9 although found to be expressed in higher levels during early ulceration process, it can still be triggered by

galectin-3 a substrate for MMPs (Ochieng et al., 1994); such substrate dependent regulation of enzyme appears to be one of the key mechanisms in the execution of tight physiological regulatory events (Dharmesh & Baenziger, 1993) to participate in the inflammatory reaction or defensive action. In other words MMP-9 although found in higher levels during ulcer aggravation, it may be acted as a part of defence machinery for reassembling of gastric mucosal epithelium. It is possible that MMP-9 may degrade the damaged layer which can be later tailored by MMP-2.

Mucosal injury as indicated previously from our studies has been shown to be due to destruction of mucosal cells producing gastric mucin. Hence, the healing process must involve either the activation of proliferation of mucosal cells to replace the injured cells that are damaged due to inflammatory response or, there could be recruitment of cells and hence the replacement of damaged cells; epithelial sheet formation and rejuvenation of damaged mucosal layer. Pertaining to this concept, in the current study role of galectin-3, an inflammatory marker (Neil & Tariq, 2009) has been addressed. Ulcer is an inflammatory disease and hence galectin-3 levels during ulcers and upon treatment with SRPP/BCPP has been investigated. Results of ELISA (Fig 3B.6), in addition to western blot analysis (Fig 3B.9A), showed upregulation of galectin-3 during ulceration; and downregulation of the same upon treatment with samples. Indeed results are supported by downregulation of galectin-3 mRNA (Fig 3B.9B) also, upon treatment with samples suggesting the gene modulatory role of dietary pectic polysaccharides, particularly SRPP and BCPP. Further, the fact that galectin-3 is a substrate for MMPs (Ochieng et al., 1994), in view of this as well as the results obtained on MMPs, it is pertinent to mention here that, galectin-3 may regulate MMP levels in the signaling cascade.

Current study also showed that SRPP/BCPP increased PGE_2 secretion (Fig 3B.6, 3B.7) and there is a positive correlation between mucin recovery and PGE_2 secretion. Thus results indicate that SRPP/BCPP enhances mucin synthesis by attenuating mucosal cell death and activating proliferation of mucosal cells via PGE_2 synthesis. We provided evidence for the ability of pectic polysaccharides - SRPP/BCPP to enhance cytoprotection and mucosal cell proliferation via PGE_2 mediated pathway. Since PGE_2 has long been known as a stimulant of cell proliferation, an essential step for production of

gastric mucin (Joh et al., 2003) which can form a cohesive comprehensive gastroprotective mucosal layer. Further, the proliferation of cells mediated by PGE_2 appears to be regulated with proportionate differentiation also. Histological data substantiates the regulated proliferation upon treatment with SRPP/BCPP since there is a defined differentiation of mucosal cells leading to mucin synthesis along with normalization of gastric mucosal architecture.

COX-2 plays more complex and wider biological roles than mere involvement in inflammation and pain (Peskar, 2001); gastric mucosal restoration is one among them. A slight increase in COX-2 expression during autohealing in ulcer control groups (Fig 3B.8) followed by demonstration of ~4 folds upregulation of COX-2 expression in sample treated groups suggest that SRPP/BCPP induces ulcer healing through COX-2 mediated upregulation of PGE_2 and cytoprotective or mucosal restoration processes such as cell proliferation, migration and re-epithelialization during ulcer healing. Results are intriguing since the current incidences of gastric ulcer have been shown to be due to the extensive use of NSAIDs that blocks COX-2 activity. SRPP/BCPP with the potentiating effect on COX-2 may therefore be able to provide a remedy for NSAIDs users, where SRPP/BCPP can curb NSAIDs induced ulceration.

We further tested the involvement of MAP kinases (ERK-1 & 2) in BCPP mediated ulcer healing process, since ERK-2 is the main signal transduction molecule for PGE_2 mediated gastroprotection (similar studies on SRPP was not possible as highlighted under materials and methods). Western blots showed two bands ERK-1 and ERK-2 of 44 and 42 kDa respectively (Fig 3B.8). There was no significant differences in 44 kDa band of ERK-1 among healthy and ulcer control groups but slightly reduced in 5 d BCPP treatment and it is further increased after 10 d of BCPP treatment. Whereas a 42 kDa band of ERK-2 was not changed in the beginning (5 d) but increased after 10 d of ulcer induction in ulcer control groups. BCPP treatment induced increased expression of ERK-2 since beginning (5d) and it increased to much higher levels in 10 d BCPP treatment (Fig 3B.8). Previous studies also had shown the necessity of ERK-2 during healing of mucosal injury (Pai et al., 1998; Kawanaka et al., 2001). The current data indicated that changes in PGE_2 and COX-2 levels were directly proportional to changes in ERK-2

expression and these changes were accelerated by BCPP treatment. Therefore the study indicates that BCPP accelerates ulcer healing mainly through ERK-2 pathway which involves COX-2 mediated PGE_2 synthesis leading to increased mucin synthesis, inhibition of mucosal cell damage, cell proliferation and regeneration of mucosa, faster than the autohealing in ulcer control groups. It is possible that SRPP and other antiulcer pectic polysaccharides act similarly through ERK-2 pathway to increase mucin secretion and regeneration of gastric mucosa.

MAP kinase associated with growth factors play a pivotal role in regulating gastric mucosal cell proliferation and in regenerating injured gastric mucosa. Results of the current study showed that activation of MAP kinase (ERK-1 & 2) play an essential role in the healing of gastric mucosal ulceration and that its inhibition or attenuation due to ulcerogenic agents leads to a significant delay in ulcer healing. These results are supported by previous studies where it is demonstrated that ERK activation is important for inducing the proliferation of gut epithelial cells (Jones et al., 1999).

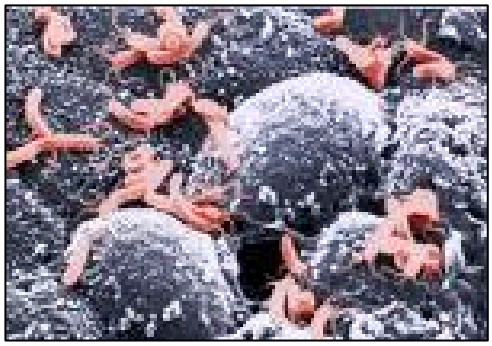
Free radical generation and inflammation are other complications reported during wound healing process which appear to aggravate ulcers. A better antiulcer compound had been believed to possess multiple properties in addition to mucosal regeneration ability, because stress induced ulcer condition generates free radicals leading to and augments oxidative stress condition. The observation by Naito et al., (1995) suggested that scavenging hydroxyl radicals generated during ulcer condition would enhances the quality of healing mucosa. SRPP/BCPP having bound phenolics showed potent antiulcer effect including the inhibition of oxidative markers such as TBARS, and protein carbonyls; normalization of depleted antioxidant molecule - GSH and antioxidant enzymes *in vivo* (Table 3B.1, 3B.2). It is possible that once ulcer healing process is triggered by polysaccharides, phenolics bound covalently to it may act directly at the given location and may favour the healing process. Increased ulcer healing capacity of SRPP than BCPP may be due to higher amount of bound phenolics in SRPP (120 mg GAE/g) when compared to that of BCPP (26 mg GAE/g). Thus polysaccharides with antioxidant nature by virtue of bound phenolics has an added advantage in ulcer healing process than either phenolics or polysaccharide alone.

In the current study it is also relevant to address the specific role and contribution of antioxidants and polysaccharides individually or in combination towards ulcer healing. Although antioxidants and polysaccharides have been implicated in ulcer prevention and healing process (Cheng et al., 1985; Gao et al., 2002 & 2004), the precise steps acted by these compounds were not clearly defined. With the current results of ulcer healing and previous reports on antioxidants as antiulcer components (Siddaraju & Dharmesh, 2007a & 2007b; Srikanta et al., 2007; Nanjundaiah et al., 2009) it may be indicated that polysaccharides may participate in the trigger of signaling cascade for ulcer healing or prevention of ulcerogenic pathways by inhibition of H^+, K^+ -ATPase enzyme which is triggered by free radical mediated stress. Phenolic antioxidants component in the polysaccharide although has been implicated in such activities, role of carbohydrates - pectins in binding to active domain of H^+, K^+ -ATPase may not be ruled out. Thus it is believed that polysaccharide may be more potent in ulcer healing ability than antioxidants which can inhibit initiation of ulcerogenic process also by inhibiting oxidative stress induced tissue damage.

Overall results provide evidence for the defining roles of antioxidants and pectic polysaccharide. For the first time the current study suggested that pectic polysaccharides – SRPP/BCPP can stimulate molecular signaling cascade by modulating the tight-regulation of signaling molecules such as MAP kinase (ERK-1 & 2), COX-2, PGE_2, galectin-3 and MMP-2 & 9 etc., as shown in Fig 3B.10. Molecular-molecular interplay of SRPP/BCPP and gastric epithelium thus imposed may augment ulcer healing process. The ability of modulation of several regulatory factors by SRPP and BCPP to heal gastric ulcers caused by acetic acid, together with the fact that acetic acid induced ulcer model mimics human ulcers, results of the study appear to be relevant and encourages the use of SRPP and BCPP against human gastric ulcers.

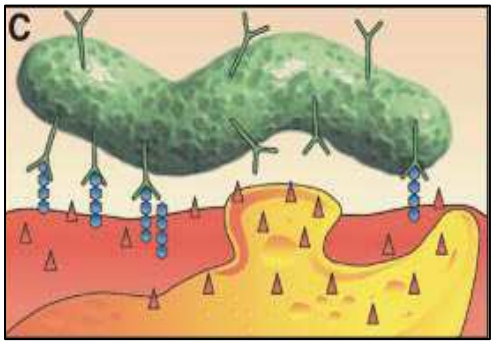
3B.7. Summary and conclusions

- Chapter 3B addresses the ulcer healing mechanisms of SRPP and BCPP.
- Studies showed ulcer healing potency of SRPP and BCPP at 200 mg/kg b.w. in acetic acid induced ulcer model in rats.
- Within 10 d of ulcer induction, SRPP showed better healing ability (90%) with fast mucin recovery (99%) when compared to BCPP which showed up to 80% healing with 90% mucin recovery at oral concentration- 200 mg/kg b.w.
- Damaged gastric mucin with molecular weight of ~55 kDa glycoprotein in ulcer control groups was recovered back to larger molecular weight (~200 kDa) gastric mucin similar to that of healthy control in pectic polysaccharide treated animals.
- The increased healing efficacy of SRPP is attributed to high phenolic content (120 mg/g) when compared BCPP (26 mg/g). Phenolics could accelerate the healing process by reducing the oxidative stress generated during ulcer condition; while polysaccharides may provide protection to mucosal cells by increasing gastric mucin synthesis.
- Study indicated upregulation of gastric mucin synthesis by SRPP and BCPP treatment, through increased synthesis of prostaglandin E_2.
- SRPP and BCPP also showed differential regulation of MMP-2 & 9 via modulation of an inflammatory molecule galectin-3 expression during ulcer healing.
- For the first time the current study suggested that pectic polysaccharides – SRPP and BCPP can stimulate molecular signaling cascade by modulating the tight-regulation of signaling molecules such as MAP kinase (ERK-1 & 2), COX-2, PGE_2, galectin-3 and MMP-2 & 9 etc.



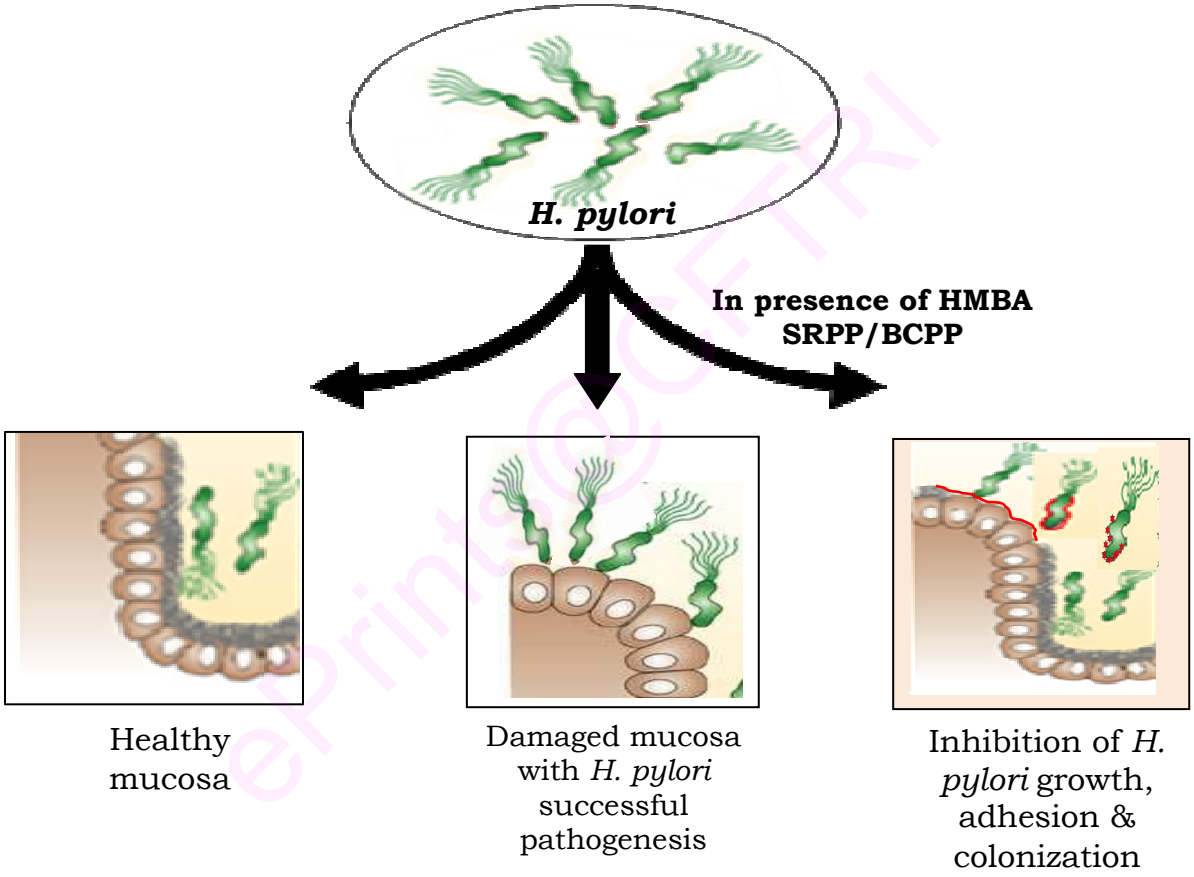
CHAPTER 4

Establishment of mechanism of action of anti-*Helicobacter pylori* compounds



4.1. Hypothesis

Can selected dietary compounds inhibit *H. pylori* growth, adhesion and colonization ?

