ANTIULCER AND ANTICANCER BIOACTIVE COMPOUNDS FROM GINGER (*Zingiber officinale*) AND MANGO GINGER (*Curcuma amada*)

A thesis submitted to the

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for the award of the Degree of

Doctor of Philosophy

in

Biotechnology

by

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CERTIFICATE

This is to certify that the thesis entitled **"Antiulcer and Anticancer Bioactive Compounds from Ginger (*Zingiber officinale*) and Mango Ginger (*Curcuma amada*)"** submitted by Mr. M. N. Siddaraju, for the award of **Doctor of Philosophy** in **Biotechnology** to the **University of Mysore** is the result of research work carried out by him in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, under my guidance during the period 2001-08

Date:
Place: Mysore

(Shylaja M Dharmesh)
(Guide)

DECLARATION

I hereby declare that the thesis entitled **"Antiulcer and Anticancer Bioactive Compounds from Ginger (*Zingiber officinale*) and Mango Ginger (*Curcuma amada*)"** submitted to the **University of Mysore** for the award of degree of **Doctor of Philosophy** in **Biotechnology,** is the result of research work carried out by me under the guidance of Dr. Shylaja M Dharmesh, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore - 570020, India, during the period 2001-08. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Date:
Place: Mysore

(M. N. Siddaraju)

Dedicated to……

My Parents, who encouraged me

My Guide, who enabled me

My Friend, who supported me

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Abbreviations and units

λ	Lambda
°C	Degree centigrade
µg	Microgram
µL	Microliter
µM	Micromolar
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AOA	Antioxidant activity
AOX	Antioxidant
ATP	Adenosine triphosphate
AU	Absorbance units
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
bw	Body weight
CAT	Catalase
CPP	Citrus pectic polysaccharide
Crtl	Control
CVD	Cardiovascular diseases
Cyt-c	Cytochrome c
Da	Dalton
DEAE	Diethylaminoethyl
DMEM	Dulbecos minimum essential medium
DMRT	Duncan's multiple range test
DNA	Deoxyribonucleic acid
DPPH	1, 1-Diphenyl-2-picryl hydrazyl
DTNB	5, 5'-Dithionitrobenzoic acid

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FCS	Fetal calf serum
g	Gram
g	g force
GAE	Gallic acid equivalent
GLC	Gas liquid chromatography
GPx	Glutathione peroxidase
GRAE	Ginger aqueous extract
GRBP	Ginger bound phenolic acids
GRFP	Ginger free phenolic acids
GRHP	Ginger hydrolyzed phenolic acids
GRP	Ginger polysaccharide
GRPP	Ginger pectic polysaccharide
GSH	Reduced glutathione
GSSG	Oxidized glutathione
h	Hour
HEPES	(4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid)
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IC_{50}	Half maximal inhibitory concentration
IU	International unit
Kg	Killogram
M	Molar
MDA	Malondialdehyde

MEM	Minimum essential medium
mg	Milligram
MGAE	Mango Ginger aqueous extract
MGBP	Mango Ginger bound phenolic acids
MGFP	Mango Ginger free phenolic acids
MGHP	Mango Ginger hydrolyzed phenolic acids
MGP	Mango Ginger polysaccharide
MGPP	Mango Ginger pectic polysaccharide
MIC	Minimum inhibitory concentration
min	Minute
mL	Milliliter
mM	Millimolar
MMP	Matrix metallo proteases
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium
N	Normality
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NBT	Nitro blue tetrazolium
NF-kB	Nuclear factor Kappa B
nm	Nanometer
NMR	Nuclear magnetic resonance
NSAID	Nonsteroidal anti-inflamatory drugs
OD	Optical density
OS	Oxidative stress
Ox	Oxidase
PB	Phosphate buffer

PBS	Phosphate buffer saline
PC	Parietal cell
PNPP	Paranitrophenyl phosphate
PPA	Proton potassium ATPase
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SD	Standard deviation
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
SOD	Superoxide dismutase
SR	Swallow root
SRAE	Swallow root aqueous extract
SRBP	Swallow root bound phenolic acids
SRFP	Swallow root free phenolic acids
SRHP	Swallow root hydrolyzed phenolic acids
SRPP	Swallow root pectic polysaccharide
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TMP	1, 1, 3, 3-Tetramethoxy propane
UV	Ultraviolet
w/v	Weight/Volume
w/w	Weight/Weight

Abstract

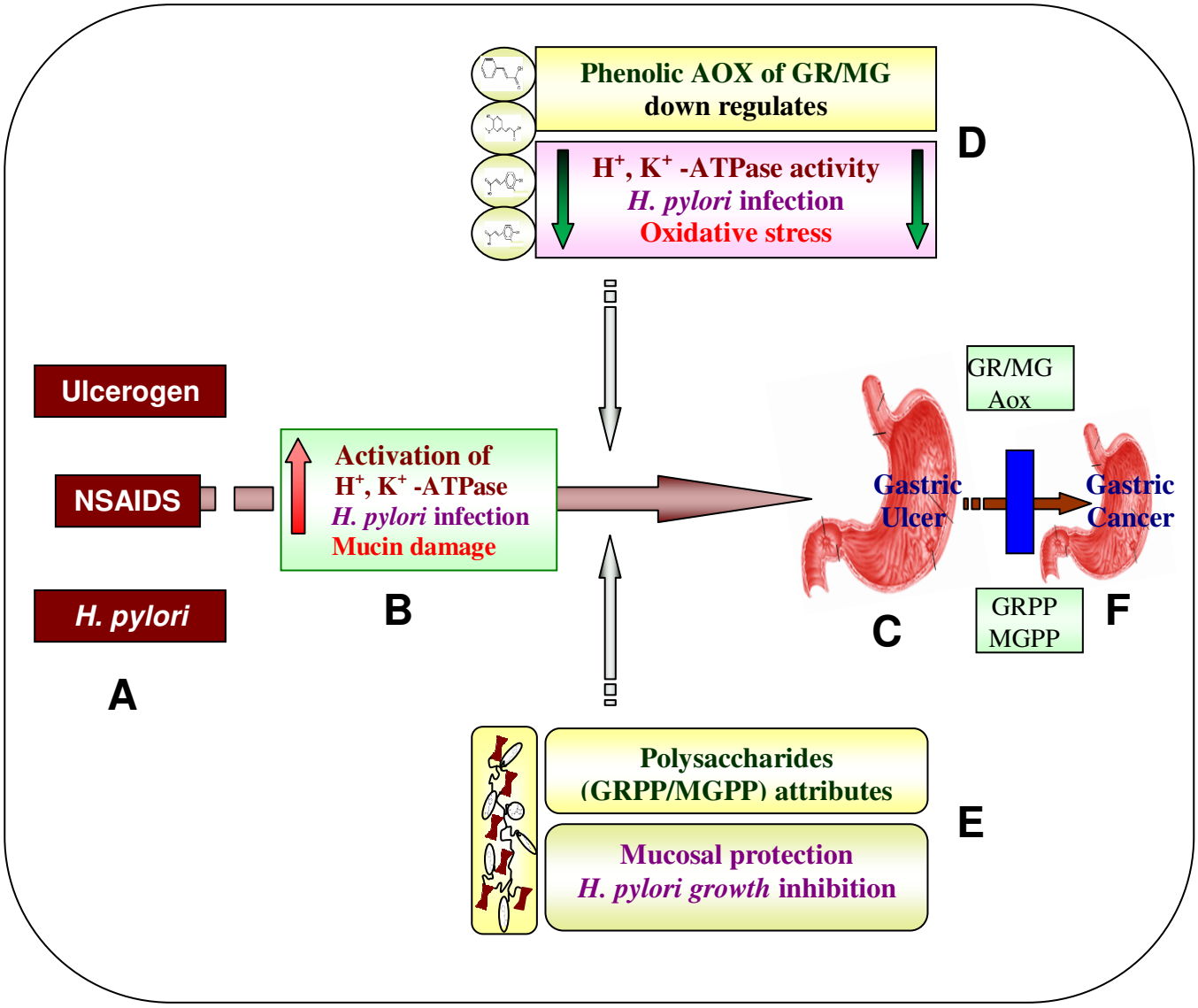
Thesis Title: **Antiulcer and anticancer bioactive compounds from ginger (*Zingiber officinale*) and mango ginger (C*urcuma amada*).**

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, and use of non-steroidal anti-inflammatory drugs (NSAIDS). Ulcers are resulted from excess secretion of hydrochloric acid from gastric parietal cells via activation of H^+, K^+-ATPase enzyme, which releases the H^+ into the lumen of the stomach leading to acidity and the released acid act on gastric mucosa leading to loss of mucosal protection. Gastric lesions thus develop due to loss of the delicate balance between gastro-protective and aggressive factors. Reduction in gastric mucin, enhanced secretion of gastric acid from parietal cells, enhanced susceptibility to *Helicobacter pylori* infection aggravates the ulcer pathogenicity leading to severe gastric ulcers with heavy bleeding, erosions, etc. Accumulated literature suggests that alcohol also play a major role in causing gastric ulcers via portal hypertension. Further, long term ulcers were found to lead to cancers.

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, Cimetidine, Famotidine) and *H. pylori* Inhibitors (Amoxicillin, Erythromycin, Metronidazole), However, majority of them have been documented with the pose of adverse effects.

In light of the above, it is pertinent to study natural products from food/plants as potentials antiulcer compounds. In the current thesis therefore dietary components have been explored as a potential effective and safer antiulcer compounds. Since stress induction has been known to cause ulcer as well as cancers via free radical induced reactive oxygen species leading to both nuclear and cellular damages, dietary sources rich in antioxidative properties have been selected. Ginger (*Zingiber officinale*) and Mango ginger (C*urcuma amada*) have been known to exhibit potential health beneficial properties against various disorders in traditional medicine.

Accordingly antiulcer and anticancer compounds have been addressed from ginger and mango ginger by proposing the research topic entitled "Antiulcer and anticancer bioactive compounds from ginger (*Zingiber officinale*) and mango ginger (*Curcuma amada*)". Results of the study suggested the role of antioxidants, particularly phenolic acids and pectic polysaccharides that are effective in prevention and healing of ulcers at multi-steps of ulcer pathogenicity as indicated in **Scheme -1.** Fractions of ginger and mango ginger showed multi-potent antiulcer (**Chapter 2 & 3**) and anticancer (**Chapter 4**) properties including inhibition of H^+, K^+-ATPase, inhibition of *H. pylori* growth, exhibiting antioxidant, antiproliferative and DNA protective potentials. Thus antioxidant and pectic polysaccharide fractions of ginger and mango ginger have been found to be effective antiulcer/anticancer alternatives



Scheme 1: summary of the work envisaged in the thesis.

Ulcerogens (A) are known to cause ulcers via activation of H^+, K^+-ATPase, inhibition of *H. pylori* infection and mucosal damage (B). Phenolic antioxidant (D) and polysaccharide (E) fractions of ginger and mango ginger have been found to interfere at multi-steps of ulcer pathogenicity and hence effective against ulcer and cancer.

Synopsis of the thesis submitted for the award of PhD degree (Biotechnology) of the University of Mysore, India.

Thesis title: Antiulcer and anticancer bioactive compounds from Ginger (*Zingiber officinale*) and Mango ginger (*Curcuma amada*).

Candidate : Siddaraju M.N.

Hyper-acidity is a common pathological condition caused due to uncontrolled hyper secretion of hydrochloric acid from parietal cells of gastric mucosa through the proton pump H^+, K^+ -ATPase. Apart from the damaging role of acid, reactive oxygen species (ROS) especially the hydroxyl radical plays a major role in causing oxidative damage of mucosa in all types of ulcers including stress related gastric mucosal damage, non-steroidal anti-inflammatory drug-induced gastric lesions and *Helicobacter pylori* mediated gastro duodenal ulcers. The modern approach to control gastro duodenal ulceration is to scavenge reactive oxygen species, inhibit H^+, K^+ -ATPase pump to control increased acid secretion and eradication of *H. pylori*. A range of drugs like histamine blockers and proton pump inhibitors although has been used for efficient management of gastric hyper secretion, many of these drugs pose adverse effects like dizziness, drowsiness, gas accumulation, headache, nausea, vomiting, inflammation of the nose, etc. Phytal sources have been popular, partly because of their low cost and minimal side effects. Excessive accumulation of free radicals during stress induced ulcers also lead to cancers.

Diet plays an important role in offering health beneficial properties with increased physiological significance beyond the pure nutritional requirement. Dietary components possess specific bioactive components which indeed constitutes the potential ingredients of functional or health promoting foods. Traditionally dietary sources have been explored for several health beneficial properties including antioxidant, antiulcer, antidiabetic, anti-hypertensive, and anticancer properties. Since oxidative stress caused either by exogenous or endogenous factors such as environmental pollutants/UV-irradiation or drugs/infectious organisms/inflammatory conditions respectively, antioxidants play a critical role in reducing such oxidative stress induced damage, ultimately offering protection against several chronic disease conditions. However, although

antioxidant property has been studied quite extensively in diet, other biochemical constituents such as polysaccharides, enzyme inhibitors, peptides, glycosides etc. still needed to be identified. Recently the involvement of such biochemical constituents in inhibiting or arresting several steps of the disease processes has been highlighted. However, the basic understanding on the role of these bioactive components in inhibiting multiple steps of different diseases, their bioefficacy, predominance, structure-function relationship and their precise contribution per se has not yet been understood. In the present investigation we attempt to understand the potency of dietary sources against ulcer and cancer based on biochemical and *in vitro* cell culture assays developed in our laboratory and *in vivo* assay systems. Our preliminary screening studies have enabled us to identify potential antioxidant/anti-ulcer and anti-cancer sources. Edible roots including swallow root / Ginger, Mango Ginger germinated wheat and *Andrographis serpyllifolia* have been identified as potent sources. Antioxidants, bioactive polysaccharides and some enzyme inhibitors have been implicated to play role against ulcer and cancer.

The main objective of the proposed research in this thesis therefore was to identify and understand the antiulcer components from ginger (*Zingiber officinale*) and mango ginger (*Curcuma amada*); and to determine their anticancer attributes and probable mode of action. Following objectives were therefore proposed.

Objectives

1. **Anti ulcerative Action of Antioxidant Fractions of Ginger (*Zingiber officinale*) and Mango ginger (*Curcuma amada*)**
2. **Studies on the Mechanism and Anti-Ulcerative Action of Antioxidant and Polysaccharide Fractions In Vitro and In Vivo; Individually and in Combination**
3. **Anticancer Attributes of Antioxidant and Polysaccharide Fractions**

These objectives were distributed in Chapter II to IV along with the relevant general introduction in Chapter I. The titles of the Chapters are as follows:

Chapter I: General Introduction; **Chapter II:** Anti ulcerative action of antioxidant fractions of ginger (*Zingiber officinale*) and mango ginger (*Curcuma amada*); **Chapter III:** Studies on the mechanism and anti-ulcerative action of antioxidant and polysaccharide fractions *in vitro* and *in vivo;* individually and in combination; **Chapter IV:** Anticancer attributes of ginger (*Zingiber officinale*) and mango ginger (*Curcuma amada*)

The research work carried towards achieving these objectives makes the subject matter of the thesis. As described earlier the thesis has been divided in to four chapters; contents provided in these chapters are highlighted as follows:

Chapter I

This chapter deals with background and general introduction; it begins with a general account on ulcer and ulcer incidences. Pathogenesis of gastric ulcers, causative factors for ulcers, mechanism of induction of ulcers, key components responsible for ulcers, currently available drugs, their limitations, need for alternative components, role of diet in offering potential anti-ulcerative agents, justification for the selection of sources – ginger and mango ginger, their potentials as safer and nontoxic uses against ulcers etc., have been addressed. This chapter also deals with the probable role of diet, traditional information on ginger and mango ginger components that are reported to be promising against ulcers etc.

Subsequent chapters II, III and IV addresses the objectives proposed. These chapters envisage a uniform format – depicting the hypothesis underlying the specific objective, work plan, a brief introduction pertaining to the respective objective. Inhibition of gastric parietal cell – plasma membrane - H^+, K^+ –ATPase activity, *H. pylori* growth inhibition, antioxidative potentials etc., were also examined. Once the *in vitro* mechanism of action was understood, the nature of phenolic compounds, which are likely to contribute to any of these, various antiulcer properties were determined. Materials and methods, results, discussion and relevant summary and conclusions highlighting the important outcome of the chapter have been described. Literature cited, references for methodologies are provided as reference at the end of the thesis.

Chapter II

This chapter deals with antiulcer activity *in vitro* of antioxidant fractions of ginger and mango ginger; their characterization and mode of action. Since aqueous extracts of ginger (GRAE) and mango ginger (MGAE) have been in effective use in traditional medicine against several health problems, in this chapter therefore we paid particular attention to determine whether there are any bioactive compounds, which can justify the traditionally observed health benefits. Since our preliminary observations indicated the role of phenolics as potential antiulcer components in ginger, we successfully prepared the aqueous extracts, isolated free and bound/hydrolyzed phenolic fractions from the selected sources ginger and mango ginger and determined initially using the *in vitro* model systems whether the isolated antioxidant fractions possesses antiulcer activity. Assays such as H^+, K^+ -ATPase inhibition, inhibition of *H. pylori*, antioxidant assays – Free radical scavenging, Reducing power ability, ability to protect DNA, anti-lipid peroxidation properties were performed and results are highlighted. Studies in this chapter indicated for the first time the predominant role of phenolic acids particularly, cinnamic, ferulic, syringic, gentisic and gallic acids in exhibiting bioactivity.