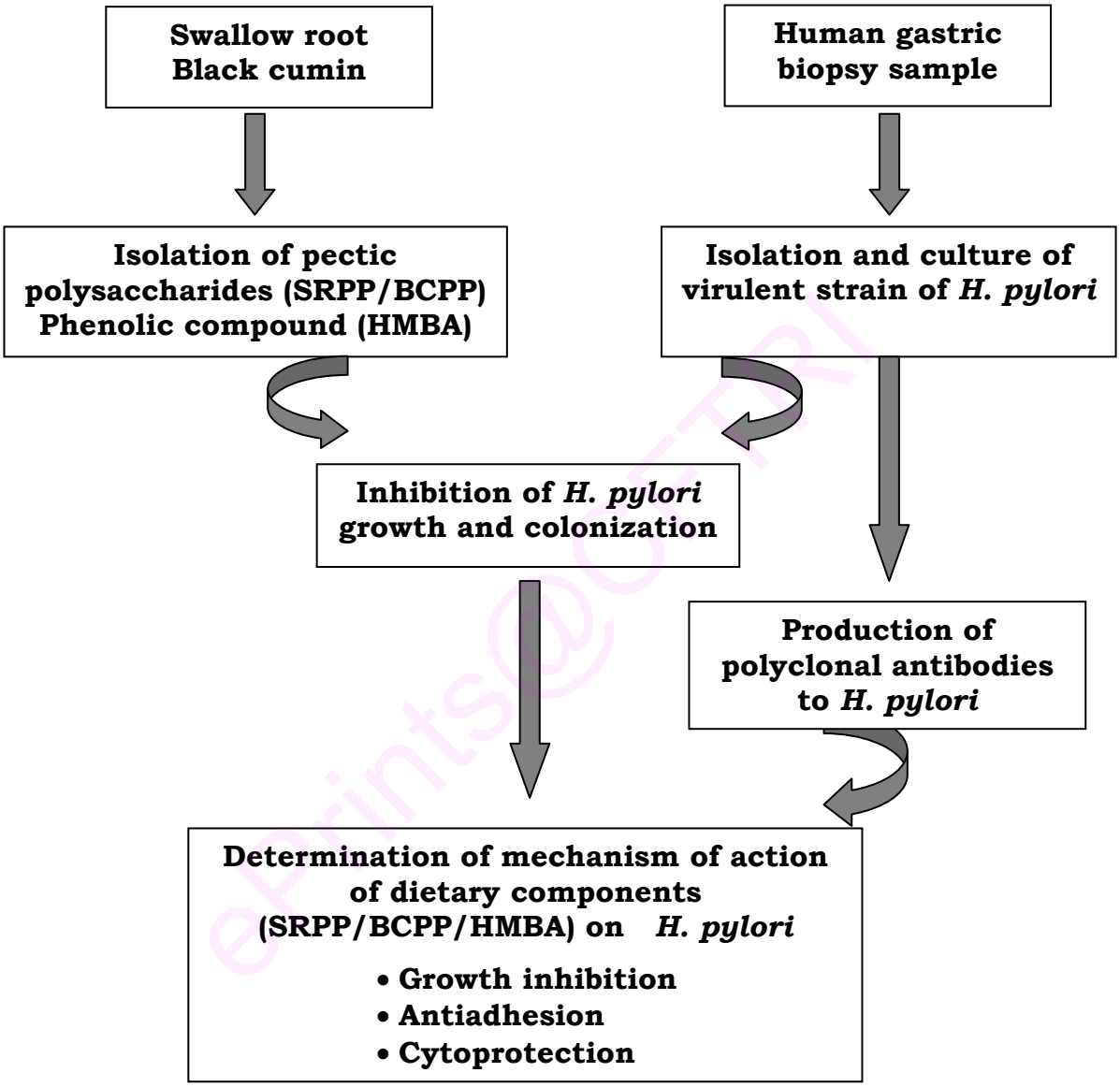


Healthy mucosa

Damaged mucosa with *H. pylori* successful pathogenesis

Inhibition of *H. pylori* growth, adhesion & colonization

4.2. Work plan



4.3. Introduction

Helicobacter pylori is a slow growing microaerophilic, highly motile, gram-negative bacterium. Colonization of virulent strains of *H. pylori* in the gastric epithelium is a major determinant in the pathogenesis of gastro duodenal ulcers and is associated with gastric carcinoma and lympho sarcoma (Warren and Marshall, 1983). Marshall and Warren's seminal discovery that a humble bacterium, *H. pylori*, causes these diseases, has opened up new avenues for research and changed the perspectives towards diagnosis and treatment of *H. pylori* mediated diseases (Mégraud, 2005).

In industrialized countries, as many as 50% of adults were found to be infected with *H. pylori*, while in the developing world, prevalence values of about 90% have been reported (Yvonne et al., 2001), probably due to poor sanitation conditions and many of them remain asymptomatic. Peptic ulcer disease arises in 10% of infected people while 1% and 0.1% of gastric adenocarcinoma and gastric MALT lymphoma respectively have been reported (Mégraud, 2005). As per recent reports the increased transition of 'passive pathogen infection' to 'successful disease manifestation' warrants the greatest concern towards this bacterium affected patients. Life style changes with increased stress appear to be responsible for this transition.

As per FDA/WHO the treatment regime for *H. pylori* infection alone includes triple therapy that includes antibiotics – amoxicillin and clarithromycin along with proton pump blockers such as omeprazole / lanzoprazole and mucoprotectants such as sucralfate etc. Growing literature enumerates that increasing number of infected individuals who harboured this bacterium exhibits resistance to first-line of antibiotics (Tokunaga et al., 2009). In areas with high antimicrobial resistance rates, new antibiotic combinations and modifications in the sequence of drug administration are being proposed as alternative treatment options to standard triple therapy (Selgrad et al., 2009). Statistically significant failure rates of triple therapy for the treatment of *H. pylori* infection and thus for *H. pylori* mediated ulcers and cancers opened up newer avenues as alternatives (Freston, 1997) against *H. pylori* infection. Requirement of additional antibiotic therapy although has been suggested by Mirbagheri et al., (2006) and Hong et al., (2006) a search for new antimicrobial substances preferably from herbal and dietary sources are

warranted to overcome multiple drug administration that potentially pose many adverse effects.

Previous chapters have reported effective antiulcer, gastroprotective, mucoprotective, H^+, K^+ -ATPase inhibitory pectic polysaccharides from selected sources - Swallow root (*D. hamiltonii)* and Black cumin (*N. sativa).* Newer compounds (phenolics and pectic polysaccharides) found in Swallow root and Black cumin have been shown to possess novel multi-step active antiulcer properties (Chapters 1 & 3). Detailed compositional analysis had revealed the presence of high amount (34.43 ± 4.0 mg GAE/g) of phenolic acids (Naik et al., 2007) with abundant level (~28% of total phenolics) of 2-hydroxy-4-methoxy benzaldehyde (HMBA) (Nayaka et al., 2010) in addition to ~6% pectic polysaccharide in Swallow root and; 12.39 ± 2.0. mg GAE/g phenolics and ~2% pectic polysaccharide in Black cumin. Systematic bioactivity studies carried out from our laboratory indicated the minimal role of HMBA despite its higher abundance than other minor phenolic constituents. HMBA displayed very poor antioxidant properties (Nayaka et al., 2010).

Further, intriguingly Chapter 3 showed potent ulcer preventive and healing properties of both SRPP and BCPP. For the first time studies also showed the molecular modulation particularly via activation of MAP kinase (ERK1 & 2) pathways. Activated ERKs subsequently stimulated PGE_2 and PGE_2 mediated proliferation of mucosal cell that was damaged during ulceration. Idea of augmenting proliferation of mucosal cells were to heal and replace the damaged layer so that mucosal defence can be enumerated and gastric protection can be ensured against acid insult or ulcerations. However studies were limiting in that one could not employ *H. pylori* induced gastric ulceration model *in vivo* due to restricted permission for the use by Institutional ethical committee as per committee for the purpose of control and supervision of experiments on animals (CPCSEA) in our institute. Nevertheless *in vitro* studies were designed to test the potentiality of selected pectic polysaccharide components and HMBA from Swallow root and Black cumin against *H. pylori* growth and adhesion. Aetiology of majority of gastric ulcer incidences revealed that invasion of *H. pylori* via a damaged mucosal layer appears to be the crucial event for successful ulcer pathogenicity in humans. Adhesion of *H. pylori* to gastric mucosal layer has been known to be via specific recognition of *H. pylori* to repertoire of affinity constituents on

gastric mucin, a layer that offers mucosal defence, an essential event to manage gastric ulcer. Further as highlighted in previous chapter (Chapter 1), HMBA in Swallow root and tannic acid in Black cumin were present in enriched amounts of ~28 and 80% of total phenolics respectively. Detailed investigation had shown less contribution of tannic acid when compared to HMBA (Fig 1.5C of chapter 1). In this chapter therefore we address to study the mechanism of action of anti-*H. pylori* compounds –SRPP, BCPP and HMBA employing cell models. Since adhesion to gastric mucin is the first step of *H. pylori* infection followed by establishment in damaged mucosal cells, inhibition of *H. pylori* growth and cell adhesion assays were performed and evaluated the effect of SRPP, BCPP and HMBA on these events.

In order to follow up the adhesion of *H. pylori* to gastric mucin, immune-microplate assay was designed for which specific antibody to the available strain/isolate of *H. pylori* was needed. This chapter therefore also addressed the production of polyclonal antibody (anti-HP-PcAb) to the confirmed isolate of *H. pylori.* Anti-HP-PcAb was characterized for sensitivity, specificity, antibody titre and its cross reactive potential to other pathogenic bacteria prior employing the same in the ELISA assay while determining the ability of selected compounds for inhibition of adhesion of *H. pylori* to gastric mucin, a natural adhesive molecule during infection.

Since the mode of action of antioxidants (phenolics) and polysaccharides in both *in vitro* and *in vivo* are depicted in chapter 1 and 3, this chapter emphasizes on the antimicrobial action of HMBA, which is neither a good antioxidant nor a pectic polysaccharide fraction which have been known for cyto/muco protective and gastroprotective properties.

The study has a greater impact since HMBA has been known to exist in higher levels in plants such as *Hemidesmus indicus* which is related to *D. hamiltonii*, which are well known in Ayurvedic system as sarva sidhaushada (Treatment for all disease) occupying higher position in the group of alternative medicines. Current chapter delineates the structure-function relationship of anti-*H. pylori* compound - HMBA and provides a probable underlying mechanism as bactericidal but host-cell protective in nature. Results of the study provide also for the first time a new anti-*H. pylori* functional attribute to HMBA particularly neutralizing effect on *H. pylori*'s vacuolating toxin.

4.4. Materials and methods

4.4.1. Materials

Ethylene diamine tetraacetic acid (EDTA), Penicillin, Streptomycin, Amoxicillin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), Triton X-100, SDS, Tween 20, TEMED, Ethidium bromide, sucrose, skimmed milk powder, Nitrocellulose membrane, Ponceau S, Porcine gastric mucin, Dimethyl sulfoxide (DMSO), Acridine orange, Ethidium bromide, Calf thymus DNA, Human serum albumin (HSA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and p-nitrophenyl phosphate were purchased from Sigma Chemical Co, St. Louis, MO, USA. ELISA plates were purchased from Nunc, Denmark. Agarose gel Electrophoresis accessories, Alkaline phosphatase conjugated-rabbit anti-mouse IgG secondary antibodies, DAB system, Freund's complete and incomplete adjuvant were procured from Genei, Bangalore, India. Ham's F12 media and bacteriological grade agar were procured from Himedia Laboratories, Mumbai, India. Protein molecular weight marker purchased from Fermentas, USA. Other chemicals such as sodium phosphate buffer, glutaraldehyde, glycine, sodium chloride, phenol, chloroform and solvents used were of the analytical grade purchased from a local chemical company, Sisco Research Laboratories, Mumbai, India.

4.4.2. Production of polyclonal antibodies (anti-HP-PcAb) to *H. pylori*

4.4.2.1. Preparation of antigen – *H. pylori* protein

A whole cell antigen was prepared from *H. pylori* cells, which were isolated from gastric ulcer patients and confirmed the strain as described in chapter-1, section-1.4.2. *H. pylori* was grown and harvested from Ham's F-12 nutrient agar plates, washed and re-suspended in 20 mM phosphate buffered saline (PBS), pH 7.4. The cell density was adjusted to 2.5 x 10^8 CFU/mL. The cells were heat inactivated at 60 ^{0}C in water bath for 45 min and cooled (Fehri et al., 2010). Protein concentration of the *H. pylori* whole cell antigen was determined and was employed for immunization, as an antigen. Bacterial viability was assessed by plating on Ham's F-12 nutrient agar for 4 days at 37 ^{0}C and colony counting to ensure complete killing of bacteria before injecting into rabbit.

4.4.2.2. Immunization of rabbit

New Zealand white male rabbit (7 months old) housed in the animal house facility of CFTRI institute was used for the experiment, according to standard operating procedures (Howard et al, 2007). The rabbit was approved by the institutional ethical committee, which follows the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. IAEC-67, 2005), Government of India, New Delhi, India.

Antigen-adjuvant mixture was prepared by mixing equal volume (0.5 mL each) of antigen (in PBS containing ~150 µg of protein) with complete Freund's adjuvant for initial 2 injections and with incomplete Freund's adjuvant for subsequent injections. Emulsified antigen was injected at multiple sites subcutaneously using a 22G needle. Four such injections were given at intermittent intervals of one week. A final booster dose was given on week 8 with Freund's incomplete adjuvant and rabbit was bled from the ear vein after 5 days of injection. Prior to immunization 1.5 mL blood was collected from the marginal vein of the ear from the same rabbit to use as pre-immune serum.

4.4.2.3. Collection of the serum

The hairs on rabbit ear were removed with a new blade and then area was swabbed with xylene and the marginal vein tapped. The tip of the ear was nicked with a sharp blade. About 5 mL of blood was collected in sterilized tubes and kept in a slanting position at room temperature for 20 min followed by storage at 4 ^{0}C for 20 min to allow the separation of serum. Serum was centrifuged at 500 *g* for 10 min and the serum was stored at –20 °C for further studies. The amount of antibody present in the serum (antibody titer) was determined by ELISA as follows.

4.4.2.4. Determination of antibody titer (anti-HP-PcAb)

An antibody titer is a measurement of how much antibody an organism has produced that recognizes a particular epitope, which is expressed as the greatest dilution ratio that still gives a positive result. ELISA is a common means of determining antibody titer.

Production of *H. pylori* protein specific antibodies in the antiserum of immunized rabbit was analyzed initially by noncompetitive ELISA (Sathisha *et al.*, 2007). A 96 well microtitre plate coated with the serially diluted antigen with a concentration range of 0.001 to 1 µg/well (100 µL volume) in 0.1 M carbonate-bicarbonate buffer, pH 9.6. Samples were incubated at 4 °C overnight; followed by washing four times (250 µL/well) with ELISA wash buffer (PBS containing 0.05% Tween-20 (PBS-T)). The wells were blocked with 200 µL of ELISA blocking buffer (5% skimmed milk powder in PBS-T) for 1 h at 37 °C. After washing, wells were incubated with 100 µL/well of polyclonal antisera diluted in ELISA dilution buffer (1% skimmed milk powder in PBS-T) (1:10,000 to 1:100,000, v/v) and incubated at 37 °C for 2 h. After washing, as mentioned above, the secondary antibody- alkaline phosphatase conjugated - Goat anti-rabbit IgG (1:5000 dilution in ELISA dilution buffer, v/v; 100 µL/well) was added and incubated at 37 °C for 1 h. Finally 100 µL of p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8 (1 mg/mL) were added to each well and incubated at 37 °C for 30 min until colour developed. The results assessed visually and quantified by reading the absorbance at 405 nm using a microtitre plate reader (Spectra Max-340, Molecular Devices, Germany). The same experiment was also done for pre-immune serum using similar dilutions for comparison.

Antibody titer is defined as the reciprocal of the highest dilution that gave a positive value in ELISA, where positive reactivity is considered when the signals are >0.5 absorbance or atleast 2 folds higher than the blank.

4.4.2.5. Determination of sensitivity

The sensitivity of anti-HP-PcAb was done by ELISA method. As described earlier in a 96 well microtitre plate, coated antigen with a concentration range of 0.001 to 1 µg/well (100 µL volume) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, incubated at 4 °C overnight; followed by washing four times (250 µL/well) in ELISA wash buffer (PBS containing 0.05% Tween-20 (PBS-T)). The wells were blocked with 200 µL of ELISA blocking buffer (5% skimmed milk powder in PBS-T) for 1 h at 37 °C. Wells were incubated with 100 µL/well of pre-immunized serum and immunized serum diluted in ELISA dilution buffer (1:20,000, v/v) and incubated at 37 °C for 2 h, followed by secondary antibody – alkaline phosphatase conjugated with goat anti-rabbit IgG (1:5000 dilution in ELISA dilution buffer, v/v; 100 µL/well).

Finally 100 µL of p-nitrophenyl phosphate was added to each well and incubated at 37 °C for 30 min and the absorbance was measured at 405 nm using a microtitre plate ELISA reader (Spectra Max-340, Molecular Devices, Germany).

4.4.2.6. Determination of specificity

To determine the cross reactivity of anti-HP-PcAb, *Escherichia coli,* ATCC-11775, a Gram negative bacteria commonly found in the gut and *Staphylococcus aureus,* ATCC-12600, a Gram positive bacteria frequently part of the skin or mucosal layer were used. Bacterial cells were heat inactivated as described for *H. pylori* and coated to 96 well microtitre plate at concentration of 0.001 to 10 µg protein/100 µL/well and performed ELISA using anti-HP-PcAb (1:20,000, v/v), secondary antibody – alkaline phosphatase conjugated with goat anti-rabbit IgG (1:5000, v/v) and 100 µL of p-nitrophenyl phosphate (1 mg/mL) as described above (Section No. 4.4.2.4). Antibody reactivity was compared with that of *H. pylori*.

4.4.2.7. Western blot analysis

Bacterial whole cell extract was prepared with 0.1% triton X-100 in PBS with brief sonication (30 s) and centrifuged at 1000 *g* for 10 min, Protein concentration in the supernatant was estimated and used for SDS-PAGE and western blot analysis. The proteins were resolved on 12.5% SDS-PAGE gel (Balasubramanian et al., 2009). 50 µg protein/well was loaded along with prestained protein molecular weight marker and electrophoresis was performed for 3 h at 50 V. Further, the proteins were electrotransferred onto nitrocellulose membrane and confirmed with ponceau S stain. The stain was removed and the membrane was incubated with blocking buffer (Tris buffered saline-TBS, pH 7.5, containing 0.05% tween-20 and 3% gelatine) for 1 h. Further membrane was incubated with primary antibody (anti-HP-PcAb serum) of 1:2000 (v/v) dilution followed by secondary antibody - goat anti-rabbit IgG conjugated with horse radish peroxidase (1:5000, v/v). The bands were detected by adding 3,3-Diaminobenzidine : H_2O_2 substrate solution (DAB system) till bands were developed (Sathisha et al., 2007).

4.4.3. Evaluation of antiadhesive property of antiulcer compounds against *H. pylori*

4.4.3.1. Inhibition of *H. pylori* induced hemagglutination

Human erythrocytes are known to contain receptors for *H. pylori* and the interaction of this with pathogen (bacteria) resulted in adhesion. This hemagglutination property of *H. pylori* was used to determine antiadhesive potential of selected antiulcer compounds such as SRPP, BCPP and HMBA.

Human erythrocytes were prepared from 10 mL of fresh blood collected in Alsever's medium (8·0 g trisodium citrate, 0·55 g citric acid, 4·2 g NaCl, 20·5 g D-glucose in 1 L water, pH 6·1), washed four times with five volumes of 0.15 M NaCl. The erythrocytes were trypsinized for 1 h at 37 °C in 0.02 M PBS, pH 7.4 containing 1 mg/mL trypsin. The trypsin treated cells were washed with five volumes of 0.15 M NaCl and fixed in five volumes of 0.02 M PBS, pH 7.4 containing 1% glutaraldehyde for 1 h at room temperature. Glutaraldehyde fixation was terminated by the addition of five volumes of 0.1 M glycine in PBS, pH 7.4 at 4 °C. Finally, erythrocytes suspended at 2% concentration in PBS were used for hemagglutination assay (Sathisha et al., 2007). *H. pylori* cell suspension was prepared in PBS to give 4 hemagglutination units (HAU). One HAU is defined as the lowest concentration of bacteria, still giving complete hemagglutination.

Hemagglutination inhibition tests were performed in U-shaped microtitre plate by mixing 50 µL of serially diluted test antiadhesives (SRPP/BCPP/HMBA) in PBS with 50 µL of *H. pylori* for 30 min at room temperature, after which 50 µL of erythrocytes were added to each well and incubated for 10 min at room temperature. Hemagglutination was visualized by microscopic observation. Erythrocytes in the bacterial suspension without antiadhesives were used as positive controls and those without bacterial suspension were considered as negative controls. Minimum inhibitory concentrations (MIC) of the substances were also determined (Lee et al., 2006b).

4.4.3.2. Inhibition of *H. pylori* adhesion to gastric mucin - ELISA method

Gastric mucin acts as a receptor for *H. pylori* adhesion and this adhesion could be inhibited by some compounds which are designated as antiadhesive

agents. Adhesion was tested by measuring *H. pylori* adhesion to gastric mucin.

100 µL of porcine gastric mucin (0.1 mg/mL) in 0.1 M bicarbonate buffer (pH 9.6) was added to each well of a 96-well immunoassay microtitre plate and incubated overnight at 4 ^{0}C. Washed 3 times with PBS and 100 µL mixture of *H. pylori* cell suspension (5 x 10^7 CFU/mL in PBS), preincubated with and without equal volume of serially diluted test antiadhesives (SRPP/BCPP/HMBA) for 30 min at room temperature was added and incubated for 1 h at 37 ^{0}C. After incubation the wells were washed with ELISA wash buffer and mucin bound *H. pylori* was detected by addition of anti-HP-PcAb (1:20,000, v/v), followed by horse radish peroxidase conjugated anti-rabbit IgG (1:5000, v/v) and p-nitrophenyl phosphate solutions as described above in section 4.4.2.4. The results were quantified by reading the absorbance at 405 nm using a microtitre plate reader (Spectra Max-340, Molecular Devices, Germany). The increase in the absorbance indicates the increased adhesion of *H. pylori* to gastric mucin and decrease in the absorbance indicates the increase potency of antiadhesives in inhibiting adhesion of *H. pylori* to gastric mucin. The wells without antiadhesives were considered as positive controls and those without *H. pylori* were considered as negative controls.

4.4.4. Determination of anti-*H. pylori* effect of antiulcer compounds

Anti-*H. pylori* effect was examined by the following methods.

4.4.4.1. Agar well diffusion assay

Inhibition of *H. pylori* growth was determined by agar well diffusion method (Ahmad & Beg, 2001). Briefly, the petriplates were prepared with Ham's F-12 nutrient agar media containing 5% FBS. After the medium was solidified, the plate was inoculated with 100 µL of *H. pylori* culture (10^5 cells/mL). Well of 7 mm was made in the agar medium and filled with 25 µL of HMBA (4 mg/mL, dissolved in DMSO). The plate was incubated at 37 ^{0}C in a microaerobic condition for 2 days. Antimicrobial activity was determined by measuring the diameter of inhibition zone around the well. For comparative evaluation, equal volume of amoxicillin and DMSO were also tested in individual wells. Results are expressed as mean value of three experiments.

4.4.4.2. Viable colony count assay

H. pylori growth inhibitory effect of HMBA was tested by viable colony count method as described earlier (Chapter 1, section 1.4.9). Stock solution of HMBA was prepared in 100 µL DMSO, diluted to 1 mL with sterile PBS at concentration range of 0.05 to 2 mg/mL and used for the assay. The effectiveness of the inhibitors killing *H. pylori* was expressed as percentage inhibition of colony growth (i.e. percentage of bacteria killed) compared to that of control (O'Mahony et al., 2005).

4.4.4.3. Determination of minimal inhibitory concentration (MIC)

MIC values were determined by serial dilution of inhibitors in 96 well microplates (Eloff, 1998) as described in chapter 1, section 1.4.11.

4.4.4.4. Scanning electron microscopic (SEM) studies

SEM has been used as a tool to understand and analyse the characteristic features of the bacteria. Lysis if any by inhibitors treatment was determined by SEM studies. This was done as described in chapter 1, section 1.4.10 and morphological changes in inhibitor treated *H. pylori* were recorded (Kai et al., 1999).

HMBA showed a potent growth inhibitory activity when compared to BCPP and SRPP against *H. pylori*. Therefore further mechanisms were studied with respect to HMBA alone.

4.4.4.5. Effect of HMBA on protein profile of *H. pylori*

If HMBA induces damage to *H. pylori*, comparative protein profiling studies of *H. pylori* culture with and without HMBA often provides the extent of damage, also target protein for HMBA. Protein profiles were thus studied. One mL of overnight grown *H. pylori* culture (10^8 cells/mL) was washed and redissolved in PBS and incubated with equal volume of HMBA (0.05 mg/mL) in PBS for 1 h at 37 ^{0}C. After incubation, cells were pelleted and protein was extracted with 0.1% SDS, and the supernatant was precipitated with trichloroacetic acid (final concentration of 5%), redissolved in SDS-PAGE buffer (Saumya & Paul, 1983) and compared between control and HMBA treated samples after estimating protein content (Bradford, 1976). Equal aliquots from 10^8 cells/mL of control and HMBA treated *H. pylori* were subjected to SDS-PAGE, which constitute 4% stacking and 12% separating

gel. The gel was stained by Coomassie brilliant blue R-250 and observed for the protein profile and compared between control and HMBA treated *H. pylori* protein.

4.4.5. Effect of HMBA on *H. pylori* mediated cytotoxicity

H. pylori has been known to cause toxicity to mammalian cells (Marina de et al., 1997) by virtue of cytotoxic factors including virulent gene products. In the current study therefore the effect of *H. pylori* on buccal cells was studied. Buccal cells were obtained from gentle scraping of the inner cheek layer of healthy person, washed 3 times with sterile PBS and suspended in Ham's F12 media containing 100 U/mL of penicillin and streptomycin. *H. pylori* pre-treated with and without HMBA (10 – 50 µg/mL) in Hams F12 nutrient broth for 1 h, then added to buccal cells at a ratio of 300:1 (bacteria/buccal cell) and co-cultured for 4 h. The cells were then stained with acridine orange and ethidium bromide and observed under microscopy.