Chapter III

In this chapter pectic polysaccharide fractions from ginger (GRPP) and mango ginger (MGPP) were isolated and determined potential *in vitro* anti-ulcerative properties as described in chapter II for GRAE and MGAE. Since GRPP showed potent *in vitro* activity it was examined for *in vivo* effectiveness in swim and alcohol stress induced gastric ulcer models along with GRAE fractions. Combinational antiulcer effects *in vivo* of both GRAE + GRPP were also determined. Both ulcer preventive and ulcer healing effects were addressed for one of the most effective antiulcer component from ginger –GRPP. Ulcer healing ability reveals the ulcer curative potentials. Mechanism of action of phenolics in GRAE and GRPP structure-function relationship of identified compounds are also depicted. Results of the study reveal the effectiveness of polysaccharides by virtue of both phenolics which are covalently bound to them and precise sugar residues – probably arabinose and glucose. Mechanism of inhibition of H^+, K^+ -ATPase activity, *H. pylori* growth to be due to the binding ability of phenolics of ginger and mango ginger both in phenolic and pectic polysaccharide fractions.

Chapter IV

This chapter deals with the anticancer attributes of anti-ulcerative antioxidant and polysaccharide fractions. This has been proposed as an objective since the initiation of both ulcers and cancers are mediated by reactive oxygen species induced oxidative stress. Antioxidant property has therefore been believed to be essential for antiulcer and anticancer properties. In chapter II and III, it was shown effectively that phenolics of ginger (GRAE) and mango ginger (MGAE) and pectic polysaccharide fractions of ginger (GRPP) and mango ginger (MGPP) with potential *in vitro* and *in vivo* antioxidant activity in addition to the inhibition of H^+, K^+ -ATPase and *H. pylori* growth. In chapter IV therefore both GRAE/MGAE and GRPP/MGPP were examined for anticancer potentials by determining the ability of the selected fractions such as inhibition of proliferation, cytoprotective ability, inhibition of oxidative stress, induction of apoptosis etc., studies thus revealed the anticancer attributes of antiulcer antioxidants and polysaccharide fractions.

Overall research of the thesis highlights the importance of phenolic antioxidants in ginger and mango ginger and the role of pectic polysaccharides (GRPP and MGPP) in effective blocking at the multi-steps of ulcer pathogenicity *in vitro* and *in vivo.* Mechanism of action appear to be via inhibition of H^+, K^+ -ATPase and *H. pylori* growth inhibition in addition to antioxidant activity. Pectic polysaccharides were very effective in protecting gastric mucosa against injury by preventing gastric mucin degradation. Enhancing mucin synthesis and preventing probable *H. pylori* invasion. Possible interaction of antioxidants with membrane domain of H^+, K^+ -ATPase and *H. pylori*; particularly arabinose and glucose rich pectic polysaccharide fractions have been highlighted. The advantages of pectic polysaccharide with covalently bound phenolics in ulcer preventive and curative ability have been depicted.

Siddaraju M.N.
(Candidate)

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(Guide)

INTRODUCTION

1.1. 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, painful spot, or ulcer." It is defined as disruption of the mucosal integrity of the stomach and/or duodenum leading to a local defect or excavation due to active inflammation. Ulcers occur within the stomach or in duodenum and are often chronic in nature. Occasionally it is also described as a break in skin or mucous membrane with loss of surface tissue, disintegration and necrosis of epithelial tissue, and often pus. It is also explained as something that festers and corrupts like an open sore. A peptic ulcer may arise at various locations (Figure 1.1).

- Stomach (called gastric ulcer)
- Duodenum (called duodenal ulcer)
- Esophagus (called esophageal ulcer)

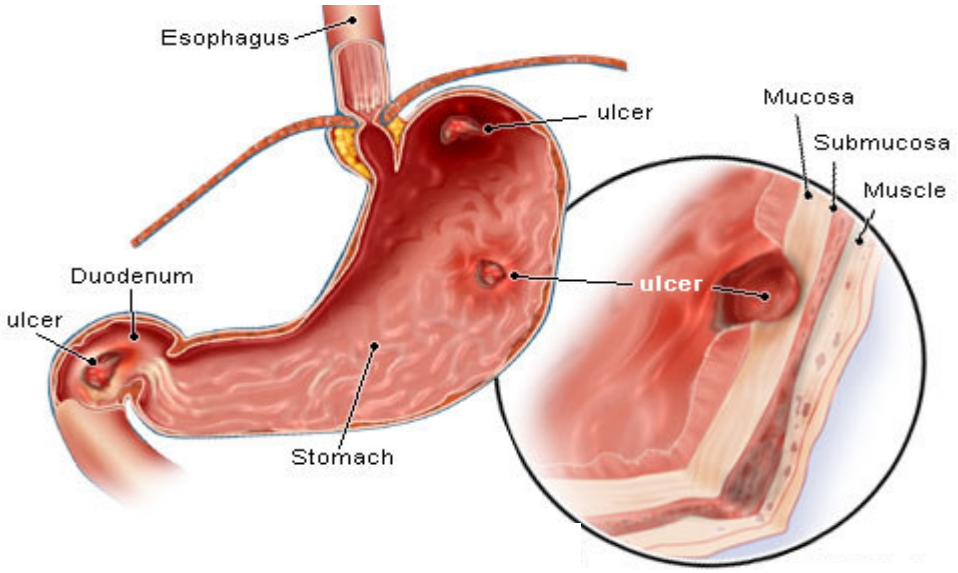


Figure 1.1. Depicting stomach ulcer (A); duodenal ulcer (B); and esophageal ulcer (C).

Pathologically, gastric or duodenal wall extends through the muscularis mucosae (the lowermost limit of the mucosa) into the deeper layers of the wall (submucosa or the muscularis propria) (Figure 1.2). It is within these layers that the ulcer may erode a major blood vessel to produce the complication of potentially life-threatening hemorrhage (Guyton AC and Hall J, 2006). Thus a peptic ulcer is a hole in the gut lining of the stomach, duodenum, or esophagus. An ulcer occurs when the acidic digestive juices that are secreted by the stomach cells corrode the lining of these organs.

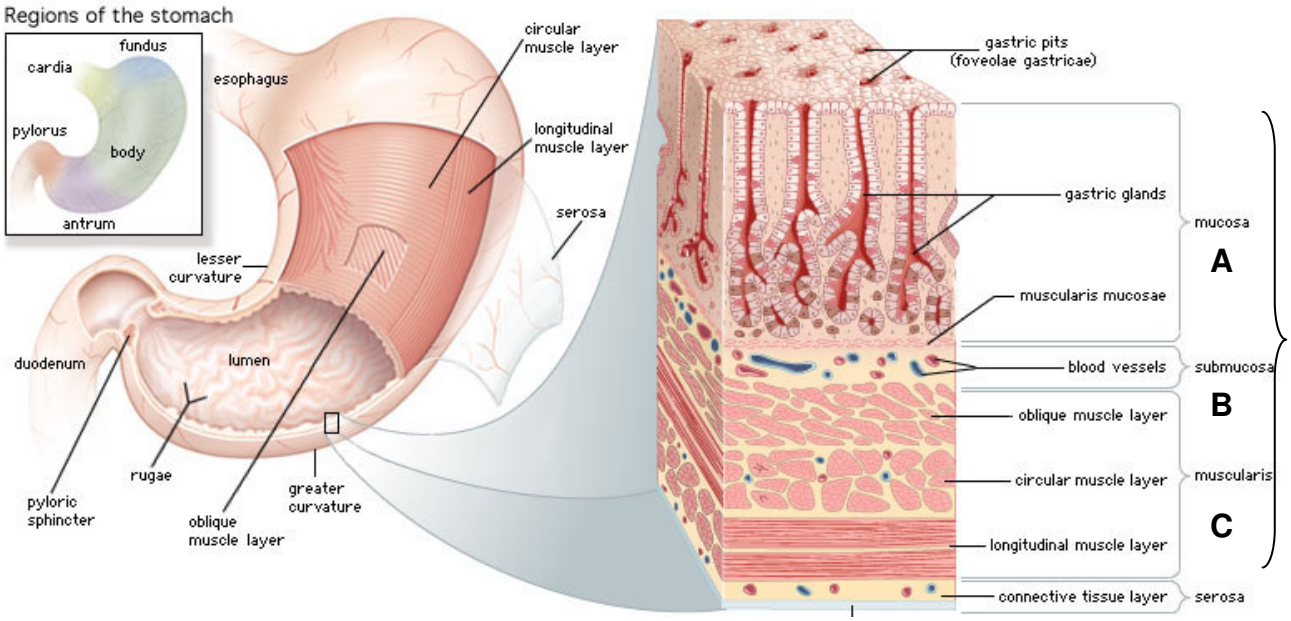


Figure 1.2. Depicting the layers of gastric or duodenal wall.

Ulcer bleeding commonly encounters in A-C regions and at times they are fatal. (Picture from Encyclopaedia Britanica, Inc 2003)

1.1. Statistics and Epidemiology

Ulcer disease is common, affecting millions of population yearly. About 2 percent of the adult populations in the United States where the statistics is maintained seriously and in other countries have active ulcers and that about 10 percent of all adults will have an ulcer at some point in their lives. Males have about three times as many ulcers as females. About 54 percent of all peptic ulcers occur in the duodenum. They are most common among males between the ages of twenty and forty-five. Gastric ulcers account for about 46 percent of all peptic ulcers

and are most common in males between the ages of fifty-five and seventy. At one time, doctors believed that ulcers were caused by too much stress. However, it is now known that bacterial infection accounts for more than three-quarters of all peptic ulcers. The medical cost of treating peptic ulcer and its complications runs in the billions of dollars annually. Recent medical advances have increased our understanding of ulcer formation. Improved and expanded treatment options are now available.

1.2. Prevalence

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 (http:// digestive.nidk.nih.gov/statistics/statistics.htm/peptic ulcer prevalence). The increasing incidence and prevalence of ulcers have been attributed to several factors encountered during day-to-day life, such as stress (Miller, 1987), exposure to bacterial infection (Ernst & Gold, 2000), and use of non-steroidal anti-inflammatory drugs (NSAIDS) (Langman et al., 1991). Indeed, NSAIDs are used daily by approximately 30 million people world wide, constituting a world market in excess of $2 billion.

1.3. Causes and pathogenicity of Ulcers

There are three major causes of peptic ulcers**: infection, certain types of medications, and other medical problems** that cause the release of too much stomach juices. Research studies have shown that most ulcers are caused by an infection by bacteria called *Helicobacter pylori* -- also referred to as *H. pylori* (Figure 1.3).

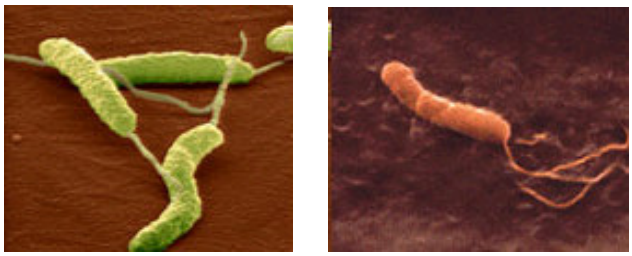


Figure 1.3. A major ulcerogen *Helicobacter pylori*

H. pylori is now considered as the cause of most ulcers (Cover & Blaser, 1992). The *H. pylori* bacterium is found in the stomach, and along with acid secretion, can damage the tissue of the stomach and duodenum, causing inflammation and ulcers. *Helicobacter pylori* is a bacterium that lives in mucous membranes in the digestive system. 95% of all duodenal ulcers and 70% of all gastric ulcers have been known to be due to *H. pylori*. (Blaser, 1990)

1.3.1. Acid and pepsin

These powerful digestive fluids are believed to contribute to the formation of ulcers. In ideal situations, the stomach can protect itself from these fluids in several ways. These are:

- The stomach produces a lubricant-like mucus that coats the stomach and shields stomach tissues.
- The stomach can produce a chemical called bicarbonate that neutralizes digestive fluids and breaks them down into less harmful substances.
- Blood circulation in the lining of the stomach, as well as cell renewal and repair, help protect the stomach.

1.3.2. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

Many drugs, especially aspirin, other NSAIDs, and corticosteroids, irritate the stomach lining and can cause ulcers (Kurata & Nogawa, 1997). Medium and highest risk recorded for different NSAIDs are depicted in Table 1.1. However, most people who take NSAIDs or corticosteroids do not develop peptic ulcers. Regardless, some experts suggest that people at high risk of developing peptic ulcers should use a type of NSAID called a coxib (COX-2 inhibitor), rather than one of the older types of NSAIDs, because coxibs are less likely to irritate the stomach. However, studies have shown that coxibs appear to increase the risk of heart attack and stroke with long-term use and, therefore, caution should be taken with their use. Because of these complications, most doctors now use a standard NSAID plus a strong acid inhibitor (such as a proton pump inhibitor) for people at high risk of developing peptic ulcers.

Table 1.1. Ulcer Risk by Specific NSAIDs

Lowest Risk	Medium Risk	Highest Risk
Nabumetone (Relafen) Etodolac (Lodine) Salsalate Sulindac (Clinoril)	Aspirin. Even low-dose aspirin (81 mg) used to protect the heart may pose some risk (although lower than standard doses). Ibuprofen (Motrin, Advil, Nuprin, Rufen) Naproxen (Aleve, Naprosyn, Naprelan, Anaprox) Diclofenac (Voltaren) Tolmetin (Tolectin)	Flurbiprofen (Ansaid) Piroxicam (Feldene) Fenoprofen Indomethacin (Indocin) Meclofenamate (Meclomen) Ketoprofen (Actron, Orudis KT)

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 NSAIDs regularly, and over 25 billion tablets of over-the-counter brands are sold each year in America. The most common NSAIDs are aspirin, ibuprofen (Advil), and naproxen (Aleve, Naprosyn), although many others are available. Patients who have an ulcer caused by NSAIDs should stop taking these drugs (Lewis et al., 2002).

NSAIDs definitely increase the risk for ulcers and gastrointestinal bleeding. The risk for bleeding is continuous for as long as a patient is on these drugs and may even persist for about a year after taking them. Taking short courses of NSAIDs for temporary pain relief should not cause major problems because the stomach has time to recover and repair any damage that has occurred.

Specific NSAIDs pose greater or lesser risks for ulcers and bleeding. No NSAIDs, however, even over-the-counter brands, should be used long-term except under a doctor's direction.

NSAIDs can make the stomach's defense mechanisms to fail in a couple of different ways:

- They can make the stomach vulnerable to the harmful effects of acid and pepsin by interfering with the stomach's ability to produce mucus and bicarbonate.
- They can affect cell repair and blood flow to the stomach.

1.3.3. Some medical problems can increase the risk of ulcers. For example, Zollinger-Ellison syndrome causes an unusually large release of digestive juices in the stomach and this excess secretion can create ulcers (Pellicano et al., 2006).

1.3.4. Other factors may also increase a person's risk for ulcers. For example, smokers are more likely to develop an ulcer and are also more likely to die from the complications of an ulcer (Pillay et al., 2007). An ulcer occurs when the natural defense mechanisms in the stomach or duodenum are overwhelmed by aggressive factors (Goel & Bhattacharya, 1991, Aase, 1989). In the stomach acid and pepsin (a digestive enzyme) are the aggressors.

Gastric mucus, bicarbonate ions (which neutralize acid), the surface cells of the stomach and compounds known as prostaglandins protect the stomach from ulceration. Other Factors responsible for stress ulcers include Ischemia, impaired mucosal blood flow, acid, free radical, Thyrotropin releasing hormone (TRH), Prostaglandin, Glucose, Angiotensin II, Nicotine and Bile salts. Other than these things there few more causes such as-

Smoking- Studies show that cigarette smoking can increase a person's chance of getting an ulcer. Smoking also slows the healing of existing ulcers and contributes to ulcer recurrence.

Caffeine- Beverages and foods that contain caffeine can stimulate acid secretion in the stomach. This can aggravate an existing ulcer, but the stimulation of stomach acid can't be attributed solely to caffeine.

Alcohol- While a link hasn't been found between alcohol consumption and peptic ulcers, ulcers are more common in people who have cirrhosis of the liver, a disease often linked to heavy alcohol consumption.

Stress- Emotional stress is no longer thought to be a cause of ulcers, but people who are experiencing emotional stress often report-increased pain of existing ulcers. Physical stress, however, is different. It can increase the risk of developing ulcers, especially in the stomach. Examples of physical stress that can lead to ulcers are that suffered by people with injuries such as severe burns, and people undergoing major surgery (William et al., 2007).

As highlighted below acid secretion and disruption in mucosal defense has been a key components attacked by ulcerogen (Figure 1.4 & 1.5). Prior understanding their targeted role during ulcerogenesis, understanding of the physiological regulation in gastric cell becomes apparent. Since H^+, K^+ -ATPase and mucosal defense has been the key factors in controlling the gastric defense, these are depicted in detail as follows.