4.4.6. Effect of HMBA on DNA damage

The DNA protective effect of HMBA was determined electrophoretically using calf thymus DNA (Rodriguez & Akman, 1998). This has been done as an effort to understand whether HMBA has any role against *H. pylori* mediated cytotoxicity. Cytotoxicity includes damage at both cytoplasm and nuclear level. Calf thymus DNA (1 µg in 20 µL) with different concentrations (2-8 µg) of HMBA was subjected to UV-irradiation for 30 min in presence of 2 mM H_2O_2. Gallic acid (2 µg) was used as a known DNA protective compound, since it is having good free radical scavenging activity. Relative difference in the migration between the native and oxidized DNA was determined by agarose gel electrophoresis and the gel stained with ethidium bromide was documented (Herolab, Germany).

4.4.7. Mechanism of DNA protection by HMBA

Ethidium bromide emits intense fluorescence light in the presence of DNA, due to its strong intercalation between the adjacent DNA base pairs. It is reported that the enhanced fluorescence can be quenched by the addition of a DNA binding molecule. The extent of quenching of fluorescence of ethidium bromide bound to DNA has been used to determine the extent of binding between the test molecule and DNA (Labieniec & Gabryelak, 2006).

Our earlier experiences had revealed that DNA protection ability could be due to either direct free radical scavenging or preventing the damaging agent to attack DNA. Interaction studies were therefore conducted to determine the mechanism of DNA protection since HMBA showed poorer antioxidant activity. Calf thymus DNA sufficiently free of protein was used. A_{260}/A_{280} of 1.8-1.9 was considered for the assay. DNA concentrations were determined spectrophotometrically with an extinction coefficient of 6600 M^{-1} at 260 nm (Labieniec & Gabryelak, 2006). All the experiments were carried out in 5 mM Tris-HCl buffer pH 7.0. DNA and ethidium bromide were dissolved in buffer at a concentration of 3 and 1 µg/mL, respectively. Phenolics (HMBA and vanillin) were used at a concentration range between 0.5-2.5 µg/mL. Vanillin was used for comparison since it is a structural analog of HMBA. The phenolics were added to ethidium bromide bound to calf thymus DNA and the intensity of fluorescence of ethidium bromide was measured. Fluorescence spectra was recorded using an excitation wavelength of 478 nm and the emission range set between 480 and 850 nm using a slit width of 5/5 nm as described previously (Nanjundaiah et al., 2009).

4.4.8. Interaction of HMBA with human serum albumin (HSA)

Interaction of HMBA with HSA was studied by spectrofluorometric method (Soares et al., 2007). Stock solution of human serum albumin was prepared to a concentration of 1.0×10^{-4} M in Tris-HCl buffer of pH 7.4 containing 150 mM sodium chloride. HMBA and vanillin were prepared to a concentration of 10 mg/100 mL in ethanol (95%). All the stock solutions were then diluted with the buffer to obtain the actual assay concentration. One mL of assay solution containing 10 µL of stock solution of HSA and varied concentrations of HMBA or vanillin (0.5 - 2.5 µg/mL) in Tris–HCl buffer of pH 7.4. Tubes were mixed thoroughly and placed in the thermostat water-bath at 37 °C for 5 min and fluorescence emission spectra were recorded in the wavelength range 290–500 nm by exciting HSA at 280 nm using a slit width of 5/5 nm. Wavelength nearer to shift observed was recorded to understand the involvement of tryptophan/tyrosine residue in HSA.

4.4.9. Determination of antioxidant activity of HMBA

In majority of the cases antimicrobial activity have been shown to be via antioxidant properties (Fung et al., 1985; Siddaraju & Dharmesh, 2007a &

2007b). In the current study therefore antioxidant activity of HMBA was determined employing 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Braca et al., 2003) as described in section 1.4.7.1 of chapter 1.

4.4.10. Statistical analysis

All the experiments were carried out in triplicates (n = 3) and the results are expressed as mean ± standard deviation (SD).

4.5. Results

4.5.1. Production and characterization of polyclonal antibodies (anti-HP-PcAb) to *H. pylori*

4.5.1.1. Antibody titre

Anti-*H. pylori* polyclonal antibody was developed in rabbit against whole cell extract of *H. pylori*. Antibody titre was determined by ELISA method (Fig 4.1). The ELISA absorbance units of ≥0.5 were considered as positive for antibody-antigen reaction. A good antibody titre of 1:50,000 (v/v) dilutions could detect the antigen concentration of 0.1 µg with the ELISA absorbance units of 1.233 which is ~5 folds higher than that of pre-immune serum.

4.5.1.2. Antibody sensitivity

The sensitivity test performed at 1:20,000 (v/v) dilutions of both immunized and pre-immunized serum showed that only immunized serum could be able to react with *H. pylori* antigen in a dose dependent manner with the absorbance units, that are about 4 to 13 folds higher than that of pre-immune serum at antigen concentration range of 0.001 to 1 µg (Fig 4.2).

4.5.1.3. Antibody specificity

The specificity of anti-HP-PcAb was tested at 1:20,000 (v/v) dilutions using *E. coli* and *S. aureus* whole cell lysates as non-specific antigens in addition to *H. pylori* at 0.001 to 1 µg concentrations. The results showed that anti-HP-PcAb was specific to only *H. pylori* and it could not react with other Gram negative *E. coli* and Gram positive *S. aureus* bacteria (Fig 4.3).

4.5.1.4. Western blot analysis

Two *H. pylori* isolates (Isolate 1 & 2) isolated from different gastric ulcer patients were used to test the reactivity with anti-HP-PcAb. These isolates were previously confirmed by PCR analysis for the presence of vacA gene. The western blot analysis using anti-HP-PcAb showed that, it could detect both the *H. pylori* isolates with some differences in detectable proteins. The vacA protein band of ~95 kDa was detected in both the isolates in additions to cleaved products of vacA of ~58 and 37 kDa proteins. The *H. pylori* isolate-2 showed more reactivity when compared to isolate-1. The *H. pylori* isolate-2 showed few more protein bands other than vacA products. The

detected protein bands could be urease or other cellular proteins of *H. pylori*. Thus the results indicate that the vacA protein is a major antibody determinant since it is detected in both the isolates (Fig 4.4).

4.5.2. Antiadhesive activity of antiulcer compounds

H. pylori adhesion to host gastric mucosal cells is the crucial step in the colonization and pathogenesis of *H. pylori*. Therefore inhibition of this process is necessary to provide gastroprotection against *H. pylori* induced gastric ulcers. Antiadhesive ability of antiulcer compounds were studied using *H. pylori* mediated hemagglutination assay and adhesion of *H. pylori* to gastric mucin *in vitro* by ELISA method. The hemagglutination assay results indicated the successful agglutination of RBCs in presence of *H. pylori* as shown in Fig 4.5A. This agglutination was inhibited in case of *H. pylori* pretreated with pectic polysaccharides – SRPP and BCPP (Fig 4.5A). HMBA although having *H. pylori* growth inhibitory property was unable to inhibit hemagglutination.

This property was further supported by *in vitro* adhesion of *H. pylori* to gastric mucin by ELISA method as explained under materials and methods. Results showed a dose dependent inhibition of *H. pylori* adhesion to gastric mucin at tested concentration range of 25 – 200 µg/mL (Fig 4.5C). Results also indicated SRPP as a potent antiadhesive compound with an IC_{50} of 49.5 ± 3.2 µg/mL, when compared to that of BCPP showing an IC_{50} of 90.0 ± 7.6 µg/mL (Fig 4.5B). Both the compounds showed dose dependent increase in the antiadhesive property in ELISA method (Fig 4.5C) and the results were correlated with that of hemagglutination assay.

4.5.3. Inhibition of *H. pylori* growth

Inhibition of *H. pylori* growth tested by agar well diffusion method shows the inhibition zone of 17 mm diameter in HMBA treated plate at 100 µg/well (Fig 4.6A). The growth inhibitory activity of HMBA was compared with other phenolic acids of Swallow root previously reported from our laboratory (Siddaraju & Dharmesh, 2007b) as shown in Table 4.1. The result shows HMBA as a best inhibitor of *H. pylori* growth next to cinnamic acid at 200 µg/well concentration. Vanillic acid and vanillin, the two structural analogs of HMBA were also tested, but they exhibited very poor growth inhibitory effect with inhibitory zone of 13 and 4 mm against 31 mm of HMBA

indicating the role of structural conformation and functional groups in exhibiting the antimicrobial activity.

The viable colony count method was also used to test the growth inhibitory effect of HMBA at different concentrations (0.05 to 0.1 mg/mL). In this assay the activity of HMBA supports the results of agar well diffusion method. This was evidenced by SEM studies of *H. pylori* treated with HMBA (Fig 4.8C,D). The MIC values determined by serial broth dilution method showed MIC of 39 ± 3.4 for HMBA, when compared to a known anti-*H. pylori* drug amoxicillin (26 ± 3.2 µg/mL) (Fig 4.6B). Thus the results indicate the potent activity of HMBA against *H. pylori* growth. Therefore further understanding on the mode of action of this anti-*H. pylori* compound was studied.

The components present in *D. hamiltonii* aqueous extract also revealed that they exhibit differential anti-*H. pylori* and antioxidant effects (Table 4.1 & Fig 4.10). Considering the abundance of these phenolic compounds (Table 4.1) as identified by HPLC and; anti-*H. pylori* and antioxidant activity (Table 4.1 & Fig 4.10), % activity has been calculated. Data revealed that >50% of anti-*H. pylori* activity has been attributed to HMBA (Fig 4.10) followed by vanillic acid (~20%) and vanillin (~12%) respectively. Similarly majority of antioxidant activity has been attributed to protocatechuic acid (~42%) followed by gallic acid (24%) and gentisic acid (12%).

4.5.4. Bacteriolytic effect of HMBA

In order to study the mode of action of HMBA on *H. pylori*, it was further tested for its cell lysis effects. The cell lysis was evidenced by protein profile of control and HMBA treated *H. pylori* cells. The relative protein content was increased to 24% in the supernatant and decreased to 64% in the pellet of HMBA treated cells when compared to pellet of control cells. The supernatant of control cells showed only 2% protein (Fig 4.7B). SDS-PAGE analysis of proteins also supported the result as indicated by decrease in the intensity of protein bands in HMBA treated pellet when compared to that of control cell pellet. But no difference was observed in the pattern of protein bands (Fig 4.7A).

The SEM study reveals the uniform rod shaped cells in control untreated *H. pylori* cells, while in case of HMBA (Fig 4.8C,D) and amoxicillin (Fig 4.8B) treatment, the morphology of the cells were altered to short rods or nearly

cocci form at lower doses (Fig 4.8C). At higher concentration (0.1 mg/mL) of HMBA the cells showed a completely distorted structure as indicated by low electron density regions, ruptured cell wall and cavity formation in the bacterial cells (Fig 4.8D). Thus the data indicated the bacteriolytic property of HMBA. Amoxicillin treatment also showed similar changes in *H. pylori* at 0.05 mg/mL concentration (Fig 4.8B).

4.5.5. Modulation of *H. pylori* mediated cytotoxicity by HMBA

Since *H. pylori* produces cytotoxicity associated proteins particularly CagA and VacA, the *H. pylori* with and without HMBA pretreatment were cocultured with buccal cells to assess *H. pylori* induced cytotoxicity in buccal cells *in vitro.* Fig 4.9G provides evidence for the presence of VacA gene which was amplified by PCR using VacA specific primers. Evident cellular changes such as cellular vacuolation, nuclear damage and apoptosis (indicated in arrows in Fig 4.9B,E was observed in buccals cells treated with control, $vacA^+$ *H. pylori* cells, whereas these changes were reduced in case of buccal cells incubated with HMBA pretreated *H. pylori* (Fig 4.9C,F).

4.5.6. Antioxidant and DNA protective ability of HMBA

Hydroxyl radicals generated from H_2O_2, under UV-irradiation induces DNA strand breaks due to oxidation reaction (Ananthaswamy & Eisenstark, 1977). The electrophoretic mobilities of DNA on agarose gel shows same pattern of DNA mobility in both native and higher doses of HMBA treated lane. Untreated but UV-exposed DNA migrated faster than native DNA indicating DNA damage (Fig 4.9H). HMBA protected DNA in a dose dependent manner at 2 to 8 µg concentrations against free radical/UV-induced DNA damage.

4.5.7. Interaction of HMBA with DNA and protein

Since the antioxidant activity was poorer for HMBA while DNA protective ability was good, it is thought that HMBA may protect DNA by binding rather than quenching UV-induced free radicals. Binding of HMBA with DNA was therefore studied. The fluorescence emission spectra of ethidium bromide bound to DNA in the absence and presence of HMBA or vanillin of *D. hamiltonii* was performed. Result showed the moderate binding ability (~10%) of HMBA to DNA when compared to its inactive isomer – vanillin, which showed only 4.5% (~2 folds less) binding (Fig 4.11A,B,E).

Results of protein (human serum albumin - HSA) binding by HMBA and vanillin substantiates the increased binding ability of HMBA with Stern-Volmer constant of 0.068 x 10^6 M^{-1} than vanillin (K_{SV} of 0.019 x 10^6 M^{-1}) (Fig 4.11C,D,E).

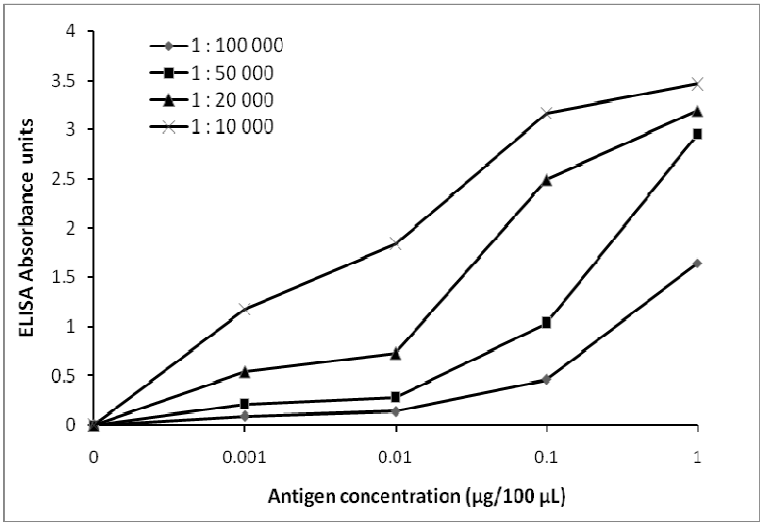


Fig 4.1. Antibody titre analysis of anti-*H. pylori* polyclonal antibody (anti-HP-PcAb).

Titration analysis performed at different concentrations of antigen – *H. pylori* protein (0.001 to 1 µg/100 µL/well) and antiserum dilutions (1:10,000 to 1: 100,000, v/v).

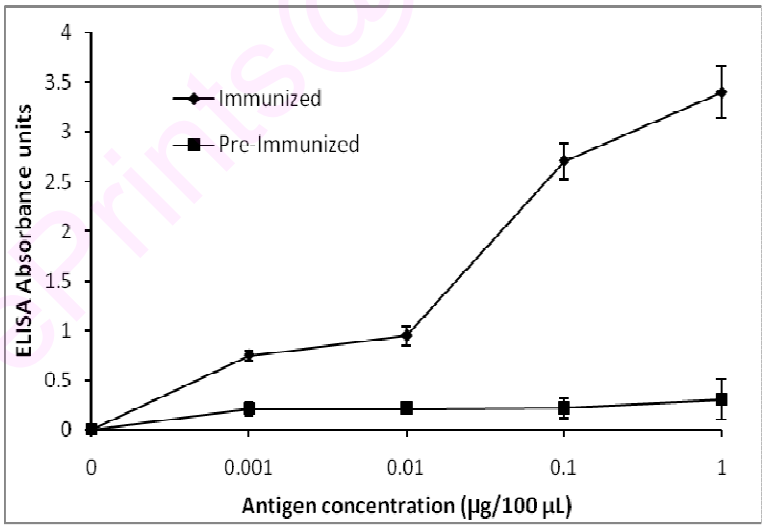


Fig 4.2. Determination of reactivity of antigen - *H. pylori* protein at different concentrations (0.001 to 1 µg/100 µL) to immunized and pre-immunized serum at serum dilutions of 1:20,000 (v/v).

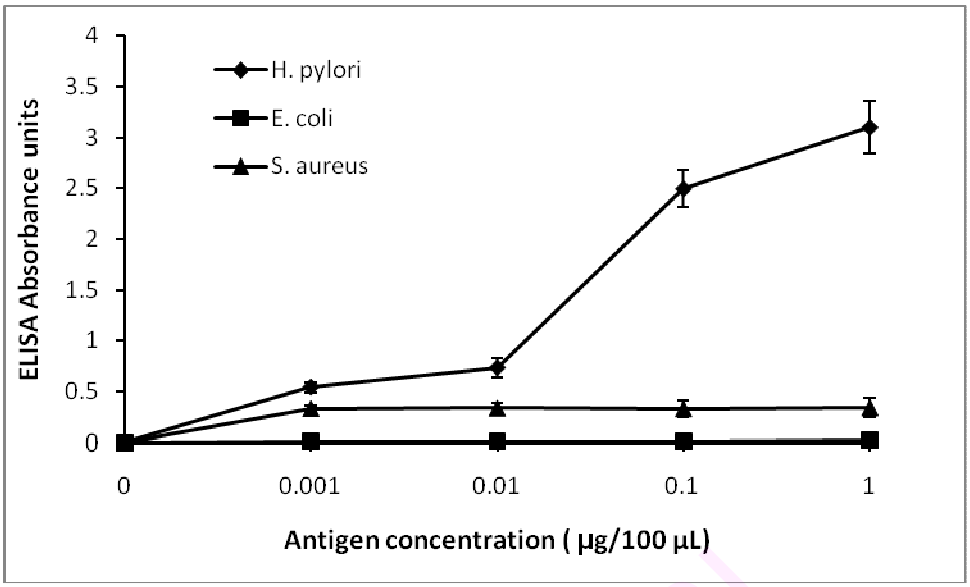


Fig 4.3. Determination of specificity of anti-HP-PcAb.

The anti-HP-PcAb (1:20,000 dilutions) reacted with only *H. pylori* protein but not with *E. coli* and *S. aureus* at different concentrations of protein (0.001 to 1 µg/100 µL) indicating the specificity of the anti-HP-PcAb in recognizing only *H. pylori.*

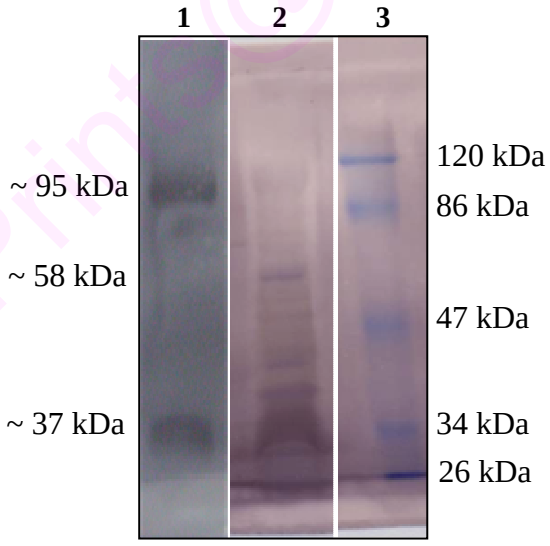
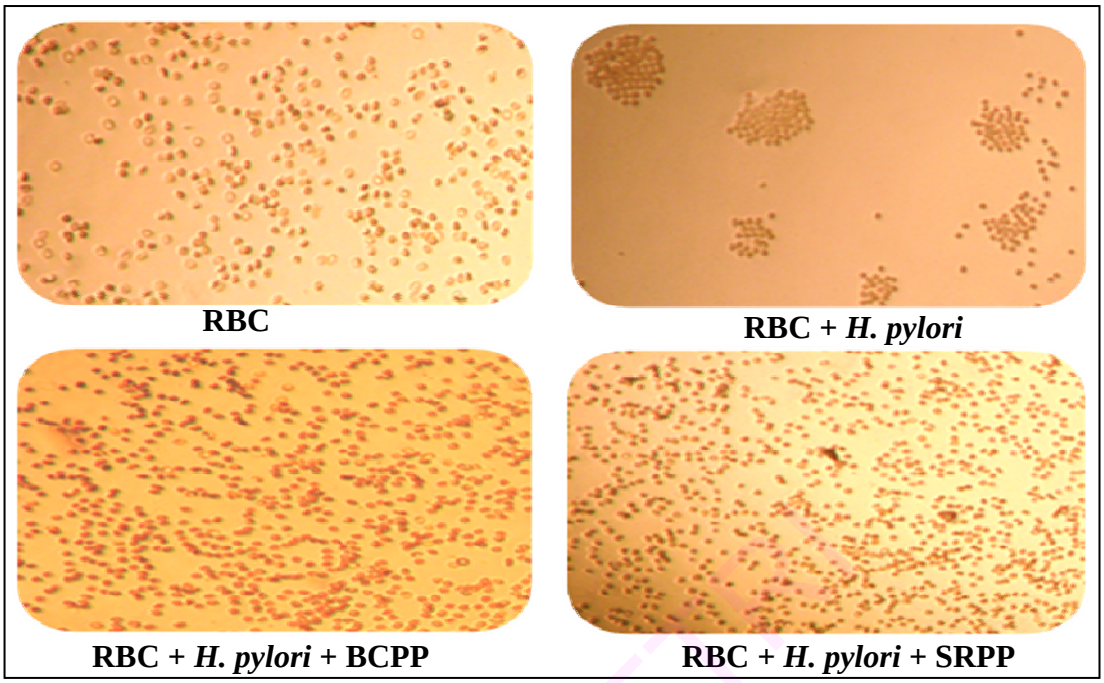


Fig 4.4. Western blot analysis of reactivity of anti-HP-PcAb to two different *H. pylori* isolates obtained from two different gastric ulcer patients.

Lane 1 – *H. pylori* isolate 1; Lane 2 – *H. pylori* isolate 2; Lane 3 – Prestained protein molecular weight marker. Approximately 95, 58 and 37 kDa protein bands were detected in two different isolates of *H. pylori* obtained from different gastric ulcer patients.

A



B

Sample	Antiadhesive activity (ELISA) IC_{50} (µg/mL)
SRPP	49.5 ± 3.2
BCPP	90.0 ± 7.6
HMBA	No inhibition

C

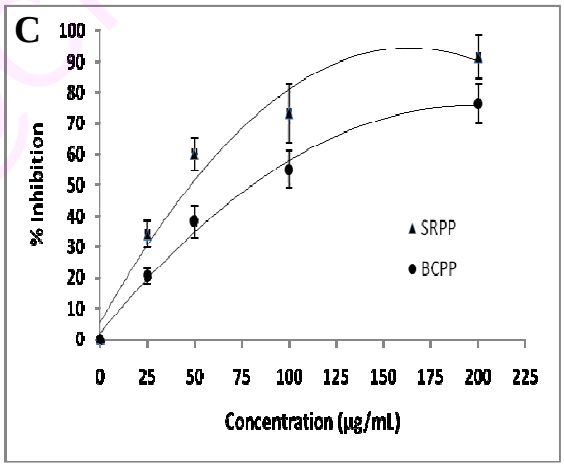
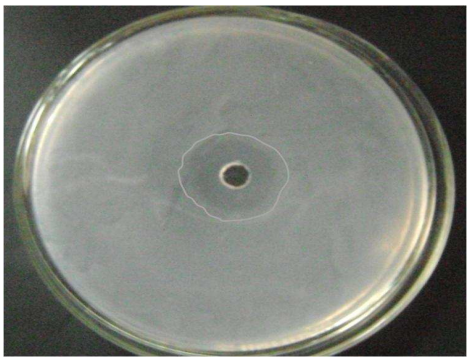


Fig 4.5. Determination of antiadhesive ability.

Fig A: Hemagglutination inhibition: Microscopic pictures of RBCs at 10X magnification. *H. pylori* induced agglutination of RBCs, which was inhibited by BCPP and SRPP (A). **Fig B** shows IC_{50} values based on inhibition of *H. pylori* adhesion to gastric mucin (ELISA) assay. **Fig C** : *H. pylori* adhesion to gastric mucin coated to 96 well microtitre wells. The adhesion was inhibited by pretreatment of *H. pylori* with SRPP/BCPP at different concentrations (25–200 µg/mL). The adherent *H. pylori* was detected by anti-*H. pylori* polyclonal antibody (anti-HP-PcAb).

A



B

Samples	*H. pylori* inhibition - MIC (µg/mL)
HMBA	39 ± 3.4
Amoxicillin	26 ± 3.2

Fig 4.6. Inhibition of *H. pylori* growth: A – Zone of *H. pylori* inhibition by HMBA (100 µg/well) in agar well diffusion method. Inhibitory activity was measured as a diameter of the inhibition zone around the well. **B** – MIC values for inhibition of *H. pylori* growth, determined by serial broth dilution method.

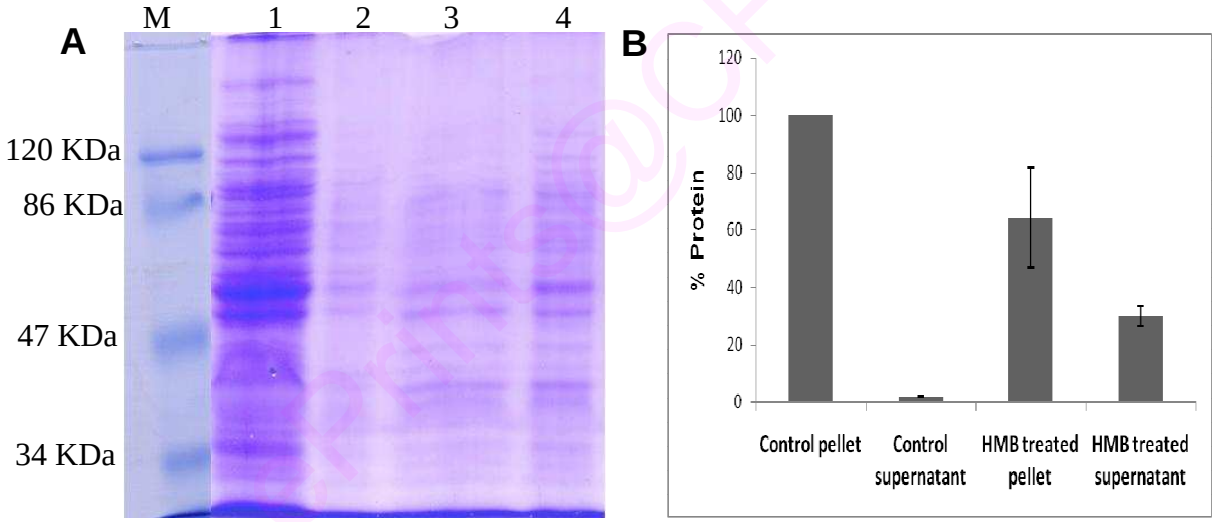


Fig 4.7. Bacteriolytic effect of HMBA: Protein profile of pellet and supernatant of control and HMBA treated *H. pylori* cells resolved on SDS-PAGE **(B)**. Lane M – pre-stained marker protein, 1 – control pellet, 2 – control supernatant, 3 – HMBA treated pellet, 4 – HMBA treated supernatant. **Fig B** shows relative percent protein content in the pellet and supernatant of control and HMBA treated *H. pylori* cells. The protein content decreased in the pellet and increased in the supernatant after HMBA treatment indicating the lysis of bacterial cell wall and release of cellular contents.