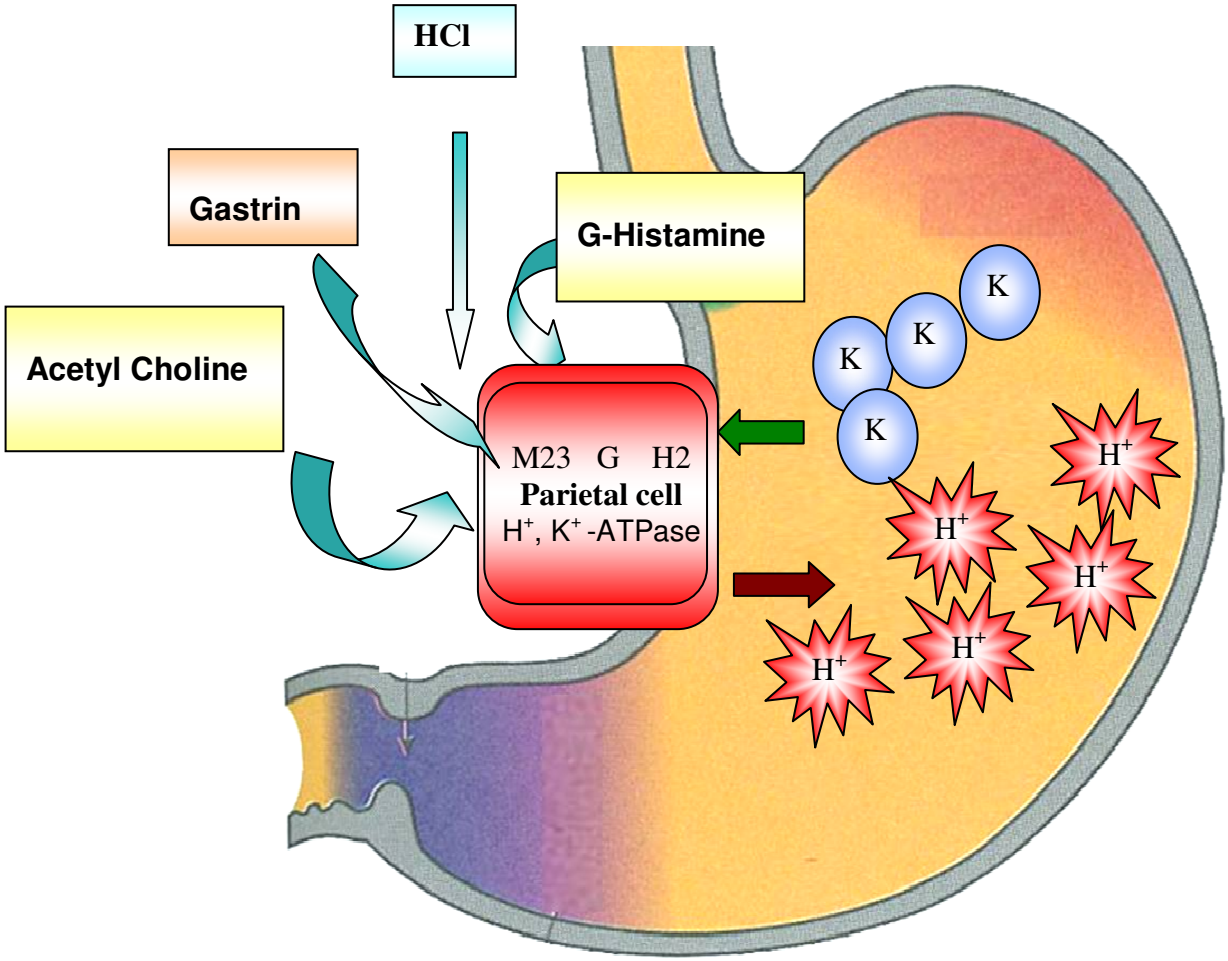


Figure 1.4. Dysregulation of Parietal cellular activity results in acidity.

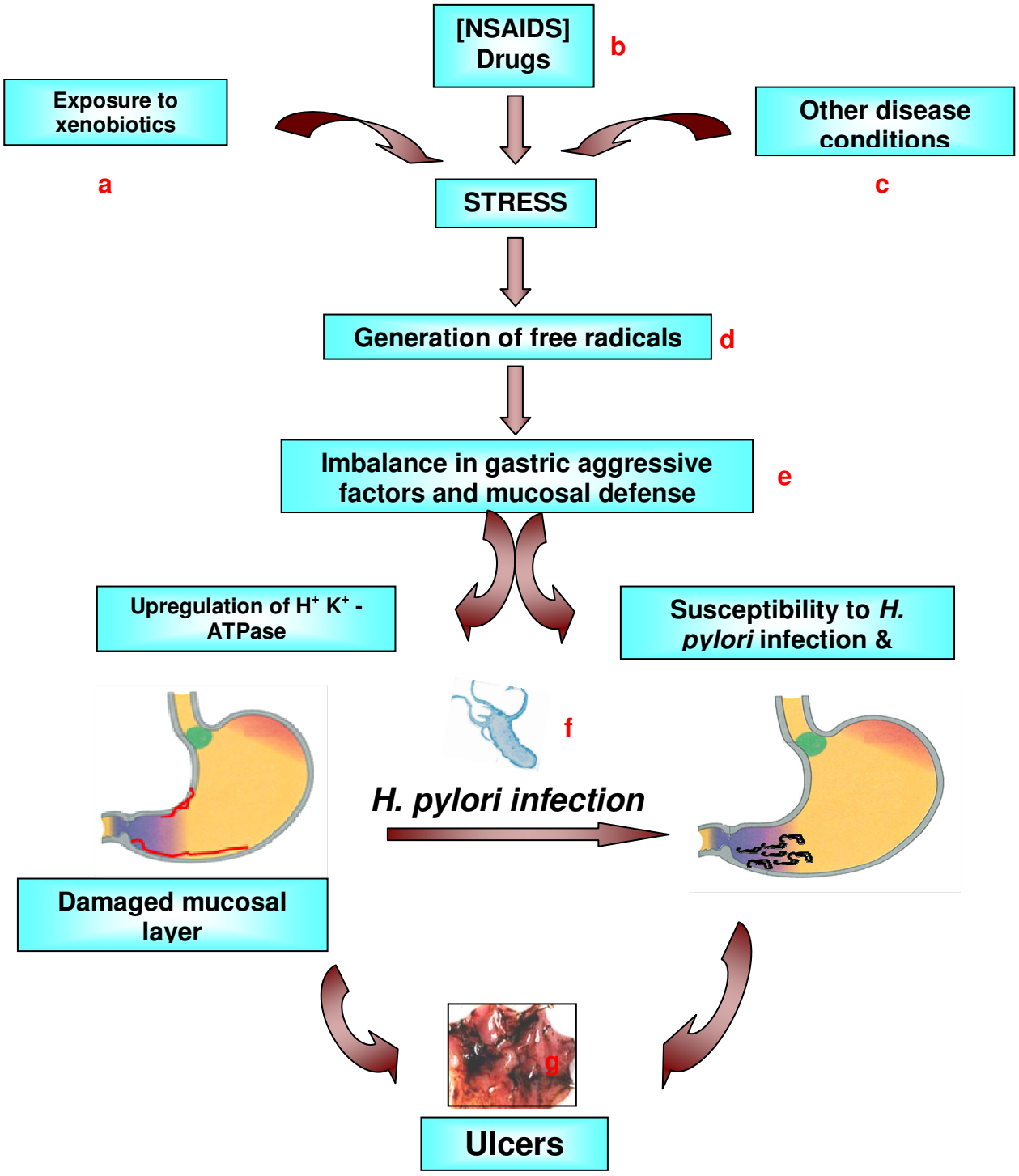


Figure 1.5. Scheme of induction of ulcerogenicity.

Drugs, xenobiotics and other stress inducing factors (a-c) generates free radical (d) leading to imbalance in mucosal defense (e) susceptibility for *H. pylori* (f) infection leading to ulcers (g).

1.4. Gastric parietal cell; role of Proton potassium ATPase

The gastric proton potassium ATPase or H^+, K^+ -ATPase is the proton pump of the stomach and as such is the enzyme primarily responsible for the acidification of the stomach contents (Wallmark et al., 1985). The H^+, K^+ -ATPase is found in parietal cells which are highly specialised epithelial cells located in the inner cell lining of the stomach, which is called the gastric mucosa. Parietal cells possess an extensive secretory membrane system and the H^+, K^+ -ATPase se is the major protein constituent of these membranes.

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. 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 (Figure 1.6).

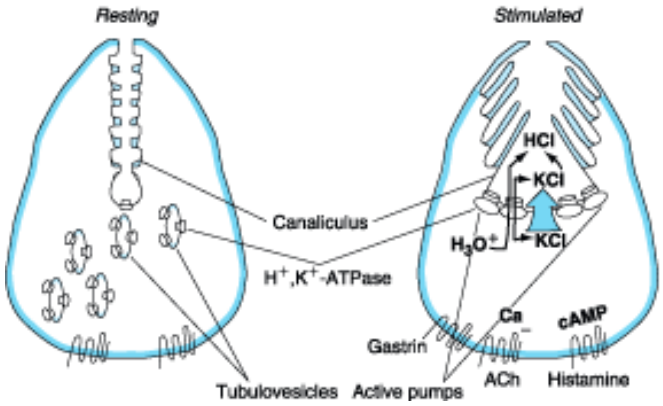


Figure 1.6. Gastric parietal cell undergoing transformation after – stimulation

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.

The H^+, K^+ -ATPase is a heterodimeric protein, the product of 2 genes. The gene ATP4A encodes the H^+, K^+ -ATPase α subunit contains and is an ~ 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 (Gottardi & Caplan, 1993).

1.4.1. Enzyme activity of the H^+, K^+ -ATPase

The 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 (Figure 1.7). 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 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 form adenosine triphosphate (ATP) to the H^+, K^+ -ATPase during the transport cycle. This phosphate transfer powers a conformational change in the enzyme that helps drive ion transport.

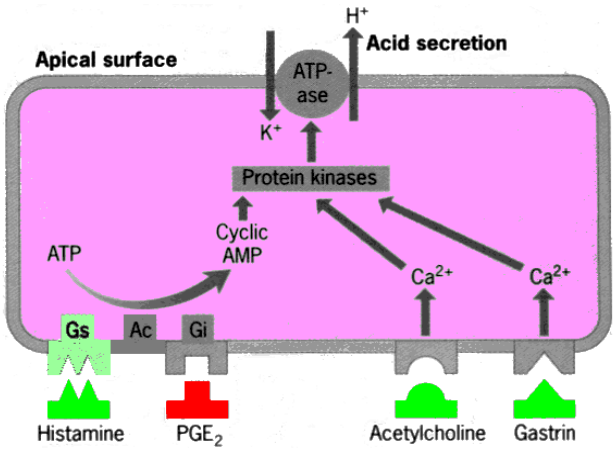


Figure 1.7. Enzyme activity of the H^+, K^+ -ATPase

1.5. Mucosal defense

However, despite these constant attack on the gastroduodenal mucosa by a host of ulcer inducing agents (acid, pepsin, bile acids, pancreatic enzymes, drugs, and bacteria), integrity is maintained by an intricate system that provides mucosal defense and repair (Gompertz et al. 1992).

Mucin, a highly glycosylated glycoprotein, which is secreted from both gastric surface mucous cells and mucous neck cells, forms a viscous gel which is adherent to the mucous surface (Neutra & Forstner, 1987). This adherent mucus gel layer exerts a protective role against (i) acid, by acting as a stable mixing barrier with the epithelium secreted bicarbonate, (ii) luminal pepsin, by forming a diffusion barrier, and (iii) gastric motility-induced mechanical damage, by acting as a lubricant (Allen et al. 1986). Various noxious factors such as NSAIDs, bile acids and *Helicobacter pylori*-derived protease and urease reduce the integrity of the gastric mucus gel layer. Maintenance of the polymeric structure of the gastric mucus glycoproteins and gastric mucus secretion is therefore essential for gastroprotection (Kinoshita et al., 1999).

The mucosal defense system can be envisioned as a three-level barrier, composed of preepithelial, epithelial, and subepithelial elements (Figure 1.8). The first line of defense is a mucus-bicarbonate layer, which serves as a physicochemical barrier to multiple molecules including hydrogen ions. Mucus is secreted in a regulated fashion by gastroduodenal surface epithelial cells. It consists primarily of water (95%) and a mixture of lipids and glycoproteins. Mucin is the constituent glycoprotein that, in combination with phospholipids (also secreted by gastric mucous cells), forms a hydrophobic surface with fatty acids that extend into the lumen from the cell membrane. The mucous gel functions as a nonstirred water layer impeding diffusion of ions and molecules such as pepsin. Bicarbonate, secreted by surface epithelial cells of the gastroduodenal mucosa into the mucous gel, forms a pH gradient ranging from 1 to 2 at the gastric luminal surface and reaching 6 to 7 along the epithelial cell surface. Bicarbonate secretion is stimulated by calcium, prostaglandins, cholinergic input, and luminal acidification.

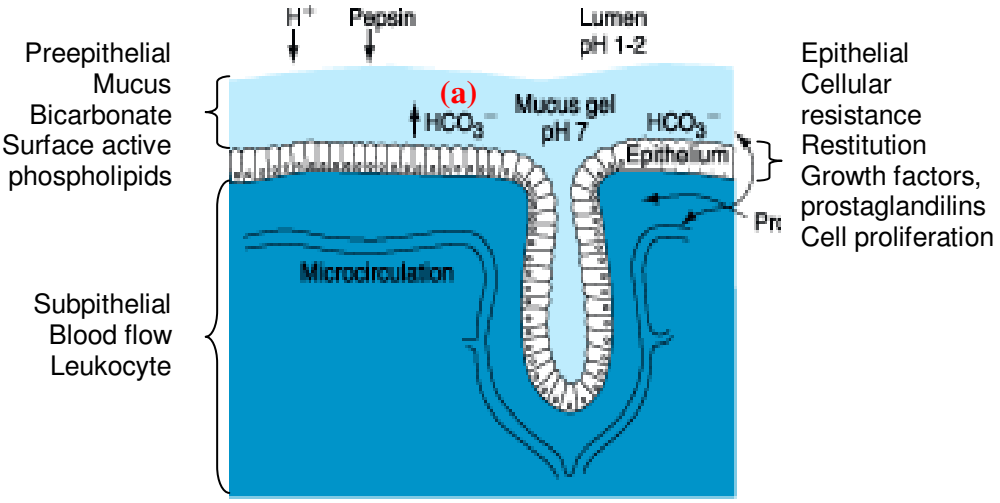


Figure 1.8. Components involved in providing gastroduodenal mucosal defense and repair; (a) mucosal gel covering entire surface of the gastric epithelium to protect from the external environment.

1.5.1. Gastric mucin sequence

Mucins are a family of high molecular-weight, heavily O-glycosylated glycoproteins that are either secreted (Allen, 1981) or are membrane-bound (Hollingsworth, 2004).

The physiochemical and biological properties of secreted mucus are largely conferred by mucins that are responsible for the rheological properties of normal mucus gels that coat and protect the epithelial cells of the internal tracts of the body (Allen, 1981). The mucin protein core consists of highly glycosylated regions (resistant to proteolysis) and regions shown to be non-glycosylated (susceptible to proteolysis) (Scawen & Allen, 1977). Cysteines in these 'naked' regions link mucin monomers by disulphide bridges to form large mucin oligomers of 2-40kDa molecular mass (Pearson et al., 1981). 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 (Figure 1.8).

1.6. *H. pylori* and its role in ulcers

In 1994, the National Institutes of Health published an opinion stating that most recurrent gastric ulcers were caused by *H. pylori*, and recommended that antibiotics be included in the

treatment regimen (NIH Consensus Conference, 1994). In 2005, Warren and Marshall were awarded the Nobel Prize in Medicine for work on *H. pylori (http://nobelprize.org/nobel_prizes/medicine/laureates/2005/)*

Since *H. pylori* is the causative factor for majority of ulcer incidences globally, role of *H. pylori* in ulcer disease is discussed at length (Figure 1.9).

Gastric infection with the bacterium *H. pylori* accounts for the majority of Ulcer disease. This organism also plays a role in the development of gastric mucosal-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (Parsonnet et al., 1991). Although the entire genome of *H. pylori* has been sequenced, it is still not clear how this organism, which is in the stomach, causes ulceration in the duodenum, or whether its eradication will lead to a decrease in gastric cancer.

1.6.1. History of the Bacterium

The bacterium, initially named *Campylobacter pyloridis*, is a gram-negative microaerophilic rod found most commonly in the deeper portions of the mucous gel coating the gastric mucosa or between the mucous layer and the gastric epithelium. It may attach to gastric epithelium but under normal circumstances does not appear to invade cells. It is strategically designed to live within the aggressive environment of the stomach. It is S-shaped (~0.5 × 3 µm in size) and contains multiple sheathed flagella. Initially, *H. pylori* resides in the antrum but, over time, migrates towards the more proximal segments of the stomach. The organism is capable of transforming into a coccoid form, which represents a dormant state that may facilitate survival in adverse conditions (Andersen et al., 1997). The bacterium expresses a host of factors that contribute to its ability to colonize the gastric mucosa and produce mucosal injury. Several of the key bacterial factors include urease (converting urea to NH_3 and water, thus alkalinizing the surrounding acidic environment), catalase, lipase, adhesins, platelet-activating factor, cytotoxin-associated gene protein (Cag A), induces cytokines and vacuolating cytotoxin (Vac A). Multiple strains of *H. pylori* exist and are characterized by their ability to express several of these factors (Cag A, Vac A, etc.) (Fauchere et al., 1990). It is possible that the different diseases related to *H. pylori* infection can be attributed to different strains of the organism with distinct pathogenic features.