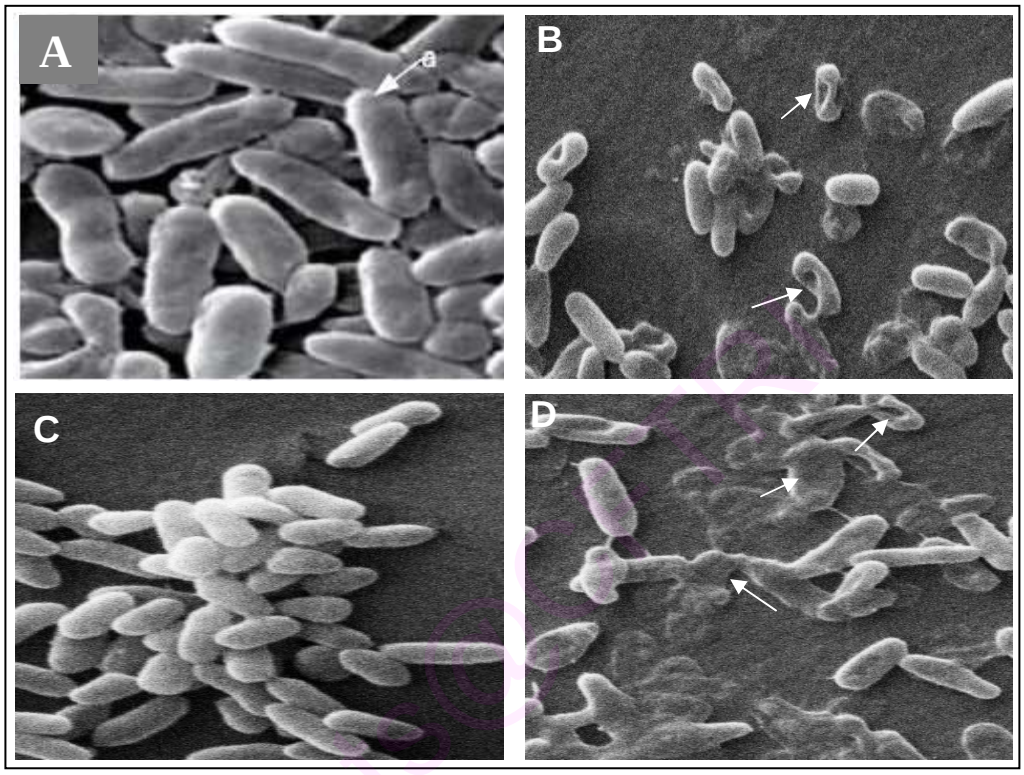


Fig 4.8. Scanning electron microscopic studies: SEM pictures of *H. pylori* cells at 15000 magnification. **A**- Control, **B-** Amoxicillin (0.05 mg/mL), and; **C & D-** HMBA (0.05 & 0.1 mg/mL) treated *H. pylori* cells. Untreated control cells are uniform in shape, whereas inhibitor treatment showed transformation of rod shaped cells to short rods or nearly cocci form at lower concentrations and completely lysed cells with disrupted structures at higher concentrations (indicated by arrows).

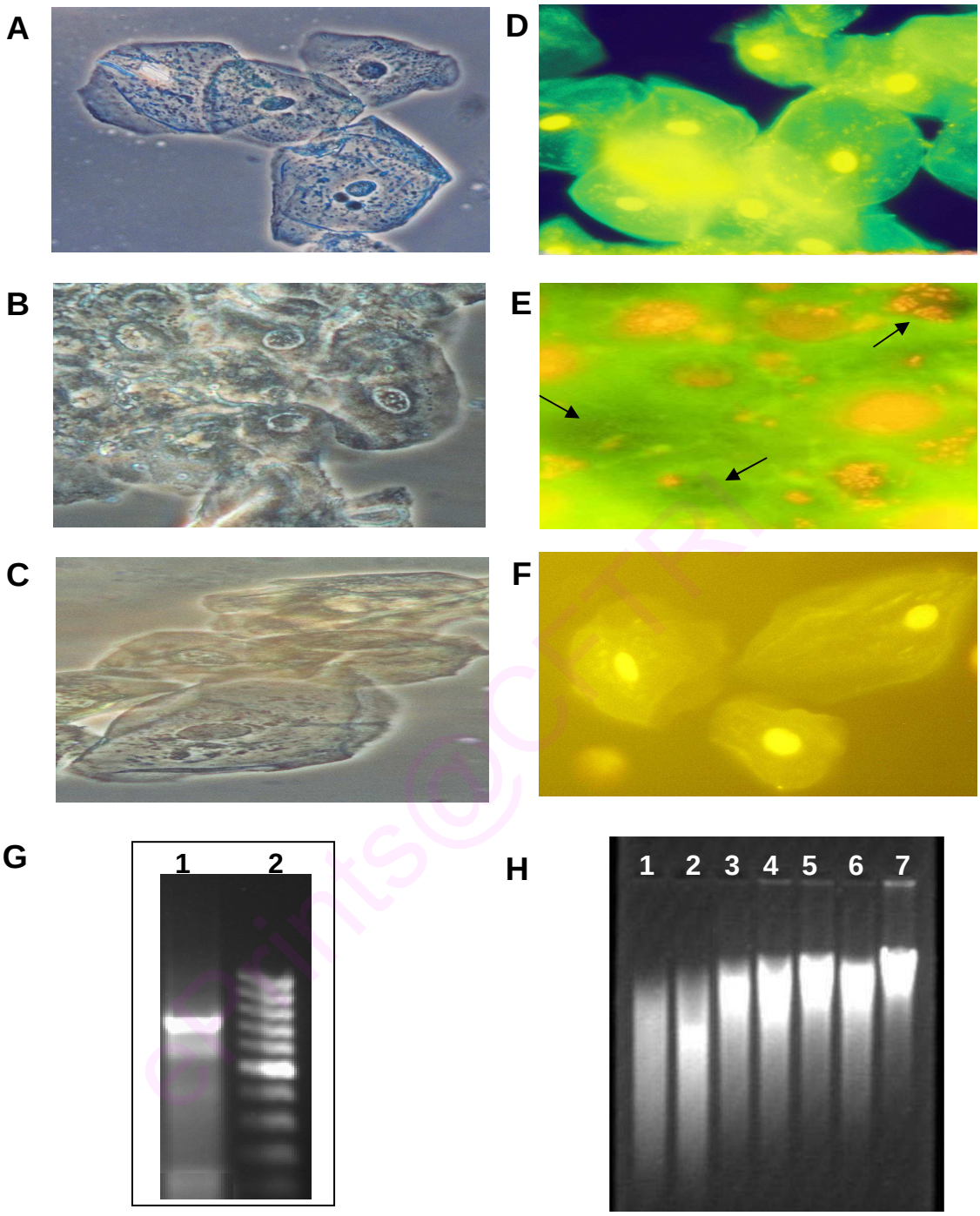


Fig 4.9. Modulation of *H. pylori* mediated cytotoxicity by HMBA: Phase contrast (**Fig A-C**) and fluorescent (**Fig D-F**) microscopic pictures of buccal cells. *H. pylori* induces cell vacuolation, nuclear degeneration and cell damage in buccal cells as indicated by arrows (**Fig 4B,E**). These changes are inhibited in buccal cells treated with HMBA (50 µg/mL) (**Fig 4C,F**). **Fig A & D** are control buccal cells without *H. pylori*. The results indicate the cytoprotective effect of HMBA against *H. pylori* induced cytotoxicity.

Fig G shows PCR amplified product of vacA gene in lane 1 the product of this gene induces host cell vacuolation and increased cell permeability during *H. pylori* pathogenesis and lane 2 -100 bp DNA ladder. **Fig 4H** shows DNA protection ability of HMBA against H_2O_2/UV-induced DNA damage, where Lane 1 – H_2O_2/UV-irradiated DNA, lane 2 to 5 are DNA pre-treated with HMBA at 2-8 µg concentration and lane 6 DNA pre-treated with gallic acid at 2 µg concentration before UV-irradiation. Lane 7 - native DNA. 1 µg of calf thymus DNA loaded/well and electrophoresed on 1% agarose gel. Ethidium bromide stained DNA bands were visualized by transilluminator. Increased mobility indicates damaged DNA.

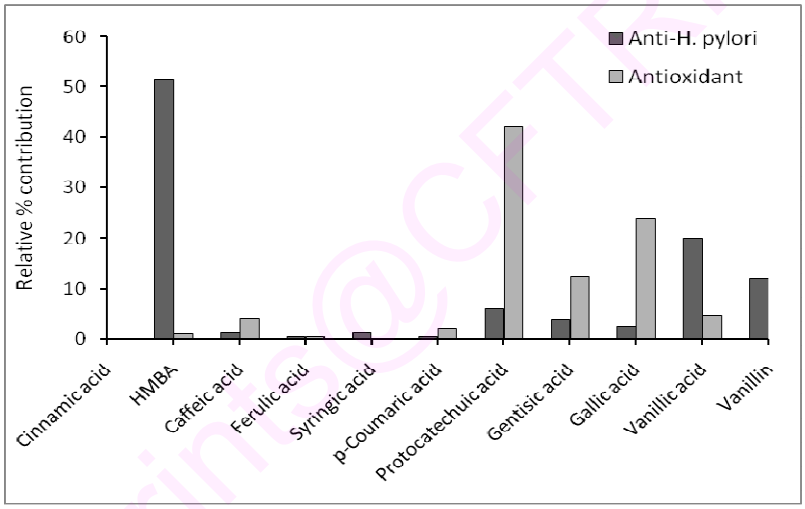
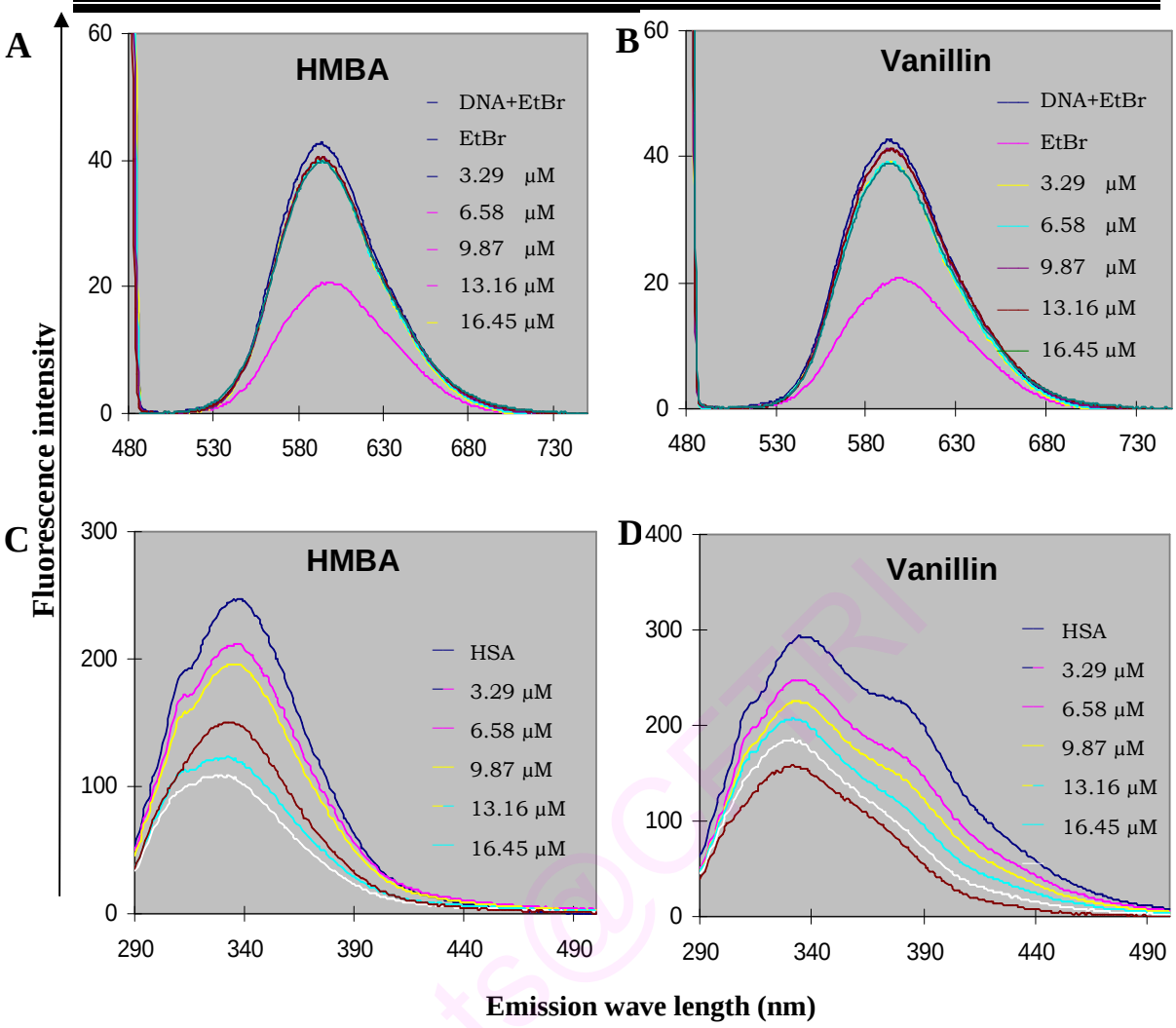


Fig 4.10. Relative % contribution of individual phenolic acids of Swallow root to anti-*H. pylori* and antioxidant activities.
Considering the relative abundance and anti-*H. pylori* & antioxidant activities suggest that HMBA and protocatechuic acid in Swallow root aqueous extract are responsible for the respective bioactivities.



E

	% DNA binding	Protein binding ability. (Stern-Volmer Constant - Ksv M^{-1})
HMBA	10.61 ± 0.36	0.068 x 10^6
Vanillin	4.53 ± 0.04	0.019 x 10^6

Fig 4.11. Interaction of HMBA with DNA and protein: Fluorescence emission spectra of ethidium bromide (EtBr) bound DNA (**Fig A,B**) and human serum albumen (**Fig C,D**) in presence of HMBA and vanillin at different concentrations. The excitation wave length was 478 nm for EtBr and 280 nm for HSA. Both excitation and emission slits widths were 5 nm. The concentrations of EtBr (3 µg/mL), DNA (5 µg/mL) and HSA (0.5 µM) were constant. The quenching of fluorescence of EtBr and HSA indicates binding of HMBA/vanillin to DNA and HSA respectively. **Fig E** shows % DNA binding and protein binding ability expressed as stern-volmer constants.