In the stomach and duodenum, despite the highly acidic environment. It takes advantage of the stomach's own mucous for protection. Any acid that does reach the bacteria is converted by *H. pylori*'s urease enzyme in the following reaction (Smoot et al., 1991):

Urea	+	Stomach acid	+	Water	⟶	Bicarbonate	+	Ammonia
$C{=}O{\cdot}2NH_2$	+	H^+	+	$2H_2O$	⟶	HCO_3^-	+	$2NH_4^+$

The products of this reaction, bicarbonate and ammonia, are strong bases that further protect the bacteria because of their acid-neutralizing capability. The body's immune system responds to the presence of *H. pylori* and sends infection-fighting cells to the area. 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.

During infection, the bacterium enters the gastric lumen where the urease allows survival in the acidic environment by producing ammonia molecules that buffer cytosolic and periplasmic pH as well as the surface layer around the bacterium. The flagella propel the helicoidal bacterium into the mucus layer and allow it to reach the apical domain of gastric epithelial cells, to which it sticks using specialized adhesins. *H. pylori* then injects the cagA protein into the host cells by a type IV secretion system and releases other toxic factors such as *H. pylori* neutrophil-activating protein (HP-NAP) and VacA.

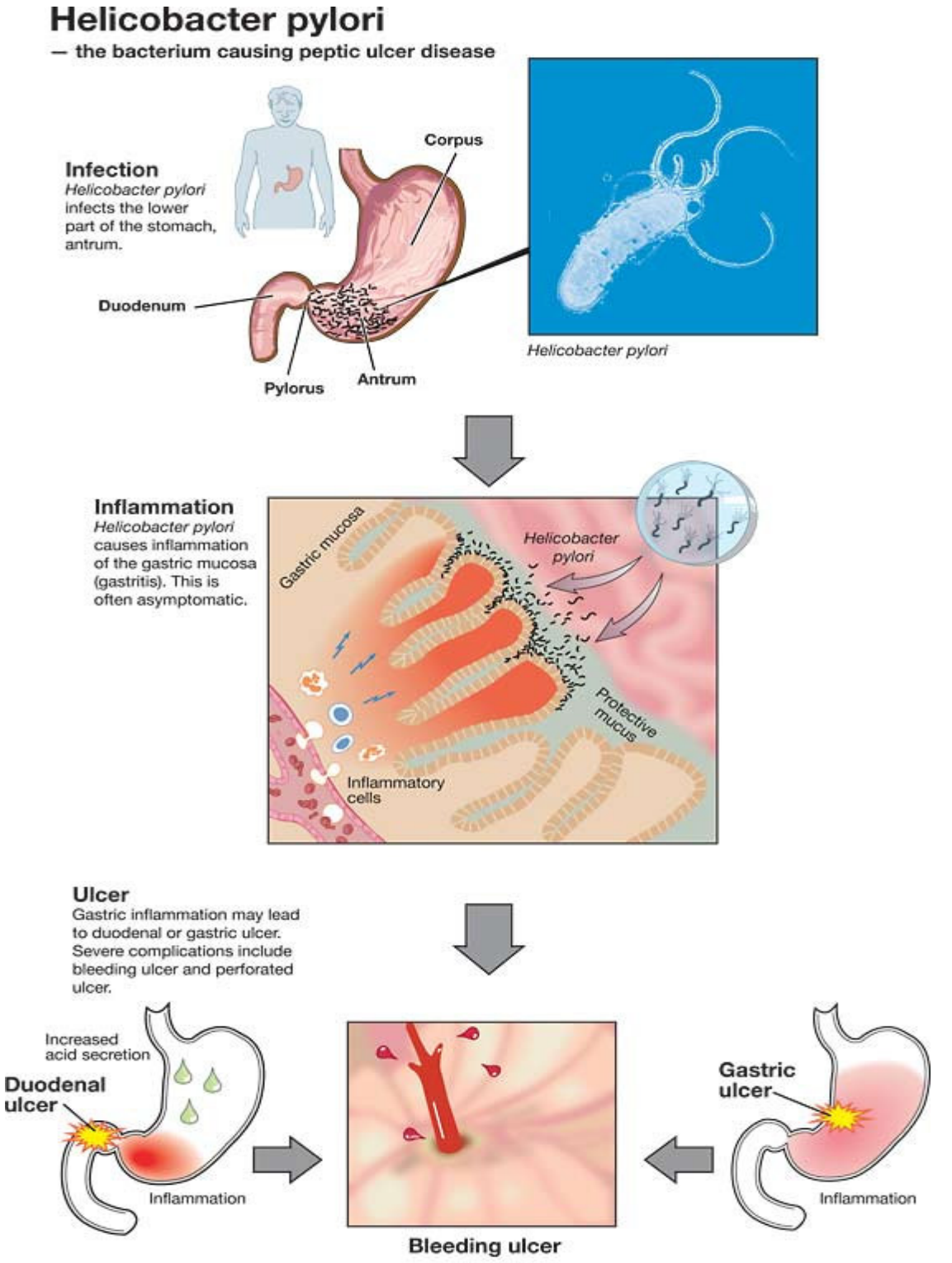
VacA induces alterations of tight junctions and the formation of large vacuoles (Cover & Blanke, 2005). Vacuoles are evident in cells in culture and in the stomach epithelial cells of human and mouse biopsies, although they are not apparent in gerbils. The neutrophil-activating protein HP-NAP crosses the epithelial lining and recruits neutrophils and monocytes, which extravasate and cause tissue damage by releasing reactive oxygen intermediates (ROIs) (Dundon et al., 2001). Injected Cag proteins cause alteration of the cytoskeleton, pedestal formation and signal the nucleus to release proinflammatory lymphokines, which amplify the inflammatory reaction with recruitment of lymphocytes and further induce the

release of ROIs. The combined toxic activity of VacA and of ROIs leads to tissue damage that is enhanced by loosening of the protective mucus layer and acid permeation.

The ability of *H. pylori* to persist in the human stomach for extended periods indicates that it is well adapted to acquire the nutrients it needs for growth in this unique niche. For example, the mucous layer of the mouse stomach contains significant amounts of molecular hydrogen (17–93 μM) originating from metabolic activity of microbial flora in the large intestine. *H. pylori* is capable of utilizing this molecular hydrogen as an electron donor for microaerobic respiration and a functional hydrogenase is required for successful colonization of mice by *H. pylori* (Maier et al. 1996). Unlike many hydrogen-oxidizing bacteria, however, *H. pylori* is not capable of autotrophic CO_2 fixation.

1.6.2. Epidemiology

The prevalence of *H. pylori* varies throughout the world 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%, with individuals born before 1950 having a higher rate of infection than those born later. About 10% of Americans <30 are colonized with the bacteria (Taylor & Blaser, 1991). This rate of colonization increases with age, with about 50% of individual's age 50 being infected. Factors that predispose to higher colonization rates include poor socioeconomic status and less education. These factors, not race, are responsible for the rate of *H. pylori* infection in blacks and Hispanic Americans being double the rate seen in whites of comparable age.



(The Nobel committee for physiology or medicine, 2005)

Figure 1.9. H. pylori infection leading to ulcer.

Risk Factors for *H. pylori* Infection are a) Birth or residence in a developing country, b) Low socioeconomic status, c) Domestic crowding, d) Unsanitary living conditions, e) Unclean food or water and f) Exposure to gastric contents of infected individual

Transmission of *H. pylori* occurs from person to person, following an oral-oral or fecal-oral route. The risk of *H. pylori* infection is declining in developing countries. The rate of infection in the United States has fallen by >50% when compared to 30 years ago.

Bacterial factors: *H. pylori* is able to facilitate gastric residence, induce mucosal injury, and avoid host defense. Different strains of *H. pylori* produce different virulence factors (Dundon et al., 2001). A specific region of the bacterial genome, the pathogenicity island, encodes the virulence factors Cag A and pic B. Vac A also contributes to pathogenicity, though it is not encoded within the pathogenicity island. These virulence factors, in conjunction with additional bacterial constituents, can cause mucosal damage (Calam et al., 1997). Urease, which allows the bacteria to reside in the acidic stomach, generates NH_3, which can damage epithelial cells. The bacteria produce surface factors that are chemotactic for neutrophils and monocytes, which in turn contribute to epithelial cell injury. *H. pylori* makes proteases and phospholipases that break down the glycoprotein lipid complex of the mucous gel, thus reducing the efficacy of this first line of mucosal defense. *H. pylori* expresses adhesins, which facilitate attachment of the bacteria to gastric epithelial cells. Although lipopolysaccharide (LPS) of gram-negative bacteria often plays an important role in the infection, *H. pylori* LPS has low immunologic activity compared to that of other organisms. It may promote a smoldering chronic inflammation.

1.7. Diagnosis of ulcers

Diagnosis can be me made by series of tests including physical examinations and differential diagnosis.

Physical Examination- Epigastric tenderness is the most frequent finding in patients with gastric or duodenal uclers. Physical examination is critically important for discovering evidence of ulcer complications. Tachycardia and orthostasis suggest dehydration secondary to vomiting or active gastrointestinal blood loss. A severely tender, boardlike abdomen suggests a perforation. Presence of a succussion splash indicates retained fluid in the stomach, suggesting gastric outlet obstruction.

Endoscopy provides the most sensitive and specific approach for examining the upper gastrointestinal tract (Figure 1.10). In addition to permitting direct visualization of the mucosa, endoscopy facilitates photographic documentation of a mucosal defect and tissue biopsy to rule out malignancy or *H. pylori*. Endoscopic examination is particularly helpful in identifying lesions too small to detect by radiographic examination, for evaluation of atypical radiographic abnormalities, or to determine if an ulcer is a source of blood loss.

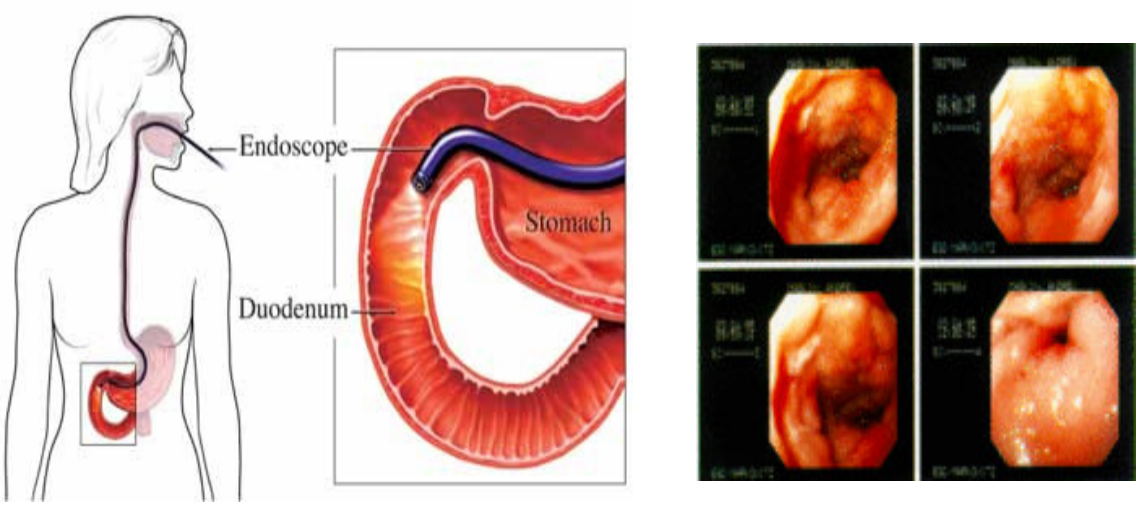


Figure 1.10. Picture depicting endoscopy A-endoscopic tube entering into the gastric region, B- endoscopic picture

Although the methods for diagnosing *H. pylori* includes a urease test in the biopsy specimen, has a sensitivity and specificity of >90 to 95%. In the interest of making a diagnosis of *H. pylori* without the need for performing endoscopy, several noninvasive methods for detecting this organism have been developed. Three types of studies routinely used include **serologic testing**, the **^{13}C- or ^{14}C-urea breath test**, and the fecal *H. pylori* antigen test. Occasionally, specialized testing such as serum gastrin and gastric acid analysis or sham feeding may be needed in individuals with complicated or refractory PUD.

1.8. Strategies developed to counteract ulcer disease

In the war against ulcer disease several drugs are developed. Table 1.2 gives the drugs Based on the target and action of the sometimes used in the treatments of peptic ulcers caused by either NSAIDs or *H. pylori*.

Table 1.2. Drugs Used to Treat Peptic Disorders

Drug Type	**Selected Side Effects**	**Comments**
Antacids		
Aluminum hydroxide Calcium carbonate Magnesium hydroxide Sodium bicarbonate	Nausea, headache, weakness, loss of appetite, constipation (aluminum hydroxide) or diarrhea (magnesium hydroxide)	Used mainly to relieve symptoms, not as a cure
Aluminum hydroxide Calcium carbonate Magnesium hydroxide Sodium bicarbonate	Nausea, headache, weakness, loss of appetite, constipation (aluminum hydroxide) or diarrhea (magnesium hydroxide)	Used mainly to relieve symptoms, not as a cure
Histamine-2 blockers		
Cimetidine Famotidine Nizatidine Ranitidine	Rash, fever, muscle pains; may cause breast enlargement and erectile dysfunction in men; may interfere with elimination of certain drugs (cimetidine); confusion (cimetidine, ranitidine)	The once-daily dose is taken in the evening or at bedtime; doses taken in the morning are less effective
Proton pump inhibitors		
Lansoprazole Omeprazole	Diarrhea, constipation, headache	Usually well tolerated; most effective means of reducing stomach acid

Pantoprazole Rabeprazole Esomeprazole		
Antibiotics		
Amoxicillin Clarithromycin Metronidazole Tetracycline	Diarrhea (amoxicillin, clarithromycin, tetracycline), altered taste, nausea	Effective for treating peptic ulcers caused by *Helicobacter pylori* infection
Miscellaneous		
Bismuth subsalicylate Misoprostol Sucralfate	Diarrhea (bismuth subsalicylate, misoprostol); darkening of the tongue and stool (bismuth subsalicylate); spontaneous abortion (misoprostol); constipation (bismuth subsalicylate); may reduce effectiveness of other drugs (sucralfate)	Bismuth subsalicylate is used in combination with antibiotics to cure *H. pylori* infection

1.8.1. Proton-Pump Inhibitors (PPIs)

PPIs are the drugs of choice for managing patients with peptic ulcers from any cause. They suppress the production of stomach acid. These drugs work by blocking the gastric acid pump – the molecule in the stomach glands that is responsible for acid secretion (Sachs et al. 2006). The final step in the secretion of gastric acid is "proton pumping", which is mediated by the enzyme known as H^+, K^+ -ATPase. This inhibition acts beyond the influence of second messengers in the parietal cell, and is independent of the action of secretogogues such as gastrin, histamine and ACH.

Proton pump inhibitors inhibit the gastric H^+, K^+ -ATPase via covalent binding to cysteine residues of the proton pump. All proton pump inhibitors must undergo acid accumulation in the

parietal cell through protonation, followed by activation mediated by a second protonation at the active secretory canaliculus of the parietal cell. The relative ease with which these steps occur with different proton pump inhibitors underlies differences in their rates of activation, which in turn influence the location of covalent binding and the stability of inhibition. Slow activation is associated with binding to a cysteine residue involved in proton transport that is located deep in the membrane. However, this is inaccessible to the endogenous reducing agents responsible for restoring H^+, K^+-ATPase activity, favouring a longer duration of gastric acid inhibition. Pantoprazole and tenatoprazole, a novel proton pump inhibitor which has an imidazopyridine ring in place of the benzimidazole moiety found in other proton pump inhibitors, are activated more slowly than other proton pump inhibitors but their inhibition is resistant to reversal. In addition, tenatoprazole has a greatly extended plasma half-life in comparison with all other proton pump inhibitors. The chemical and pharmacological characteristics of tenatoprazole give it theoretical advantages over benzimidazole-based proton pump inhibitors that should translate into improved acid control, particularly during the night (Sachs et al. 2006).

1.8.2. Targets for inhibiting acid secretion by the parietal cell

The gastric acid pump is an ATPase present in cytoplasmic membranes of the resting parietal cell. On activation, the pump is translocated to the canalicular membrane, where it pumps out H+ ions into the canalicular space in exchange for K+ ions (Figure 1.11). Gastric acid secretion by the parietal cell is controlled through food-stimulated and neuroendocrine pathways involving the activity of gastrin, histamine, pituitary adenylate cyclase-activating peptide and acetylcholine. There are, therefore, several potential ways in which gastric acid secretion might be modified (Sachs 2003). Targeting the muscarinic receptors through which acetylcholine stimulates gastric acid secretion is one possible approach, but muscarinic antagonists (e.g. atropine) are not specific to the gastrointestinal system and have adverse effects such as dry mouth and blurred vision.

Competitive antagonists such as cimetidine and ranitidine can be used to block the binding of histamine to H2 receptors, but the parietal cell can still respond to other activating signals such as acetylcholine. Although histamine antagonists have reasonable efficacy at night, all patients quickly develop tolerance, perhaps as a result of upregulation of other pathways. (Hatlebakk &

Berstad, 1996). Given the redundancy inherent in the physiological control of gastric acid secretion, targeting the final effector in the secretion pathway – the gastric H^+, K^+ -ATPase – is likely the most effective pharmacological approach. The potassium-competitive acid pump antagonists (APAs), which inhibit the gastric H^+, K^+ -ATPase via K+-competitive binding, are a promising new class of agent but their efficacy has yet to be demonstrated in clinical trials (Andersson & Carlsson, 2005). At present, proton pump inhibitors (PPIs) remain the most effective available therapy.

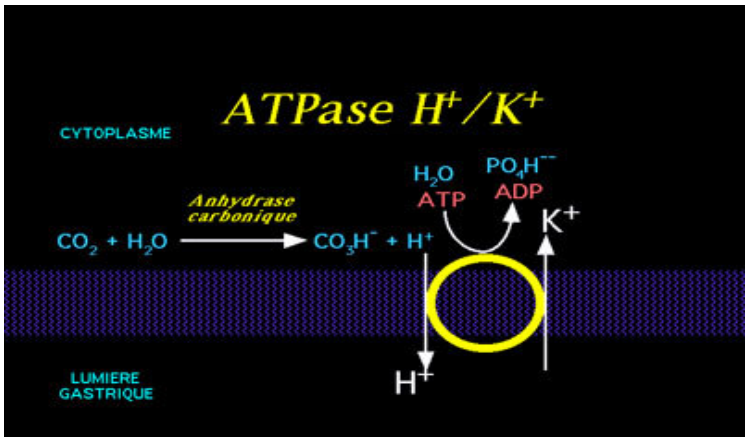


Figure 1.11. Diagram showing translocation of H^+ ions into the canalicular space in exchange for K^+ ions

1.8.3. Effects of different PPIs on the parietal cell

The benzimidazole derivative omeprazole was the first clinically useful PPI. Other benzimidazole PPIs subsequently introduced include lansoprazole, pantoprazole and rabeprazole. All these agents consist of two heterocyclic moieties – a pyridine and a benzimidazole moiety – linked via a methylsulfinyl group (Figure 1.12). A new PPI in development, tenatoprazole, has an imidazopyridine ring in place of the benzimidazole moiety. Proton pump inhibitors are weak bases carried in the circulation and delivered to the parietal cell as prodrugs. In this form, PPIs are capable of crossing cell membranes. The parietal cell is the only membrane- enclosed space in the body with a pH below 4.0. In this acidic environment of pH 1.0, PPIs accumulate in the secretory canaliculus of the parietal cell – at the luminal side of the gastric H^+, K^+ -ATPase – as a result of protonation of the pyridine moiety, which renders them less membrane permeable. It is likely that the monoprotonated species binds directly to the pump. Once on the acidic surface of the pump (or in the acid

compartment), PPIs undergo a second protonation on the benzimidazole or imidazopyridine moiety that effects a chemical rearrangement involving nucleophilic attack on the (unprotonated) pyridine by the now electrophilic 2C of the protonated benzimidazole, producing a planar cationic sulfenic acid. (Lindberg et al. 1986, Shin et al. 2004)

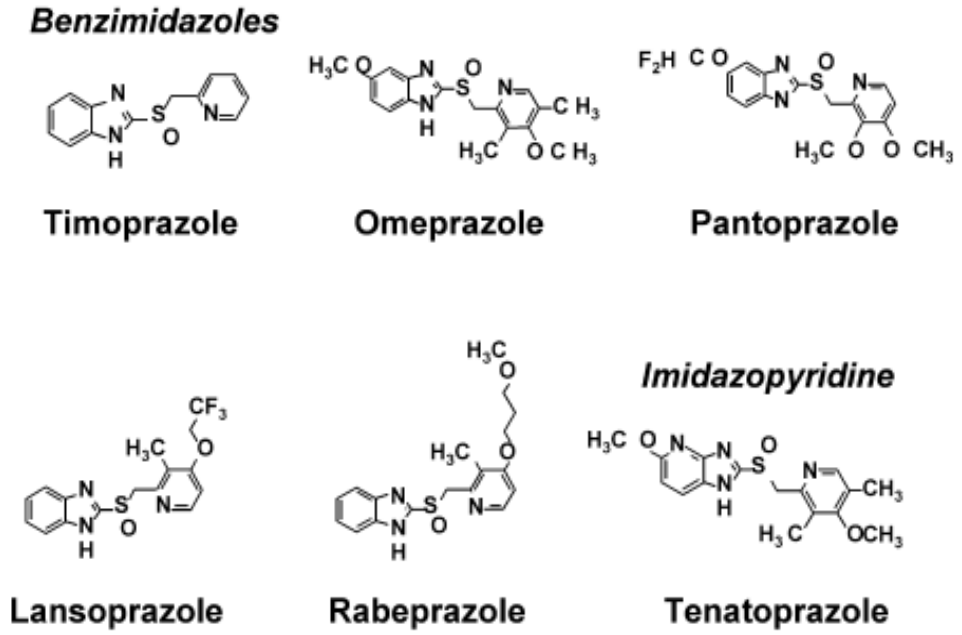


Figure 1.12. Proton pump inhibitors

This thiophilic cation, or the sulfenamide form produced by dehydration of the sulfenic acid, is the active form of the drug that reacts with cysteine sulfhydryls on the pump to form one or more covalent disulphide bonds, thus inhibiting its activity. The need for these two protonation steps in the accumulation and activation of PPIs and the particular chemical requirements underlying them mean that the covalent reaction that inhibits the ATPase is specific to the active gastric H^+, K^+-ATPase with a very large margin of safety given the pH of activation (2.0–2.5) (Shin et al. 2004).

The Substituted benzimidazoles are potent proton pump inhibitors. One has been approved for use in the US, **omeprazole (Prilosec)**. Interestingly, omeprazole rearranges (in the presence of acid) to a sulfenamide analogue, which acts as an irreversible inhibitor of the ATPase by forming a covalent disulfide bond with a crucial sulfhydryl group in the active site (Figure1.13).

The enantiomerically pure S-isomer, esomeprazole, has been marketed seperately as Nexium. (Wallmark et al. 1985b)

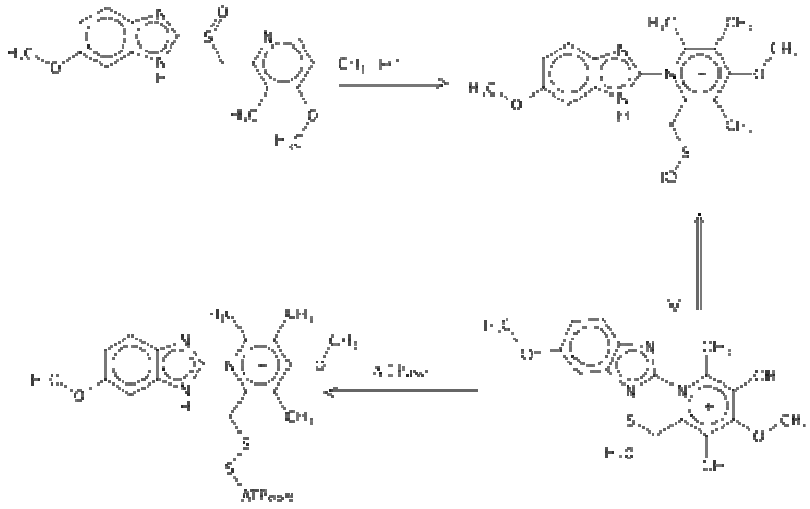


Figure 1.13. Action of omeprazole

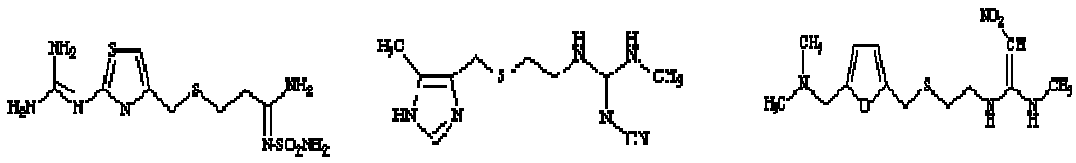
1.8.4. H_2 Receptor Antagonists

H_2 blockers impede acid production by blocking the actions of histamine, a substance produced by the body that encourages acid secretion in the stomach. H_2 blockers were the standard treatment for peptic ulcers until the development of antibiotic regimens against *H. pylori*. These drugs cannot cure ulcers, but in certain cases they are useful. They are effective only for duodenal ulcers, however, and have little effect on stomach (gastric) ulcers. Four H_2 blockers are currently available over the counter in the US: famotidine (Pepcid AC), cimetidine (Tagamet), ranitidine (Zantac), and nizatidine (Axid). All have good safety profiles and few side effects. Each is discussed below. H_2 blockers can interact with other drugs, so the doctor should be made aware of any other drugs a patient is taking. There are some differences among these drugs (Figure 1.14).

Famotidine *(Pepcid AC)* is the most potent H2 blocker. The most common side effect of famotidine is headache, which occurs in 4. 7% of people who take it. Famotidine is virtually free of drug interactions but it may have significant adverse effects in patients with kidney problems.

Cimetidine (Tagamet) has few side effects; approximately 1% of people taking cimetidine will experience mild temporary diarrhea, dizziness, rash, or headache. Cimetidine interacts with a number of commonly used medications, such as phenytoin, theophylline, and warfarin. Long term use of excessive doses (more than 3 grams a day) may cause impotence or breast enlargement in men; these problems resolve after the drug is discontinued.

Ranitidine (Zantac) interacts with very few drugs. In one study, ranitidine provided more pain relief and healed ulcers more quickly than cimetidine in people younger than 60, but there was no difference in older patients. A common side effect of ranitidine is headache, which occurs in about 3% of the people who take it.



A B C

Figure 1.14. structures of H_2 blockers A- famotidine, B- ranitidine, C- cimetidine

1.8.5. Prostaglandins and Cytoprotective Agents (Figure 1.15)

It has been known for a number of years that prostaglandin synthetase is present in the gut wall, and that prostaglandins of the E and F series are synthesized and secreted in gastric juice. Prostaglandins (and in particular PGEs) can inhibit histamine-stimulated HCl release without interrupting mucosal flow, and are thus capable of protecting the mucosa, a phenomenon termed **cytoprotection**. Other effects include enhancement of mucosal blood flow, stimulation of bicarbonate release and increased mucus production. Because prostaglandins are short-lived intermediates in vivo, it is generally not possible to use these agents as cytoprotectants. However, there are now a number of analogues of PGE1 which are useful as cytoprotectants. The stable analogue **misoprostol** was recently marketed for this purpose. Misoprostol has two structural features which make it more stable to metabolism than PGE1: the hydroxyl substituent is moved from the 15 to the 16 position, where it resides geminal to a methyl group, greatly reducing metabolism by oxidation, and it is administered as

the methyl ester. The methyl ester is a pro-drug form which must be cleaved prior to activity. Misoprostol retains all of the cytoprotective attributes of PGE1.

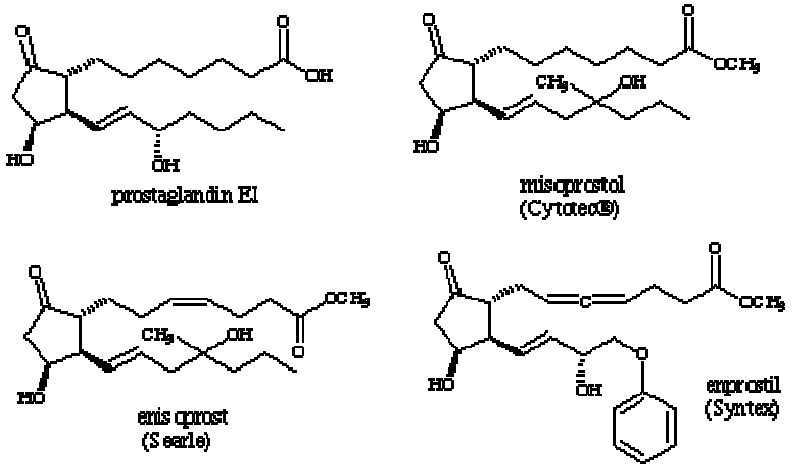


Figure 1.15. Structures of prostaglandins

1.8.6. Mucosal protectant

There is one additional cytoprotective agent which acts by a completely different mechanism. Sucralfate (Carafate) protects the gastric mucosa by forming a sticky, viscous gel that adheres to mucosal tissue and protects it from the action of gastric acid and pepsin. Sucralfate has no inherent acid-neutralizing activity. Sucralfate (Carafate- Figure 1.16) seems to work by adhering to the ulcer crater and protecting it from further damage by stomach acid and pepsin. It also promotes the defensive processes of the stomach. Sucralfate has an ulcer-healing rate similar to that of H2 blockers. Other than constipation, which occurs in 2.2% of patients, the drug has few side effects. Sucralfate does interact with a wide variety of drugs, including warfarin, phenytoin, and tetracycline.

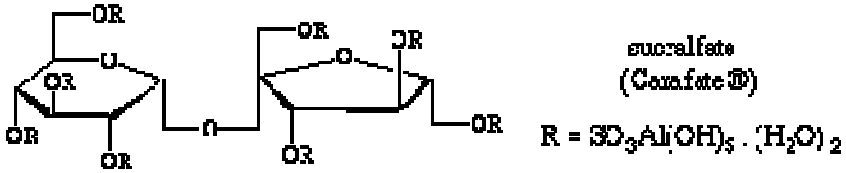


Figure 1.16. Structures of sucralfate

In addition to, as indicated earlier, the use of inevitable use of NSAIDs, exposure to xenobiotics and other disease conditions also manifests as ulcers (Figure 1.5).

The above mentioned factors create stress in the body releasing generated free radicals (FR). Free radicals are deleterious to health since they are active chemically, interacts with biomolecules of cellular components. Such type of free radical attack on the parietal cell membrane (Figure 1.4) perturbs the membrane by oxidative process and delocalizes the membrane components leading to loss of co-ordination in the activity. ROS may initiate the process of gastric damage as indicated in Figure 1.5.

1.9. Reactive oxygen species: role in Oxidative damage

Reactive oxygen species are molecules like hydrogen peroxide, ions like the hypochlorite ion, radicals like the hydroxyl radical (It is the most reactive of them all), hydroxyl ion and the superoxide anion which is both ion and radical.

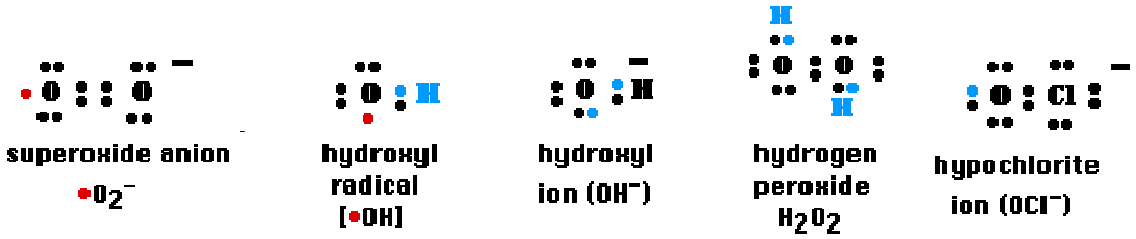


Figure 1.17. Reactive oxygen species

A radical (also called a "free radical") is a clusters of atoms one of which contains an unpaired electron (shown in red in figure 1.17) in its outermost shell of electrons. This is an extremely unstable configuration, and radicals quickly react with other molecules or radicals to achieve the stable configuration of 4 pairs of electrons in their outermost shell (one pair for hydrogen). Link to discussion of electron organization in atoms.

1.9.1. ROS Formation

Reactive oxygen species are formed by several different mechanisms:

- the interaction of ionizing radiation with biological molecules
- as an unavoidable byproduct of cellular respiration. Some electrons passing "down" the electron transport chain leak away from the main path (especially as they pass through

ubiquinone) and go directly to reduce oxygen molecules to the superoxide anion (#2 above).

- synthesized by dedicated enzymes in phagocytic cells like neutrophils and macrophages –
  - NADPH oxidase (in both type of phagocytes)
  - yeloperoxidase (in neutrophils only)

1.9.2. ROS Activity

Strong oxidants like the various ROS can damage other molecules and the cell structures of which they are a part.

Among the most important of these are the actions of free radicals on the fatty acid side chains of lipids in the various membranes of the cell, especially mitochondrial membranes (which are directly exposed to the superoxide anions produced during cellular respiration).

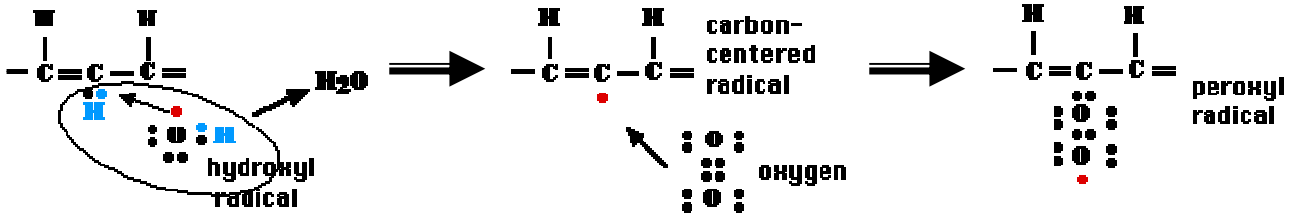


Figure 1.18. Reactive oxygen species in action

A hydroxyl radical removes a hydrogen atom from one of the carbon atoms in the fatty acid chain (only a portion of which is shown) forming a molecule of water and leaving the carbon atom with an unpaired electron (in red); thus now a radical. One of the most likely (Figure 1.18) is to react with a molecule of oxygen (O2) forming a peroxyl radical.