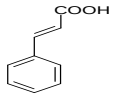
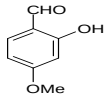
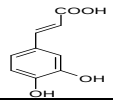
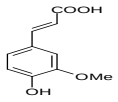
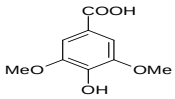
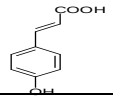
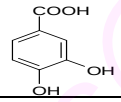
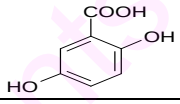
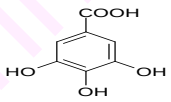
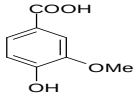
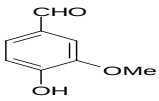
Phenolic compounds	Structures	Abundance (mg/g)	*H. pylori* inhibition zone (mm)	Antioxidant activity (IC_{50} in µg/mL)
Cinnamic acid (3-phenyl-2-propenoic acid)		0.02 ± 0.001	34 ± 4.1	4.6 ± 0.3
HMBA (2-hydroxy-4-methoxy benzaldehyde)		5.06 ± 0.239	31 ± 3.5	213 ± 5.12
Caffeic acid (3,4-dihydroxy cinnamic acid)		0.15 ± 0.008	28 ± 3.4	1.8 ± 0.14
Ferulic acid (4-hydroxy-3-methoxy cinnamic acid)		0.08 ± 0.017	25 ± 2.6	6.6 ± 0.51
Syringic acid (4-hydroxy-3,5-dimethoxy bezoic acid)		0.21 ± 0.007	18 ± 2.1	64.9 ± 5.4
p-coumaric acid (p-hydroxy cinnamic acid)		0.08 ± 0.01	18 ± 2.8	1.9 ± 0.20
Protocatechuic acid (3,4-dihydroxy benzoic acid)		1.16 ± 0.045	16 ± 1.8	1.35 ± 0.16
Gentisic acid (2,5-dihydroxy benzoic acid)		0.76 ± 0.036	16 ± 2.1	3.0 ± 0.28
Gallic acid (3,4,5-trihydroxy benzoic acid)		0.54 ± 0.052	14 ± 1.6	1.1 ± 0.09
Vanillic acid (4-hydroxy-3-methoxy benzoic acid)		4.72 ± 0.104	13 ± 1.4	49.5 ± 2.4
Vanillin (4-hydroxy-3-methoxy benzaldehyde)		4.61 ± 0.101	< 4.0	80.00 ± 2.01

Table 4.1. Anti-*H. pylori* potential of HMBA in comparison with other phenolic acids of Swallow root.

Anti-*H. pylori* and antioxidant activity of HMBA compared with other known phenolic acids of Swallow root for understanding structure-function relation. 200 µg/well individual phenolic acids were used for *H. pylori* inhibition study. Inhibition zone for *H. pylori* is expressed in millimetres. Antioxidant activity was measured as free radical scavenging activity and expressed as IC_{50} values (µg/mL). All data are the mean ± SD of three replicates.

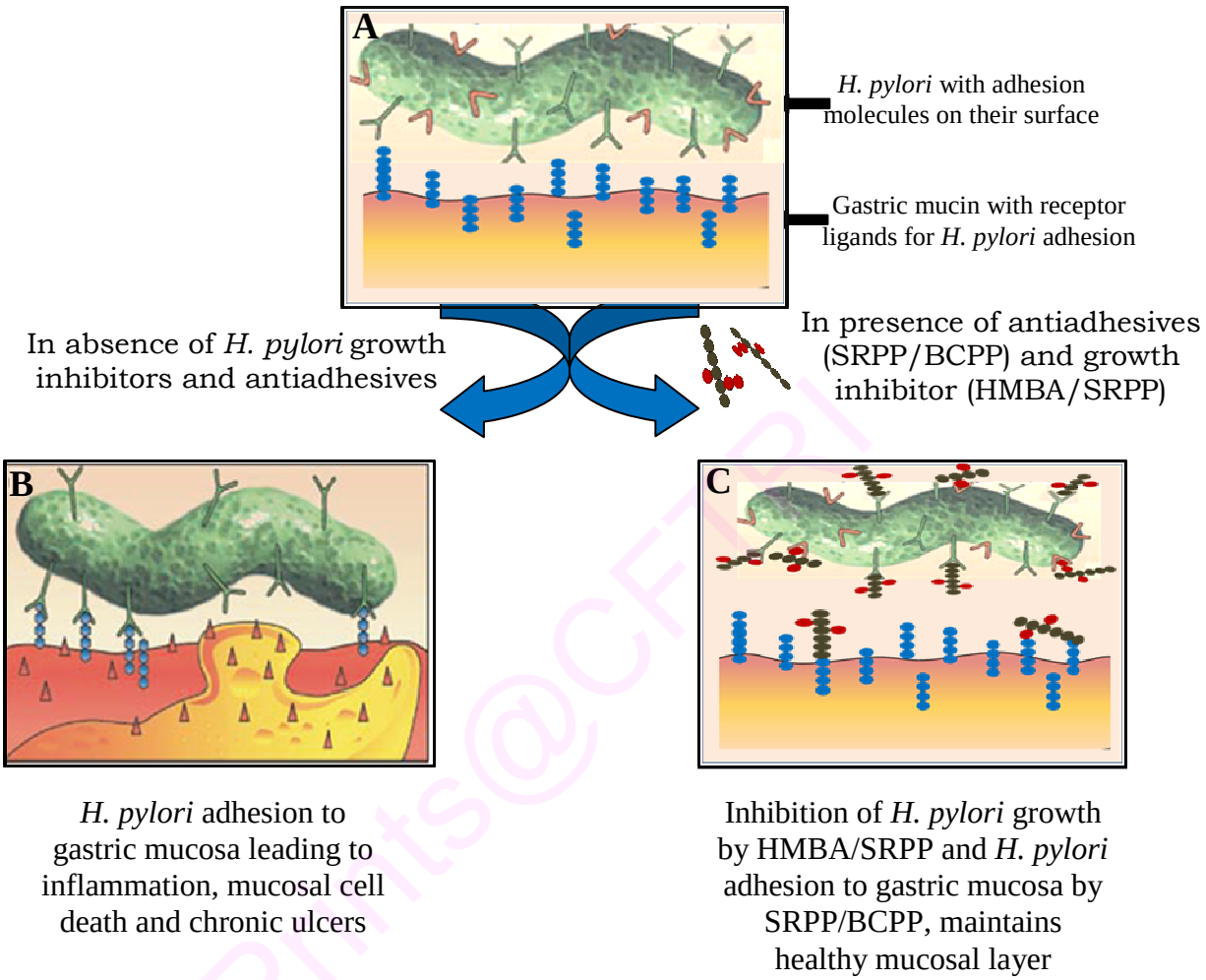


Fig 4.12. Interaction between *H. pylori* and gastric mucosa in presence and absence of dietary components (SRPP/BCPP/HMBA): *H. pylori* and gastric mucin interaction (A) leading to potential *H. pylori* mediated inflammation, mucosal cell death and ulcers (B). Glycosylated components on the *H. pylori* surface appear to interact with carbohydrate domains of gastric mucin is responsible for *H. pylori* adhesion followed by invasion and pathogenesis. Dietary components- SRPP/BCPP results in inhibition of adhesion of *H. pylori* to gastric mucin and inhibition of growth and colonization; while HMBA appears to contribute significantly towards *H. pylori* growth inhibition and protect mucosal cells against cytotoxicity of vacA of *H. pylori* (C).

4.6. Discussion

H. pylori mediated gastric ulcer and cancers are common global problems since it was found to colonize in ~50% of gastric ulcer/cancer patients. *H. pylori* has been considered as a major threat not only because they cause several gastric pathologies but also pose challenges in therapy. World Health Organization has also proposed *H. pylori* as a class I carcinogen in humans, since it has been demonstrated that chronic infection is strongly associated with the development of gastric malignant diseases (Schmaußera et al., 2000; Obst et al., 2000). Further, although treatment regimens have been well established as a combination of antibiotics, bactericides and proton pump inhibitors, eradication is not always successful. A few problems have been observed in the use of these drugs which includes the emergence of drug resistance and low compliance, in addition to the occurrence of a number of harmful side effects (Gerrits et al., 2006). These observations warranted therapeutic agents with highly selective antibacterial activity against *H. pylori*, which can work without the risk of resistance and other untoward effects.

Recent studies focused more on host cytoprotective compounds in addition to antimicrobials which can pose problem of not only the development of resistance but may also potentially kill intestinal beneficial microflora (Nord & Edlund, 1990). In the light of the above, our investigations were on identifying anti-*H. pylori* compounds from natural sources which inhibit *H. pylori* growth and adherence in addition to inhibition of H^+, K^+ -ATPase (an enzyme that causes acidity in the stomach) effectively and possessing antioxidant properties so that the compound can be a potential antiulcer agent.

Results presented in previous chapters of the thesis revealed several dietary sources with potential anti-*H. pylori* properties. Multi-potent gastroprotective property was reported in extracts of ginger (*Zingiber officinale*) and Swallow root (*D. hamiltonii*) (Siddaraju & Dharmesh, 2007a; Nanjundaiah et al., 2009; Naik et al., 2007). As per our recent report, cinnamic acid, a major constituent in ginger together with abundant gallic acid contributed significantly to antiulcer properties of ginger (Nanjundaiah et al., 2009). However, potential anti-*H. pylori* compounds in *D. hamiltonii* had not been

reported, although novel phenol-bound polysaccharide had been depicted to inhibit multiple steps of ulcer pathogenesis including the growth of *H. pylori* (Chapter 1 & 3).

Systematic analysis of *D. hamiltonii* extract which showed potential antiulcer effect was initiated. Since HMBA was found to be a predominant component, it was isolated from the roots of *D. hamiltonii* by hydrodistillation and cold crystallization method; and characterized by HPLC, ESI-MS, FT-IR and NMR studies as depicted in chapter 2. It was further tested for growth inhibitory activity against *H. pylori*. Isolated HMBA inhibited the growth of *H. pylori* in a dose dependent manner with MIC of ~ 39 ± 3.4 µg/mL as opposed to that of amoxicillin (MIC 26 ± 3.2 µg/mL) for which *H. pylori* is susceptible. Results were further substantiated by electron microscopy and electrophoretic studies indicating lysis of *H. pylori* by HMBA (Fig 4.7 & 4.8).

Adhesion of bacteria to host epithelial surface is a first requisite step in the colonization process. It is also shown that adhesion and colonization may trigger the virulent gene which subsequently can act on the host cell leading to invasion and establishment of pathogen in the host cell (Mahdavi et al., 2002). Successful establishment of pathogen in the host cell is a result of interaction of pathogen with that of the host cell (Fig 4.12) (Mahdavi et al., 2002). Insertion of *H. pylori*'s virulent products (cagA and vacA) into host cells by type IV secretion system leading to cytotoxicity and successful pathogenesis (Naumann, 2001). The localization of *H. pylori* to its unique niche in the human stomach may be mediated by number of specific adhesion ligands, which have been identified in a variety of experimental systems (Mahdavi et al., 2002). During persistent infection and chronic inflammation (gastritis), *H. pylori* triggers the host tissue to retailor the gastric mucosal glycosylation patterns to up-regulate the inflammation-associated sLex antigens (ligand for *H. pylori* adhesion) which are needed for successful adhesion (Mahdavi et al., 2002).

It is well known that gastric ulcers are caused due to increase in oxidative stress leading to mucosal epithelium damage. Evans et al., (1988) first described *H. pylori* mediated hemagglutination that was inhibited by bovine milk derived sialyl lactose, which contains 85% of 3' sialyl lactose and 15% of 6' sialyl lactose by weight. They also observed the abolishment of adherence activity by treatment of host cell mimicking monolayer cells with

neuraminidase enzyme and also by the preincubation of bacteria with fetuin, a sialic acid rich glycoprotein. These studies point towards the fact that sialic acid is an important adhesion site for *H. pylori* and thus sialic acid rich polysaccharide/glycoprotein domain may be essential for the effective inhibition of *H. pylori* adhesion to host cells. Effective inhibition by sialyl lactose substantiate this observation (Evans et al., 1988).