This might then steal a hydrogen atom from a nearby side chain making it now a radical.
One of the insidious things about free radicals is that in interacting with other molecules to gain a stable configuration of electrons, they convert that target molecule into a radical. So a chain reaction begins that will propagate until two radicals meet each other and each contributes its unpaired electron to form a covalent bond linking the two.

The peroxyl radical may interact with:

- another peroxyl radical on a nearby side chain crosslinking them with a covalent bond.
- another nearby carbon-centered radical crosslinking them covalently.

In both these latter cases, radical formation comes to an end but with the result that the fatty acid side chains of membrane lipids may have become so deformed as to damage the membrane.

Cellular antioxidant enzymes and the free-radical scavengers normally protect a cell from toxic effects of the ROS. However, when generation of the ROS overtakes the antioxidant defense of the cells, oxidative damage of the cellular macromolecules (lipids, proteins, and nucleic acids) occurs, leading finally to various pathological conditions (Figure 1.19) (Bandyopadhya et al 1999).

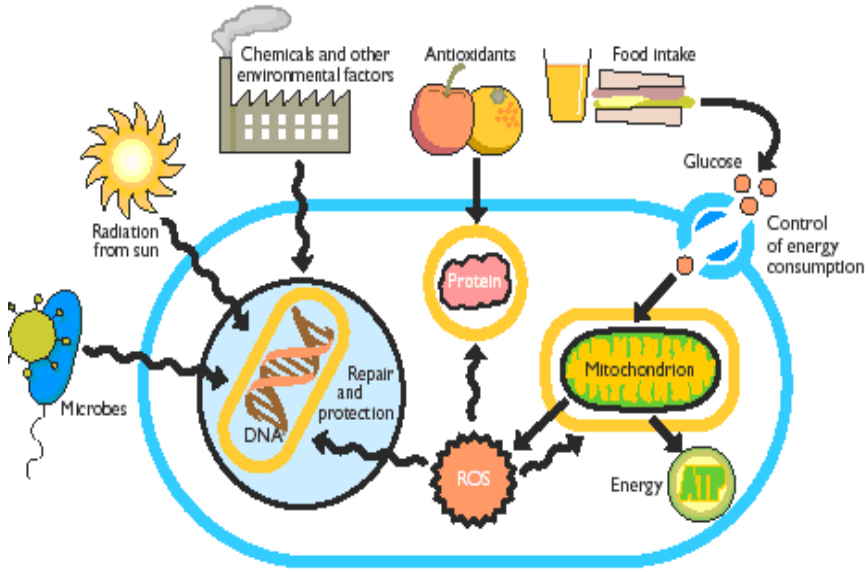


Figure 1.19. ROS damages the cellular macromolecules (lipids, proteins, and nucleic acids)

ROS-mediated lipid peroxidation, oxidation of proteins, and DNA damage are well-known outcomes of oxygen-derived free radicals, leading to cellular pathology and ultimately to cell death (Figure 1.20). The mechanism of ROS-mediated oxidative damage of lipids, proteins, and DNA has been extensively studied (Halliwell & Hulliridge, 1990). The site-specific oxidative damage of some of the susceptible amino acids of proteins is now regarded as the

major cause of metabolic dysfunction during pathogenesis. ROS have also been implicated in the regulation of at least two well defined transcription factors which play an important role in the expression of various genes encoding proteins that are responsible for tissue injury. One of the significant benefits of the studies on ROS will perhaps be in designing of a suitable antioxidant therapy to control the ROS mediated oxidative damage, and the disease processes.

Normal cellular homeostasis is a delicate balance between the rate and magnitude of oxidant formation and the rate of oxidant elimination. Oxidative stress can, therefore, be defined as the toxicity outcome of the overproduction of oxidants.

1.9.3. Free Radicals vs. Oxidants

The term free radicals have been equated with reactive species or oxidants. By definition, a radical is a molecule possessing an unpaired electron. Superoxide, nitric oxide, hydroxyl, alkoxyl and alkyl-peroxyl (lipid) are radicals. However, with the exception of hydroxyl radical none of these radicals are strong oxidants. Thus, not all radicals are strong oxidants and not all oxidants are radicals.

However, experimental evidence has implicated reactive species in the pathogenic mechanism of several diseases (Aruoma, 1998). It is, therefore, important to understand the biochemical pathways for the induction of oxidative stress by reactive species. The most reasonable biochemical hypothesis is the reactive species-mediated modification of critical cellular targets.

Iron-sulfur enzymes are direct targets for superoxide and toxicity can be derived from the inactivation of these enzymes (Premysl et al, 2006). Hydrogen peroxide at low µM levels does not react with many biological targets at an appreciable rate. However, the reaction of hydrogen peroxide with reduced divalent redox active metals such as iron can lead to the formation of strong oxidants. This reactivity of hydrogen peroxide may be important in biological oxidations of proteins and lipids that take place at the sites of metal binding. Divalent redox active metals can also catalyze the formation of the highly reactive hydroxyl by the metal-catalyzed Haber-Weiss reaction (Halliwell & Gutteridge, 1984).

However, hydroxyl radical reacts with almost all biological targets at rates exceeding 10^9 M^{-1} sec^{-1} and therefore its diffusion distance inside a cell is minimal. Thus, in order for hydroxyl radical to cause toxicity it must be formed within a few Angstroms from a biological target.

$$O_2^{\bullet-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + -OH + Fe^{3+}$$

An alternative pathway of superoxide toxicity is the formation of peroxynitrite by the reaction with nitric oxide. Nitric oxide is a radical but a weak one electron oxidant. Since both •NO and O2• are radicals they react rapidly to form peroxynitrite:

$$\bullet NO + O_2^{\bullet-} \rightarrow ONOO^-$$

Peroxynitrite, the reaction product of nitric oxide (NO) and superoxide, is a potent biological oxidant that mediates tissue injury in diverse pathological conditions, including ischemia-reperfusion injury, immunocomplex-mediated pulmonary edema, acute endotoxemia, neurological disorders, and atherosclerosis (Moncada et al., 1991). At the cellular level, peroxynitrite causes deleterious effects on various biomolecules; indeed, an extensive literature documents its ability to promote lipid peroxidation (Radi et al., 1991), protein nitration and nitrosylation (Patel et al., 1999), DNA damage (Guidarelli et al., 2000) and oxidation of thiols (Salgo et al., 1995).

1.9.4. Cellular Responses to Reactive Species:

The flux and the time of exposure are critical factors in determining the outcome of oxidative stress. Although the antioxidant networks maintain the critical balance towards physiology, a few reactive species escape the surveillance of the antioxidant network and react with biological targets. Oxidation of biological targets will not necessarily translate to expression of a phenotype because repair processes may sustain normal physiologic function. However, as the frequency of oxidation of biological targets increases (and possibly as repair processes slow), detection of oxidized proteins, lipids and even DNA becomes apparent with aging and other reactive-species mediated pathologies.

Severe oxidative stress results in necrotic cell death (Xue et al, 2003). Generation of reactive species during hyperoxia (breathing of >95% oxygen) or reperfusion of an ischemic tissue leads to tissue necrosis. A moderate exposure to reactive species can also result in cell death that usually occurs 20-24 hours after the initial insult. In most cases delayed cell death resembles apoptosis since DNA fragmentation and other features of apoptosis are evident. It is not clear how reactive species can induce delayed cell death or apoptosis. Potential pathways that once altered by reactive species will lead to delayed cell death include energy sources (mitochondria, activation of Poly- ADP ribosyl synthase), ionic homeostasis, signal transduction and membrane structural integrity.

Overall, the inherent ability of cells to withstand oxidative stress is dependent upon several factors: their antioxidant capacity, the ability to sustain metabolic requirements by deriving energy from alternate pathways, efficiency to repair oxidatively modified biomolecules, and availability and utilization of trophic support.

1.10. Primary defense against ROS

1.10.1. Catalytic removal of ROS by antioxidant enzymes

Superoxide dismutase (SOD), catalase, and peroxidases constitute a mutually supportive team of defense against ROS. While SOD lowers the steady state level of O^{2-}, catalase and peroxidases do the same for H_2O_2. The first enzyme involved in the antioxidant defense is the superoxide dismutase: a metalloprotein found in both prokaryotic and eukaryotic cells (Jamieson 1998). The iron-containing (Fe-SOD) and the manganese-containing (Mn-SOD) enzymes are characteristic of prokaryotes. In eukaryotic cells, the predominant forms are the copper-containing enzyme and the zinc-containing enzyme, located in the cytosol. The second type is the manganese containing SOD found in the mitochondrial matrix4. The biosynthesis of SOD is mainly controlled by its substrate, the O_2 (Ji et al. 1994). Induction of SOD by increased intracellular fluxes of O^{2-} has been observed in numerous microorganisms, as well as in higher organisms.

1.10.2. Secondary defense against ROS: Free-radical scavengers

In addition to the primary defense against ROS by antioxidant enzymes, secondary defense against ROS is also offered by small molecules which react with radicals to produce another

radical compound, the 'scavengers'. When these scavengers produce a lesser harmful radical species, they are called 'antioxidants'. For example, *a*-tocopherol, ascorbate, and reduced glutathione (GSH) may act in combination to act as cellular antioxidants. *a*-Tocopherol, present in the cell membrane and plasma lipoproteins, functions as a chain-breaking antioxidant. Once the tocopherol radical is formed, it can migrate to the membrane surface and is reconverted to *a*-tocopherol by reaction with ascorbate or GSH. The resulting ascorbate radical can regenerate ascorbate by reduction with GSH, which can also directly scavenge ROS, and the resulting GSSG can regenerate GSH through NADPH-glutathione reductase system.

1.11. Diet and ulcer

As we understand from the previous literature, increase in the ulcer incidence is alarming. Life style is the biggest reason. In recent days, due to excessive stress in the society, lack of excerscise, untimely food, intake of food for convenience (not for health purpose), occassionaly also lack of awareness in intake of balanced diet etc., results in gastric disturbances. This together with the ineffectiveness of ulcer drugs warrants to evaluate diet as a potential to manage and cure ulcers.

There is an abundance of evidence that regular consumption of fruits and vegetables is associated with a reduced risk of chronic and degenerative diseases, such as cancer, ulcer disease and cardiovascular disease (Gerber et al. 2002, Saito et al. 1998 and Middleton et al. 2000). When the mechanism of antioxidant protection becomes unbalanced by exogenous factors such as smoking, ionising radiation, certain pollutants, organic solvents and pesticides and endogenous factors such as normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes may occur, resulting in above-mentioned diseases and accelerating ageing. However, antioxidant supplements or foods rich in antioxidants may be used to help the human body in reducing oxidative damage by free radicals and active oxygen (Halliwell and Gutteridge, 1984). A predominantly plant-based diet reduces the risk for development of several chronic diseases. It is often known that antioxidants contribute to this protection.

1.12. Antioxidants

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells.

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols.

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Sies, 1997). These compounds may be synthesized in the body or obtained from the diet (Vertuani et al, 2004). The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. The relative importance and interactions between these different antioxidants is a very complex question, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another (Chaudière & Ferrari-Iliou 1999, Sies 1993). The action of one antioxidant may therefore depend on the proper function of other members of the antioxidant system (Vertuani et al, 2004). The amount of protection provided by any one antioxidant will also depend on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts.

Some compounds contribute to antioxidant defense by chelating transition metals and preventing them from catalyzing the production of free radicals in the cell. Particularly important is the ability to sequester iron, which is the function of iron-binding proteins such as transferrin and ferritin (Imlay, 2003). Selenium and zinc are commonly referred to as

antioxidant nutrients, but these chemical elements have no antioxidant action themselves and are instead required for the activity of some antioxidant enzymes.

1.12.1. Ascorbic acid

Ascorbic acid or "vitamin C" is a monosaccharide antioxidant found in both animals and plants. As it cannot be synthesised in humans and must be obtained from the diet, it is a vitamin (Smirnoff, 2001). In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalysed by protein disulfide isomerase and glutaredoxins (Meister, 1994). Ascorbic acid is a reducing agent and can reduce and thereby neutralize reactive oxygen species such as hydrogen peroxide (Padayatty, 2003) In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants.

1.12.2. Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life (Meister 1994). It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants Due to its high concentration and its central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants (Meister & Anderson, 1983).

1.12.3. Tocopherols and tocotrienols (vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera & Barbas, 2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolising this form. It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber & Atkinson 2007). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidised α-tocopheroxyl radicals

that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol. However, the roles and importance of the various forms of vitamin E are presently unclear and it has even been suggested that the most important function of α-tocopherol is as a signaling molecule, with this molecule having no significant role in antioxidant metabolism (Sen et al., 2006). The functions of the other forms of vitamin E are even less well-understood, although γ-tocopherol is a nucleophile that may react with electrophilic mutagens and tocotrienols may be important in protecting neurons from damage (Sen et al., 2006).

1.13. Phytochemicals

A phytochemical is a natural bioactive compound found in plant foods that works with nutrients and dietary fiber to protect against disease. Research suggests that phytochemicals, working together with nutrients found in fruits, vegetables and nuts, may help slow the aging process and reduce the risk of many diseases, including cancer, heart disease, stroke, high blood pressure, cataracts, osteoporosis, and urinary tract infections Inverse associations between fruit and vegetable intake and chronic diseases, such as different types of cancer and cardiovascular disease, have been demonstrated in numerous epidemiological studies.

Phytochemicals in Fruits and Vegetables (Figure 1.20)

Carotenoids: Carotenoids are the pigments responsible for the colors of many red, green, yellow and orange fruits and vegetables. Carotenoids are a large family of phytochemicals which include alpha-carotene, beta-carotene, lutein, lycopene, cryptoxanthin, canthaxanthin, zeaxanthin, and others.

Beta-Carotene may help to slow the aging process, reduce the risk of certain types of cancer, improve lung function, and reduce complications associated with diabetes. Beta-carotene is found in yellow-orange fruits and vegetables such as mangoes, cantaloupe, apricots, papaya, kiwifruit, carrots, pumpkins, sweet potatoes, and winter squash, and green vegetables, such as broccoli, spinach, and kale.

Lutein is essential for maintaining proper vision as we age. It has been shown to reduce the risk of cataracts and macular degeneration, the leading causes of blindness in older people

and may help reduce the risk of certain types of cancer. Kale, spinach and collard greens contain the most lutein of any fruit or vegetable. Other sources of lutein include kiwifruit, broccoli, collard greens, brussels sprouts, swiss chard, and romaine lettuce.

Lycopene, diets rich in lycopene have been shown to reduce the risk of prostate cancer and heart disease. Lycopene is found in red fruits and vegetables such as tomatoes and cooked tomato products, red peppers, pink grapefruit, watermelon.

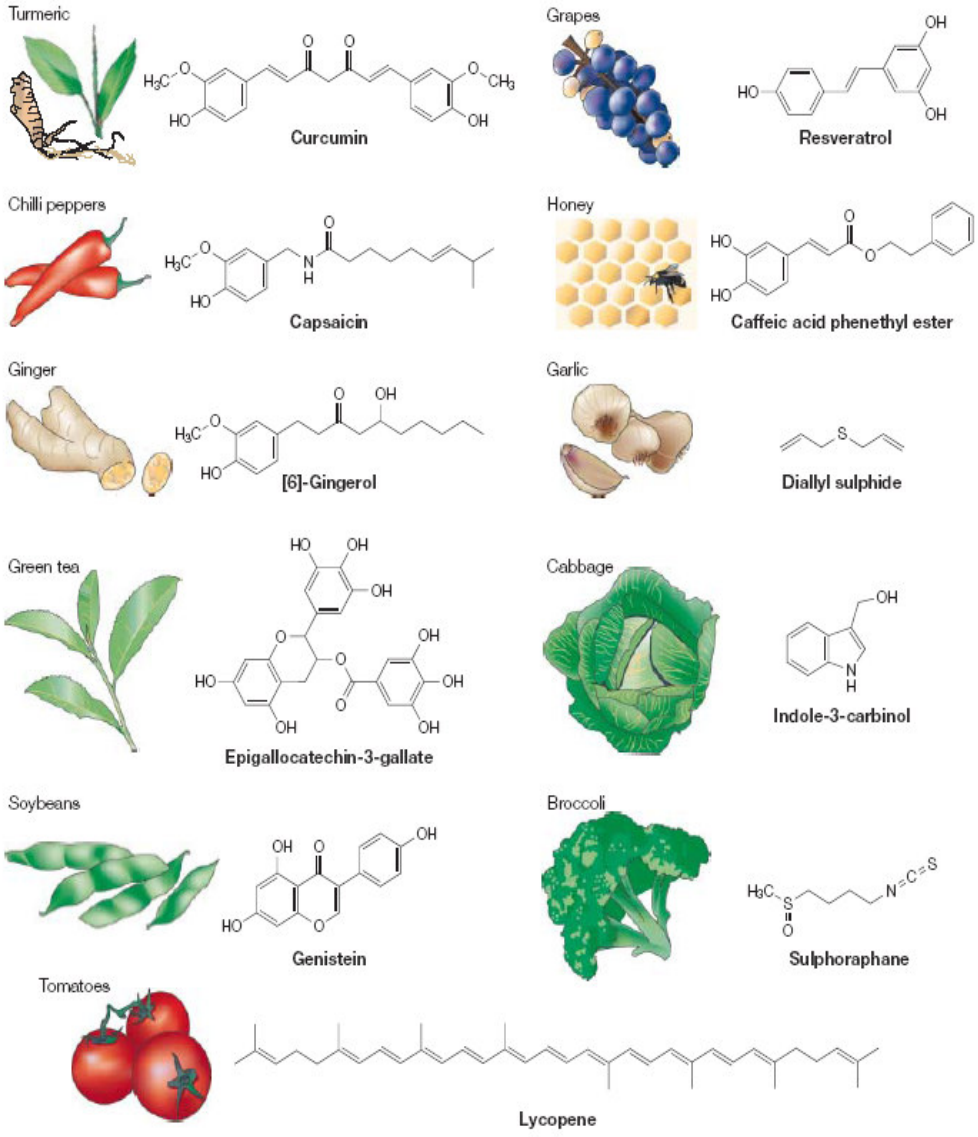


Figure 1.20. Representative phytochemicals and their dietary sources

Zeaxanthin may help to prevent macular degeneration, which is the leading cause of visual impairment in people over 50. It may also help to prevent certain types of cancer. Corn, spinach, winter squash, and egg yolks contain zeaxanthin.

Flavonoids are another large family of protective phytochemicals found in fruits and vegetables. Flavonoids, also called bioflavonoids, act as antioxidants. There are many different types of flavonoids and each appears to have protective health effects. Some of the better known flavonoids include resveratrol, anthocyanins, quercetin, hesperidin, tangeritin, kaempferol, myricetin, and apigenin. Flavonoids are found in a variety of foods, such as oranges, kiwifruit, grapefruit, tangerines, berries, apples, red grapes, red wine, broccoli, onions, and green tea. The five primary flavonoids found in fruits and vegetables are:

Anthocyanins, which are particularly high in blueberries, have been shown to protect against the signs of aging. In one study, elderly rats that ate the equivalent of a half-cup of blueberries daily for eight weeks improved balance, coordination, and short-term memory. Scientists think these results may apply to humans as well. Anthocyanins in blueberries and cranberries have also been shown to help prevent urinary tract infections. Blueberries, cherries, strawberries, kiwifruit, and plums contain anthocyanins.

Quercetins may reduce inflammation associated with allergies, inhibit the growth of head and neck cancers, and protect the lungs from the harmful effects of pollutants and cigarette smoke. Apples, pears, cherries, grapes, onions, kale, broccoli, leaf lettuce, garlic, green tea, and red wine contain quercetins.

Phenolic Compounds (Polyphenols):

Polyphenols another class of antioxidants, have also been “associated with a lower risk of some diseases, including ulcer and cancer (Ruggiero et al. 2006, Yuichi, 2007).” Polyphenols are a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule (Figure 21).

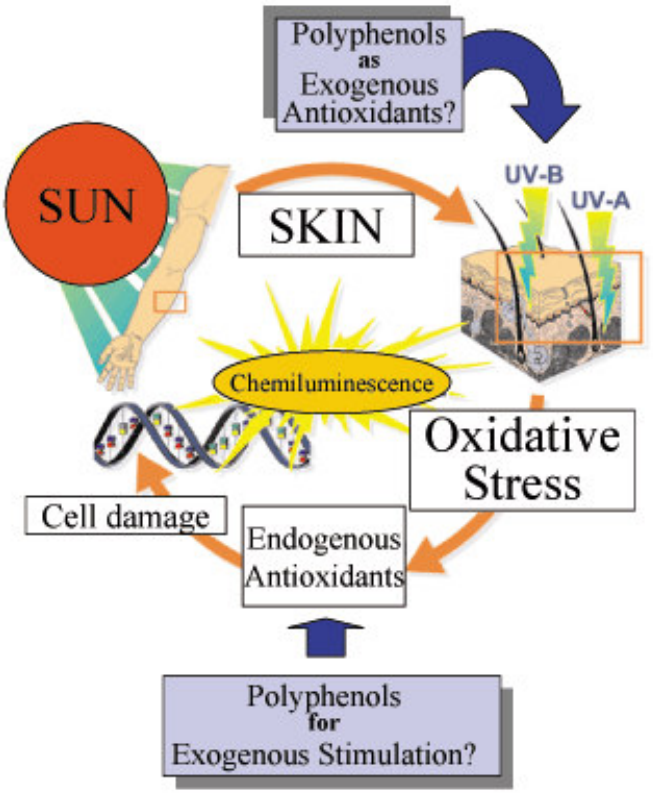


Figure 1.21. Polyphenols protect oxidative stress

Polyphenols are known to be stimulated in plants in response to exogenous factors that generate free radicals such as UV radiation (A). This may create oxidative stress (B) in the cell and damage cellular components (C). enhanced polyphenolic endogenous antioxidants (D) protects system against oxidative damage. Exogenously supplied polyphenols (E) also reduces the damage effectively.

1.14. Dietary fibers

Dietary factors that have been investigated against ulcers include dietary fibres apart from antioxidants. Antioxidant potentials have been discussed earlier Dietary fibre was yet another molecule reported to act against ulcer. Although Medline search was done from 1966 till present, evidence that diet and lifestyle are associated with duodenal ulcers arose mainly from only three case – control and three prospective studies. Antioxidants to some extent has been known to protect ulcers. Precise mechanisms through which it protects ulcers are not reported. Recently few reports shown that, diets rich in phenolic acids particularly free and bound phenolics constituting different phenolic acids have been shown to possess antiulcer

activity (Saito et al. 1998, Galati et al. 2003). Phenolic acids such as caffeic, ferulic, cinnamic and protocatecheuic acids have recently been shown from our laboratory to exert antioxidant and anti-microbial activity (Siddaraju & Shylaja 2007). 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 for ulcer (Das et al. 1997). However data still needs to be elucidated for the complete understanding of the potentials of dietary antioxidants against ulcer.

Dietary fibers are another source implicated in gut from time immemorial. Fibers are part of the die, important component from plant foods. They vary considerably in its physical properties and chemical composition. Crude fiber consists of cellulose and lignin. Dietary fiber includes crude fiber and non cellulose polysaccharides, hemicelluloses, pectins, gums etc.

Dietary fibers are generally classified according to its water solubility properties. The structural fibers, cellulose, lignin and some hemicelluloses, are insoluble, the natural gel forming fibres, pecans, gums mucilages and remaing hemicellulose, are soluble. In the past, a bland, low-fibre diet was recommended to patients with ulcers, but in the late 1970s, evidence that, a higher-fibre diet was beneficial in ulcer treatment began to accumulate. A small clinical trial in India (Malhotra 1978) compared a rice diet with unrefined wheat diet among ulcer patients over a 5- year period. Results showed that 81% patients on the rice diet had ulcers recur, while only 14 % of patients on the unrefined wheat diet had ulcers recur. Results of a small clinical trial 19 showed that incidence of Duodenal ulcer recurrence was lower among those who has been on a high dietary fiber diet (28.2g/d) for 6 months than among those on a lower –fiber diet. (11.4 g/d). Authors of a 1990 case –control study (Katschinski et al, 1990) attributed the protective effect not to dietary fiber, but to low intake of refined sugar because a relatively higher intake of unrefined carbohydrate has been believed to might provide a gastric acid buffer system. In that study, however, relative risk of ulcer disease, though not consistent are significant, but found to be reduced by high vegetable fiber

A prospective cohort study, the health professional's follow-up-study (Aldoori et al, 1997), which looked at 51529 US male health professionals aged 40 – 75 years showed that fiber from legumes had the greatest influence on duodenal ulcers (DU) risk reduction. Legumes include beans, tofu, peanut butter, and nuts, which are good sources of soluble fiber. Soluble

fiber reduced risk of DU significantly more than an insoluble fiber. Cereal fiber intake was associated with an upward trend in risk of DU, but this result was not statistically significant.

Data from the current study suggest that fiber of different types (soluble or insoluble) and sources (fruit, vegetable, legume or cereal) affects risk of DU to varying, but not necessarily clinically significant, degrees. Pectic polysaccharides are also reported from several plant sources similar to that found in dietary sources. 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.

A water soluble crude polysaccharide fraction prepared from the root of falcatum L. (Japenese name = Saiko) has been shown to prevent HCl/ethanol induced ulcerogenesis in mice significantly (Yamada et al. 1991). One of the potent pectic polysaccharide which showed ulcer inhibitory activity had a sugar composition – galacturonic acid with small proportions of arabinose, rhamnose and galactose, and its average relative molecular mass was estimated to be 63 kDa. polygalacturonan region was found to be important for the activity (Sun et al 1992). Another water soluble pectic polysaccharide with a molecular mass of 15 kDa from Panax ginseng C composed mainly of galactose and galacturonic acid with small proportions of rhamnose, arabinose, mannose, glucose and glucuronic acid showed potential inhibition of ulcerogenesis.

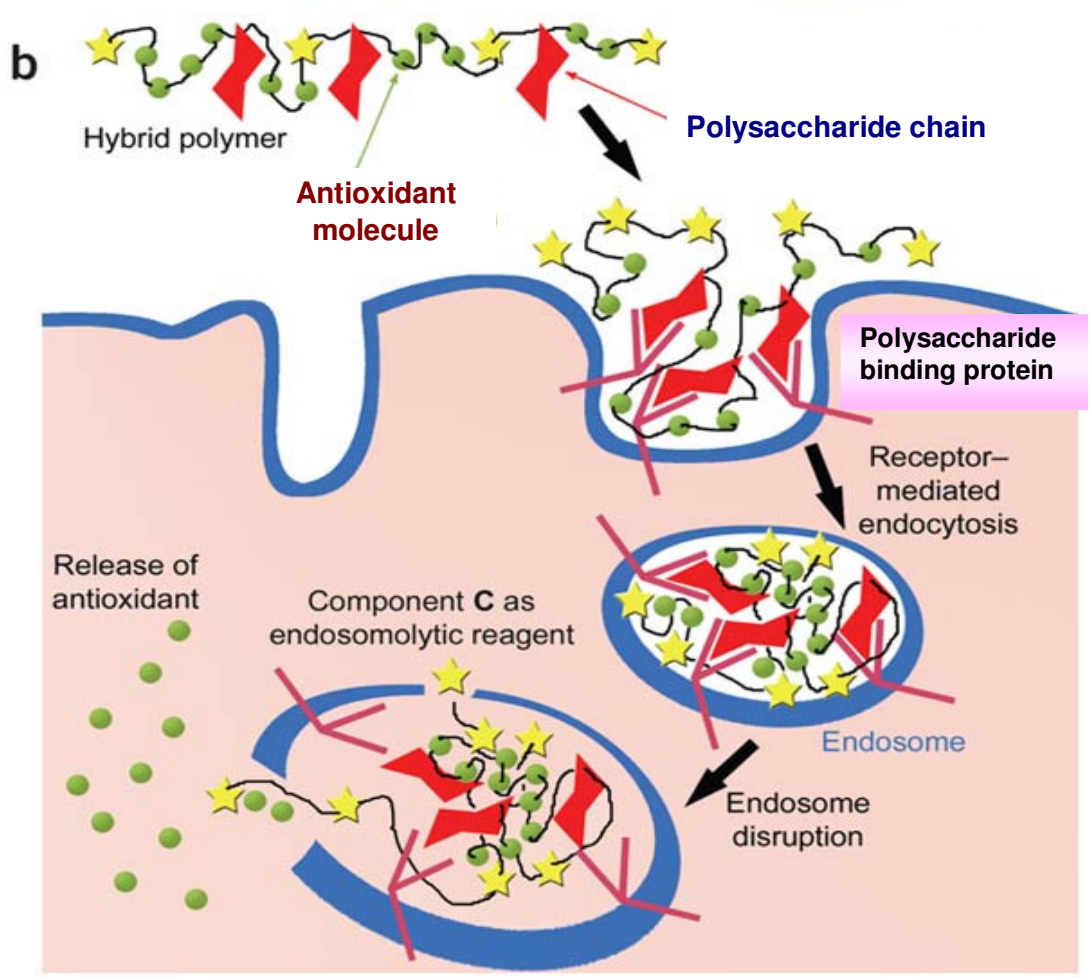
1.14.1. Phenolic compounds as cross-links of plant derived polysaccharides

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 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 (Waldron et al, 1997), on the gelling properties of sugar beet pectins (Oosterveild et al, 2000) 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, 1998). 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 (Ishi, 1997). Similarly,sinapic acid is thought to be bound to polysaccharides via ester-linkages (Bunzel et al, 2002).

In the light of the functional role of polysaccharide with bound phenol, investigators on designing of 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 (Figure 1.22) 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)

Figure 1.22. Role of phenol-polysaccharide

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

1.15. How ulcers can lead to cancers?

Any repeated insult in the body for a long period of time operates the repair mechanism constar to ensure protection. In this case since ulcerogens due to stress or alcohol or drug is a cor.....ous process leading to gastric mucosal damage, gastric cell has been known to be driven towards the making up the loss of mucin cells to proliferate to produce more and more mucin. The insult being constant, defense mechanism is also constant and some time point cell loses its control and this is what defined as precancerous condition (unregulated proliferation of cells). From this stage cancer cell adaptation adds to the pathogenicity scheme of aggressive cancer progressing towards the severity of the disease- metastasis. Many times long term usage of antiulcer drugs also lead to cancer (Figure 1.23).

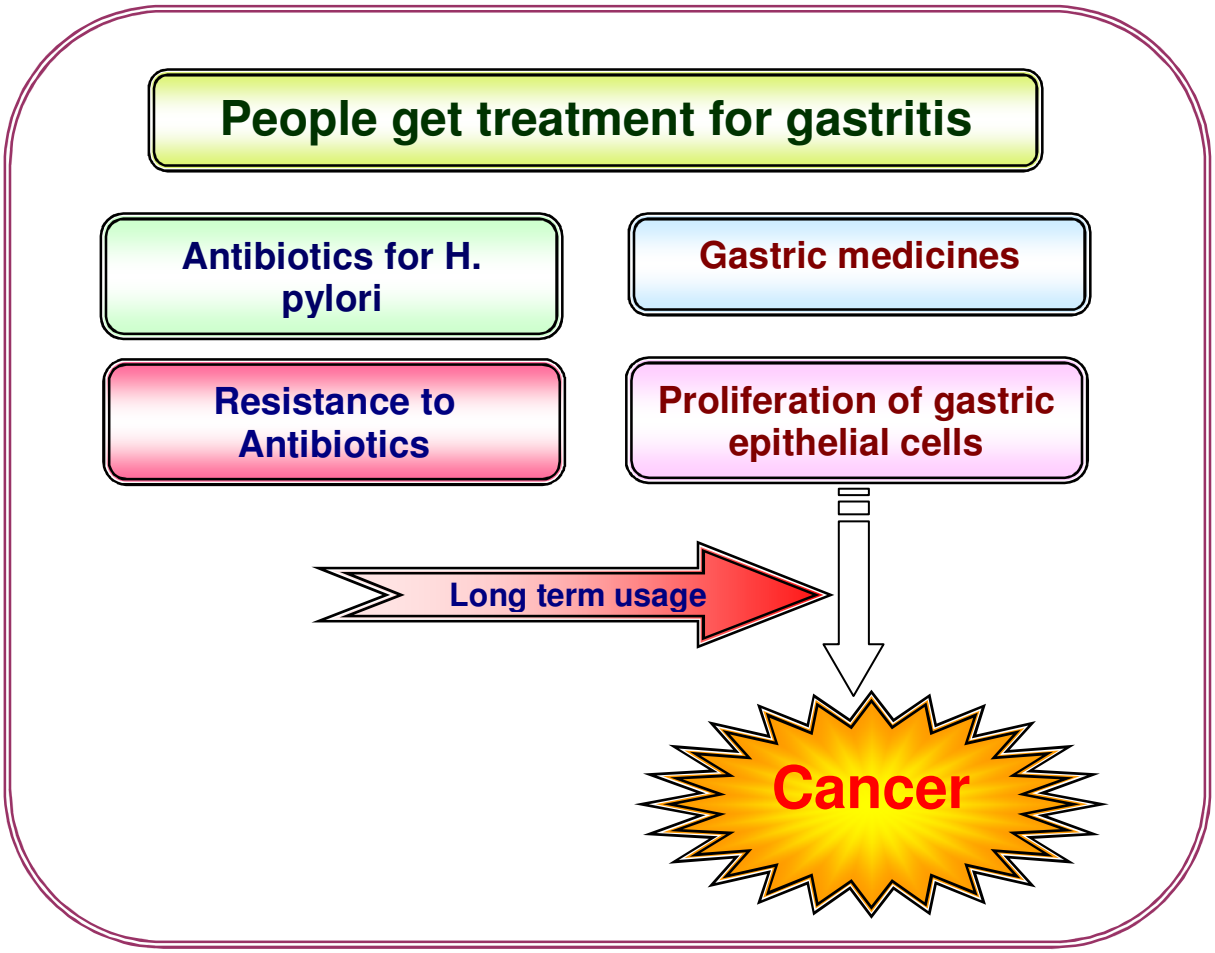


Figure 1.23. Long term usage of antiulcer drugs also lead to cancer.

This is the background in brief about why we included the anticancer attribute of antiulcer components (Chapter- 4). Review below highlights some of the characteristic feature of cancer cell and mechanism of advancement of the disease.