In this scenario we questioned the possibility of SRPP/BCPP which are dietary/plant pectic polysaccharides acting as antiadhesive compounds. Addition of sialic acid to a carbohydrate/glycoprotein is a complex process and is synthesized only in animal/mammalian system and not in the plant system. However, in view of the observation made by Saitoh et al., (1991) and Tsouvelekis et al., (1991), who could show with elaborative studies with various isolates of *H. pylori* and various heterogeneous glycoproteins, glycolipids not necessarily having sialic acid as a competitive ligand for inhibition; enabled us to still explore our studies on antiadhesive properties of dietary pectic polysaccharide. Studies were encouraged also by the observation of results by Simon et al., (1997) that long passaged isolates of *H. pylori* bind to epithelial monolayers, but are not sensitive to inhibition by either 3' Sialyl lactose or 6' Sialyl lactose. Data thus describe a heterogenous adherence repertoire for these bacteria, apart from sialic acid domain on the gastric mucin. In addition to this, observations made by Lee et al., (2006a; 2006b) also enabled us to study the role of dietary/plant pectic polysaccharides against *H. pylori* adhesion and colonization. They showed the inhibition of adherence of *H. pylori* and other pathogenic bacteria to gastric cells by a pectic polysaccharide of *Panax ginseng,* which contains mainly galacturonic and glucoronic acids (93%). They also suggested that antiadhesive activity might be strongly associated with high uronic acid content, implying the potential role of carbohydrates harboring negatively charged groups in host–bacterial interaction. Indeed these observations enabled us to move towards the understanding of possible role of pectic polysaccharide against *H. pylori.* In fact previous observation made in the laboratory showed that there is a *H. pylori* growth inhibition by pectic polysaccharide (GRPP), also supports the view that sugar residue of pectic polysaccharides other than sialic acid can also inhibit gastric mucin and pathogen interaction and hence ulcer preventive properties. Glycoproteins

fetuin, gastric mucin and bovine submaxillary mucin inhibited *H. pylori* binding to epithelial monolayers *in vitro,* whereas transfetuin, which has oligosaccharides terminating only in α, 2-6 linked NeuAc consistently failed to inhibit binding. In the current study therefore we elaborated the investigation to work for potentiality of selected dietary pectic polysaccharides to inhibit *H. pylori* adhesion to RBC or gastric mucin employing inhibition of hemagglutination and anti-HP-PcAb based immunoassays. *H. pylori* binds to RBCs by virtue of its binding ability to glycoprotein, which is present in abundant levels in RBC. Pretreatment of *H. pylori* with SRPP/BCPP inhibited *H. pylori* induced RBC agglutination (Fig 4.5A). Results were further précised with the ELISA model, where gastric mucin was coated to microwell and allowed *H. pylori* to bind to gastric mucin. Binding of *H. pylori* was monitored by employing anti-HP-PcAb. ELISA absorbance was less in SRPP than with BCPP treated and controls wells indicated that SRPP inhibited *H. pylori* adhesion to mucin more effectively than BCPP. SRPP inhibited *H. pylori* binding with an IC_{50} of 49.5 µg/mL; while BCPP could inhibit with an IC_{50} 90 µg/mL. Data thus confirmed the SRPP and BCPP binding to *H. pylori,* there by not allowing *H. pylori* to bind to gastric mucin (Fig 4.5). In other words *H. pylori* pretreated with SRPP and BCPP abolished ELISA reactivity suggesting that probably SRPP/BCPP are binding to the antigenic determinant of *H. pylori.* Antigenic determinant being vacA protein or vacuolating toxin of *H. pylori* which causes virulence, it is possible that SRPP/BCPP may bind to the vacuolating toxin hence may neutralize the toxic effect. Thus, blocking of *H. pylori* virulence posed advantage of eliminating cytotoxicity of *H. pylori* by treatment with selected pectic polysaccharides. The fact that anti-HP-PcAb has reacted with vacA protein of ~95 kDa mature protein and its cleaved products of 58 and 37 kDa proteins (Fig 4.4) revealed that antibody could detect not only the intact virulent gene product but also its degraded products of molecular size 58 and 37 kDa proteins. It is also supported by observations of Islam et al., (2007), who showed the detection of vacA and its degraded products by serum of vacA+ *H. pylori* infected patients. Data thus substantiates the fact that both the degraded products of vacA protein are antigenic determinants. Data thus confirms the inhibition of *H. pylori* adhesion, a prerequisite for *H. pylori* colonization and causing ulcers. It is

important to mention here that HMBA did not show *H. pylori* adhesion to host cells although it could inhibit *H. pylori* growth, suggesting that affinity binding of test compounds may be required for complete inhibition of *H. pylori*.

Among SRPP and BCPP, SRPP appear to be a potent inhibitor of *H. pylori* adhesion than BCPP. As depicted in chapter 2, SRPP possessed an arabinogalacturonan domain while BCPP is a rhamnogalcturonan type-I with arabinan and galactan in the side chain (Fig 2.6 & 2.7 of chapter 2). Results are supported by the observation made by Maruyama et al., (2000) and Nagaoka et al., (1994), which showed antiulcer potential of rhamnose rich polysaccharides. In addition to this, the difference in the uronic acid content is also important in exhibiting differences in the antiadhesive activity between SRPP and BCPP. SRPP contains uronic acids of 141 mg/g, while BCPP contained 30 mg/g respectively (Table 2.2 of chapter 2) and these observations are also supported by previous investigators (Lee et al., 2006a; 2006b). These differences in the structural composition of SRPP and BCPP may highlight differences in bioactivity. The data further confirms that carbohydrate mediated adhesion may play a critical role in *H. pylori* infection through gastric mucin as shown in Fig 4.12. Presence of arabinan, structure in the selected pectic polysaccharide which showed potential inhibition has a greater insight towards the understanding of carbohydrate–carbohydrate interaction between *H. pylori* and test polysaccharides. Thus the current data may suggest food formulations which may work against ulcer.

It has to be pointed out here that our earlier reports on anti-*H. pylori* compounds from dietary sources were believed to be due to antioxidant potential (Siddaraju & Shylaja, 2007a & 2007b). Anti-*H. pylori* compounds from dietary sources particularly phenolics were thought to exert their antimicrobial effect by causing i. hyperacidification at the plasma membrane interface of the microorganism or ii. intracellular acidification, resulting in disruption of H^+, K^+-ATPase required for ATP synthesis of microbes or iii. may be related to inactivation of cellular enzymes causing membrane permeability changes. Our recent observation that cinnamic acid acting as a potent inhibitor of H^+, K^+-ATPase, and also *H. pylori* growth substantiated the results reported by Vattem et al., (2005). In view of these findings the contribution of HMBA was predicted to be different since it was showing very

poor antioxidant activity. The results suggested that antimicrobials need not possess antioxidant properties; however, in order to confirm and elucidate the probable mechanism of action of HMBA, further aspects such as the cytotoxic effects of *H. pylori* / *H. pylori* toxins on target cells including DNA damage (Nuclear fragmentation) and the influence of HMBA on *H. pylori* toxins induced cyto and DNA toxicity were examined.

It is well established that *H. pylori* envisages successful infection by invading on to the host cells via the release of cytotoxin (vacuolating cytotoxin - VacA) that creates vacuoles in the target cells. Presence of vacA gene has been confirmed by PCR (Fig 4.9G). It is also evident from our results (Fig 4.9B,E) where hollowness or vacuoles were created in addition to severe fragmentation of nucleus and the cellular lysis in buccal cells upon inoculation with *H. pylori*. Effective inhibition of cytotoxicity, in addition to DNA protection has triggered a greater impact in eradication of *H. pylori* induced pathogenic symptoms. In fact cytotoxicity initiated by vacA of *H. pylori* play a critical role in gastroduodenal disease, particularly in causing gastric erosion leading to severe ulcerations. Gastric erosion has been identified as a key step in leading to disease severity since erosion not only cause imbalance in mucosal defence but also favours increased invasion of *H. pylori*. HMBA thus may be a key component in the control of *H. pylori* induced ulcerations besides suppressing the growth of *H. pylori*. Further, it is important to highlight here that DNA damage caused by *H. pylori* either through *H. pylori* toxin or via reactive oxygen species has been found to be one of the major steps in gastric cancer that resulted from *H. pylori* (Schmaußera et al., 2000; Obst et al., 2000). Our data on effective protection to DNA against oxidation induced DNA damage (Fig 4.9H) will be useful in preventing DNA damage which otherwise would lead to aberrations in cells and genes that can cause gastric cancer.

Our observation that HMBA can be a good DNA protectant against oxidative damage but not an antioxidant, raised questions regarding the probable mechanism of action of HMBA. This data is similar to the property observed by andrographolide – an active component recently reported from our laboratory in *Andrographis serpillifolia* (Sathisha et al., 2009) and from *A. paniculata* by other investigators (Das, et al., 2009). It is believed to be due to the efficient binding of HMBA to DNA molecule thereby not allowing the

oxidant to act on it; rather than quenching reactive oxygen species that can hit DNA to cause DNA damage. It is intriguing here that generally antioxidants show better cytoprotectivity and DNA protectivity (Nayaka et al., 2010; Nishio et al., 2008). However, in the current study, the antioxidant activity of HMBA was poorer while exhibited potential cytoprotectivity and DNA protectivity. Significant cytoprotectivity and DNA protectivity thus could be due to the binding ability of these compounds. Possible binding of HMBA to DNA was therefore examined comparing the binding ability with vanillin, another component found in *D. hamiltonii* but showing poorer anti-*H. pylori* activity. Results of the data presented in Fig 4.9A,B,E indicated that, HMBA binds to DNA much stronger than vanillin which may be responsible for anti-*H. pylori* effect.

Generally increased protein binding efficiency also indicates the increased bioavailability which may potentiate the effect *in vivo*. It is well established that the binding of phenolics to proteins which depicts the binding to plasma proteins is an important pharmacological parameter, since it frequently affects the distribution and elimination of these compounds, which dictates the duration and intensity of physiological action (Ulrich et al., 2002). The effect is especially significant for highly protein bound drugs, where only a small alteration in bound fraction can produce a profound change in the pharmaco dynamically active free antioxidant concentration. The results of HSA-phenolics interaction study (Fig 4.11C,D,E) showed direct correlation between binding ability of these compounds with anti-*H. pylori* activity. Compounds those are able to bind to HSA strongly showed better anti- *H. pylori* activity than those that have poorer binding.

Studies are of significance to arrive at structure-function relationship between various phenolic acids including hydroxy cinnamates and benzoates which we had shown previously that their chemistry play a significant role in exhibiting either antioxidant or antimicrobial activity (Chethan et al., 2008; Siddaraju & Dharmesh, 2007b; Nanjundaiah et al., 2009). Results of the study indicated that similar molecules behave differently depending on the target organism. It was therefore essential to analyze the mechanism of specified compound - HMBA against the target organism - *H. pylori*. Results of the data indicated different chemical structural requirement for anti-*H. pylori* effect and provides for the first time the role of HMBA in *D. hamiltonii.*

Better anti-*H. pylori* activity with poorer antioxidant property may indicate that anti-*H. pylori* activity may be mediated by mechanisms other than antioxidant mechanism. This observation infact has been supported from our previous work (Siddaraju & Dharmesh, 2007a & 2007b) that gallic acid with most active antioxidant property (IC_{50} 1.1 ± 0.09) was poorer in inhibiting *H. pylori* growth and H^+, K^+ -ATPase activity (IC_{50} 132 ± 14) while; cinnamic acid with poorer antioxidant activity (IC_{50} 4.6 ± 0.3) exhibited potent anti-*H. pylori* activity together with good inhibition of H^+, K^+ -ATPase activity (IC_{50} 15.1 ± 1.8). Observed results were attributed to the possibility of interaction of hydrophobic constituent with the membrane domain of the *H. pylori* or H^+, K^+ -ATPase (Nanjundaiah et al., 2009).

It was reported from the literature that aldehyde group and hydroxyl group expresses antibacterial activity (Friedman et al., 2003). Since HMBA contains these groups it was presumed to contribute to anti-*H. pylori* activity. Structure-function relationship hence believed to provide a detailed mechanism of understanding of *H. pylori* infection and possible protection from dietary components. Current data when compared to the data generated by Friedman et al., (2003) on similar aspects suggests some commonality and some contradictory results. For example, our observation that aldehydes (HMBA) expressing better anti- *H. pylori* activity than their counterpart hydroxy benzoic acid talleys with the earlier observations. However, the effect regarding more hydroxyl groups yielding better antimicrobial activity differs with *H. pylori* activity which could be due to the differential interaction of *H. pylori* to such compounds than other organisms tested in the earlier report. It is possible that microbes with different surface molecules may interact differently with similar molecules.

Comparison of the chemical structures of the test compounds and their activities revealed that 1) the aldehyde (-CHO) group was more active than the carboxyl (-COOH) group, whether or not OH groups were present since hydroxy benzoic acid showed 2 - 3 folds lower activity than HMBA. ii) On comparison with vanillin which is an isomer of HMBA showed ~8 folds poorer anti-*H. pylori* activity indicating the importance of chemical groups rearrangement which takes place during the synthesis in plants, which has an impact on differential interaction with biomolecules. Also the enzymes involved are probably responsible for the same. Iii) Better anti-*H. pylori*

activity was observed with monosubstituted hydroxy > disubstituted hydroxy > trisubstituted hydroxy benzoic acids, since 3,4,5 trihydroxybenzoaic acid showed poorer activity than 2,5 dihydroxy benzoaic acid. The observation made in this chapter thus is highly significant since it provides a functional attribute to one of the major compounds found in *D. hamiltonii* and *H. indicus* which are known as microbial fighters in traditional knowledge.

4.7. Summary and conclusion

- Chapter 4 addresses the *H. pylori* inhibitory and host cell protective mechanism of HMBA.
- Polyclonal antibody produced against virulent strain of *H. pylori* indicated vacA and its degraded products as major antigenic determinants.
- SRPP and BCPP are good antiadhesive compounds preventing *H. pylori* adhesion to gastric mucin with an IC_{50} of 49.5 and 90 µg/mL respectively; however HMBA did not show antiadhesive activity indicating the efficacy of pectic polysaccharides, particularly arabinogalacturonan of SRPP and rhamnogalacturonan of BCPP.
- Phenolic compound - HMBA showed potent *H. pylori* growth inhibitory activity with MIC of 39 µg/mL, as opposed to 150 µg/mL of SRPP, which contained higher amounts of phenolics (120 mg/g). BCPP did not show any *H. pylori* inhibitory effect indicating that SRPP by virtue of bound-phenolics may exhibit anti-*H. pylori* effect and BCPP with very less bound-phenolics (26 mg/g) therefore be ineffective.
- *H. pylori* has been shown to induce host cell vacuolation probably as a mechanism of pathogenesis. Presence of vacA gene has been confirmed in the test *H. pylori* strain.
- HMBA could inhibit $vacA^+$ -*H. pylori* induced vacuolation suggesting cytoprotective ability of HMBA.
- The protein and DNA binding efficacy of HMBA has been attributed to potent *H. pylori* inhibitory and cytoprotective role of HMBA.
- HMBA being a poorer antioxidant exhibiting better anti-*H. pylori* activity may indicate that anti-*H. pylori* activity could be effectively executed by mechanisms other than antioxidant mechanisms also.

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