1.16. Cancer

Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. Cancer may affect people at all ages, even fetuses, but the risk for most varieties increases with age. Cancer causes about 13% of all deaths. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007 (American Cancer Society 2007). Cancers can affect all animals.

1.16.1. Origins of cancer

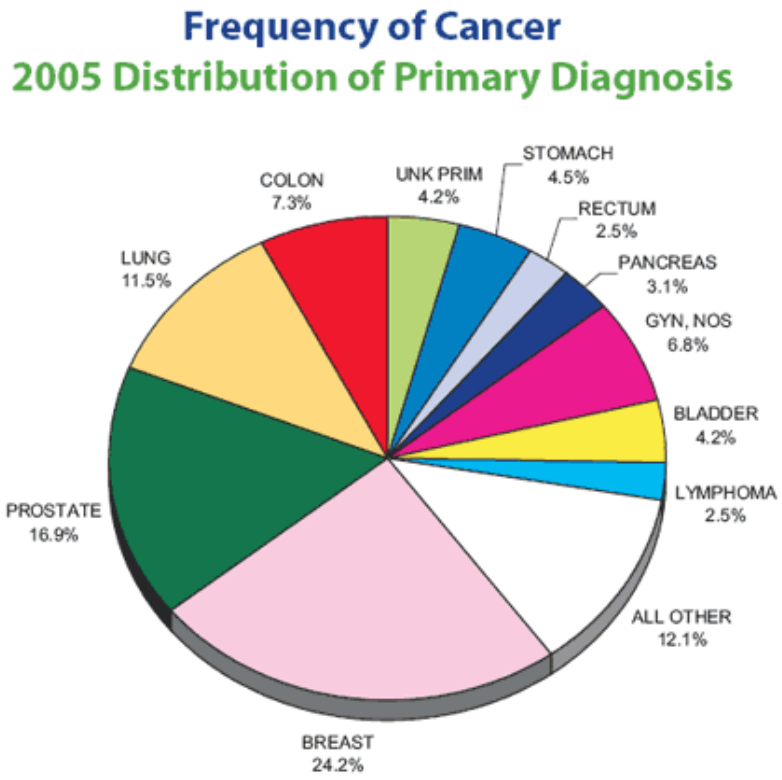
Cell division or cell proliferation is a physiological process that occurs in almost all tissues and under many circumstances. Normally the balance between proliferation and programmed cell death is tightly regulated to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer disrupt these orderly processes. The uncontrolled and often rapid proliferation of cells can lead to either a benign tumor or a malignant tumor (cancer). Benign tumors do not spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they extrinsically compress vital structures. Malignant tumors can invade other organs, spread to distant locations (metastasize) and become life-threatening.

1.16.2. Types of Cancers

Classified based on the cellular origin of cancer cell. They are listed as follows.

- **Carcinoma:** malignant tumors derived from epithelial cells. This group represents the most common cancers, including the common forms of breast, prostate, lung and colon cancer.

- **Lymphoma and Leukemia**: malignant tumors derived from blood and bone marrow cells
- **Sarcoma**: malignant tumors derived from connective tissue, or mesenchymal cells
- **Mesothelioma**: tumors derived from the mesothelial cells lining the peritoneum and the pleura.
- **Glioma:** tumors derived from glia, the most common type of brain cell
- **Germinoma**: tumors derived from germ cells, normally found in the testicle and ovary
- **Choriocarcinoma:** malignant tumors derived from the placenta



The leading causes of death in the United States are cardiovascular diseases and cancers. Similarly, in Taiwan, around 27% of deaths are from cancer and 18% of deaths are from cardiovascular and heart diseases (Department of Health Web, 2004). It was estimated by Willet (1994) that roughly 32% (range of 20%–42%) of deaths from cancer could be avoided by dietary modification. Epidemiological studies have strongly suggested that diet plays an important role in the prevention of chronic diseases (Bauman, 2004; Willet, 1995). Polyphenolics, thiols, carotenoids, tocopherols, and glucosinolates commonly found in fruits,

vegetables and grains, provide chemoprotective effects to combat oxidative stress in the body and maintain balance between oxidants and antioxidants to improve human health (Adom & Liu, 2002; Jia, and Wu, & Liu, 2003).

An imbalance caused by excess oxidants leads to oxidative stress, resulting in damage to DNA and protein and increased risk of degenerative diseases such as cancer (Farombi et al, 2004, Sander et al, 2004).

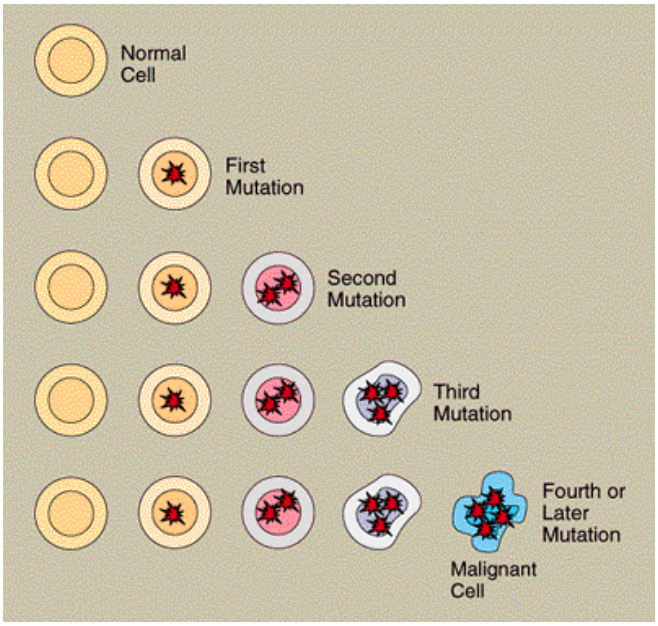


Figure 1.24. Cancer usually arises in a single cell.

The cell's progress from normal to malignant to metastatic appears to follow a series of distinct steps, each controlled by a different gene or set of genes. Persons with hereditary cancer already have the first

1.16.3. Causes of cancer

- Cancers are caused by a series of mutations. Each mutation alters the behavior of the cell somewhat.
- Carcinogenesis, which means the initiation or generation of cancer, is the process of derangement of the rate of cell division due to damage to DNA. Cancer is, ultimately, a disease of genes. In order for cells to start dividing uncontrollably, genes which regulate cell growth must be damaged. Proto-oncogenes are genes which promote cell growth and mitosis, a process of cell division, and tumor suppressor genes discourage

cell growth, or temporarily halt cell division in order to carry out DNA repair. Typically, a series of several mutations to these genes are required before a normal cell transforms into a cancer cell. (Figure 1.24)

Radiation: High levels of radiation like those from radiation therapies and x-rays (repeated exposure) can damage normal cells and increase the risk of developing leukemia, as well as cancers of the breast, thyroid, lung, stomach and other organs.

Ultraviolet (UV) Radiation: UV radiation from the sun are directly linked to melanoma and other forms of skin cancer. These harmful rays of the sun cause premature aging and damage the skin. Artificial sources of UV radiation, such as sun lamps and tanning booths, also increase the risk of skin cancer. By wearing protective clothing and sunscreens and by avoiding prolonged exposure to the sun, one may reduce the risk of skin cancer. Many of the 1.3 million skin cancers diagnosed in the year 2000 could have been prevented by protection from the sun's rays.

Viruses: Some viruses, including hepatitis B and C, human papillomaviruses (HPV), and the Epstein Barr virus, which causes infectious mononucleosis, have been associated with increased cancer risk. Immune system diseases, such as AIDS, can make one more susceptible to some cancers.

Chemicals: Long term exposure to chemicals such as pesticides, uranium, nickel, asbestos, radon and benzene can increase the risk of cancer. Such carcinogens may act alone or in combination with another carcinogen, such as cigarette smoke, to increase the risk of cancer and other lung diseases.

Tobacco: Cigarette smoking and regular exposure to tobacco smoke greatly increase lung cancer. Cigarette smokers are more likely to develop several other types of cancer like those of the mouth, larynx, esophagus, pancreas, bladder, kidney and cervix. Smoking may also increase the likelihood of developing cancers of the stomach, liver, prostate, colon and rectum. The use of other tobacco products, such as chewing tobacco, are linked to cancers of the mouth, tongue and throat. The risk of cancer decreases soon after a smoker quits, while precancerous conditions often diminish after a person stops using smokeless tobacco.

Alcohol: Heavy drinkers face an increased risk of cancers of the mouth, throat, esophagus, larynx and liver. Some studies suggest that even moderate drinking may slightly increase the risk of breast cancer. All cancers caused by cigarette smoking and heavy use of alcohol could be prevented completely. The American Cancer Society (ACS) estimated that in the year 2000 about 171,000 cancer deaths were expected to be caused by tobacco use, and about 19,000 cancer deaths were to be related to excessive alcohol use, frequently in combination with tobacco use.

Diet: High-fat, high cholesterol diets are proven risk factors for several types of cancer such as those of the colon, uterus and prostate. Obesity may be linked to breast cancer among older women as well as to cancers of the prostate, pancreas, uterus, colon and ovary. Many cancers that are related to dietary factors could be prevented. Healthy food choices and a well balanced diet including fiber, vitamins, minerals and low fat items may help to reduce cancer risk.

Others: Regular screening examinations by a health care professional can result in the detection of cancers of the breast, colon, rectum, cervix, prostate, testis, oral cavity, and skin at an earlier stage, when treatment is more likely to be successful. Self-examinations for cancers of the breast and skin may also result in detection of tumors at early stages. The screening-accessible cancers listed above account for about half of all new cancer cases.

1.16.4. Malignant tumor cells have distinct properties

- evading apoptosis
- unlimited growth potential (immortalitization) due to overabundance of telomerase
- self-sufficiency of growth factors
- insensitivity to anti-growth factors
- increased cell division rate
- altered ability to differentiate
- no ability for contact inhibition
- ability to invade neighbouring tissues
- ability to build metastases at distant sites

Tissue can be organized in a continuous spectrum from normal to cancer.

Cancer tissue has a distinctive appearance under the microscope. Among the distinguishing traits are a large number of dividing cells, variation in nuclear size and shape, variation in cell size and shape, loss of specialized cell features, loss of normal tissue organization, and a poorly defined tumor boundary. Immunohistochemistry and other molecular methods may characterise specific markers on tumor cells, which may aid in diagnosis and prognosis.

1.16.5. Steps involved in Cancer Development

Biopsy and microscopical examination can also distinguish between malignancy and hyperplasia, which refers to tissue growth based on an excessive rate of cell division, leading to a larger than usual number of cells but with a normal orderly arrangement of cells within the tissue. This process is considered reversible. Hyperplasia can be a normal tissue response to an irritating stimulus, for example callus Figure 1.25.

Dysplasia is an abnormal type of excessive cell proliferation characterized by loss of normal tissue arrangement and cell structure. Often such cells revert to normal behavior, but occasionally, they gradually become malignant. The most severe cases of dysplasia are referred to as "carcinoma in situ." In Latin, the term "in situ" means "in place", so carcinoma in situ refers to an uncontrolled growth of cells that remains in the original location and shows no propensity to invade other tissues. Nevertheless, carcinoma in situ may develop into an invasive malignancy and is usually removed surgically, if possible.

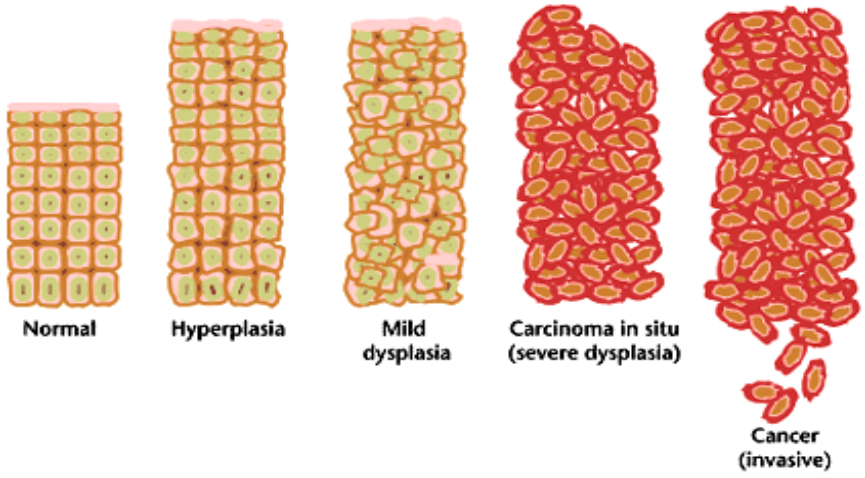


Figure 1.25: Represents the scheme of cancer which depicts morphological changes encounter during development.

Metastasis

Metastasis is a complex series of steps in which cancer cells leave the original tumor site and migrate to other parts of the body via the bloodstream or the lymphatic system (Figure 1.26 & 1.27). To do so, malignant cells break away from the primary tumor and attach to and degrade proteins that make up the surrounding extracellular matrix (ECM), which separates the tumor from adjoining tissue. By degrading these proteins, cancer cells are able to breach the ECM and escape. When oral cancers metastasize, they commonly travel through the lymph system to the lymph nodes in the neck. The body resists metastasis by a variety of mechanisms through the actions of a class of proteins known as metastasis suppressors, of which about a dozen are known. (Yoshida et al, 2000).

Cancer researchers studying the conditions necessary for cancer metastasis have discovered that one of the critical events required is the growth of a new network of blood vessels, called tumor angiogenesis (Weidner et al, 1991). It has been found that angiogenesis inhibitors would therefore prevent the growth of metastases

Invasive cancer cells migrate from the primary tumor site into the surrounding tissue towards blood vessels. These cells then penetrate the vessel wall (intravasate), gaining access to the vessel lumen. Cells are then carried to distant organs by normal blood flow, where they lodge in small capillaries of various organs. These cells can then extravasate through the vessel wall into the surrounding tissue, where they form secondary tumors. This process is the major cause of death in cancer patients and there are no therapeutic agents to prevent the spread of cancer.

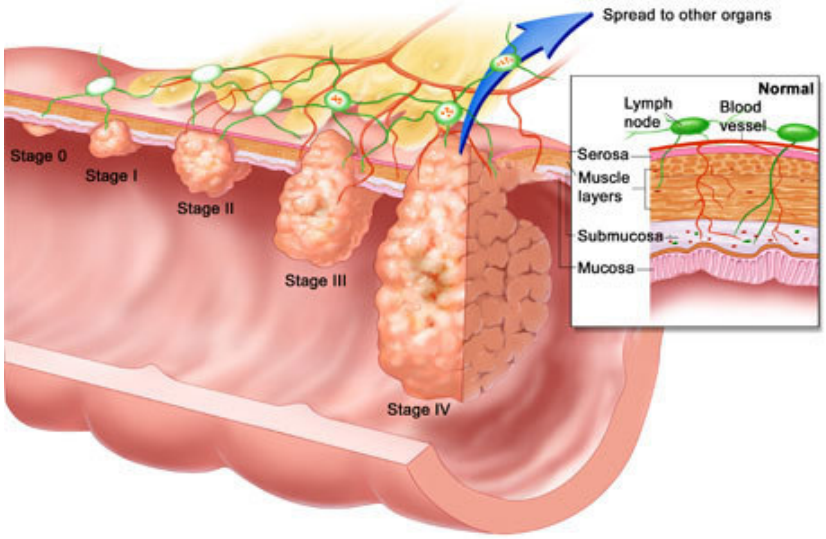


Figure 1.26. Represents the scheme of cancer which depicts morphological changes encounter during development.

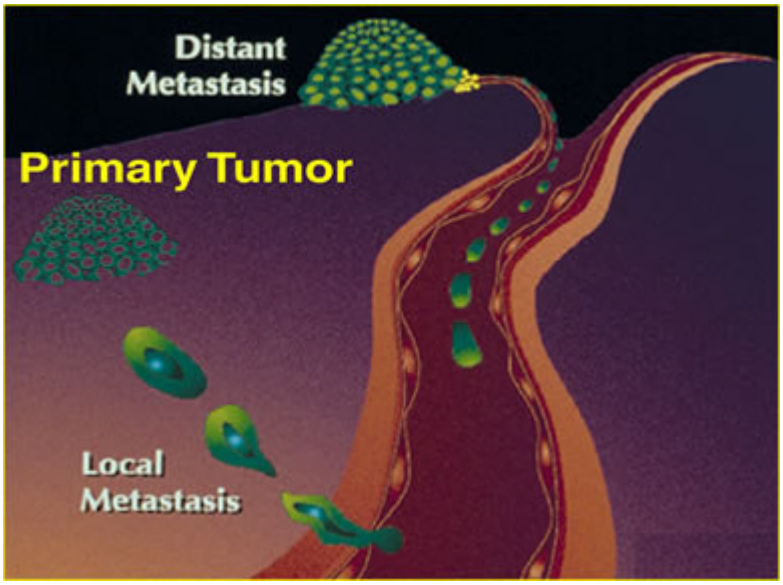


Figure 1.27. Schematic showing tumor cell metastasis.

1.17. Need for the current study

Based on the literature cited, it is clear that ulcer incidences are increasing, ulcer therapeutics are not satisfactory since they pose side effects and cannot be prescribed in a pregnancy condition, alcoholic condition although those are the factors that makes human body susceptible for oxidative damage and ulcer induction. There are certain leads in the potential alternatives from plants and dietary sources, but precise components responsible for potential ulcer prevention and their mechanism of action was not clearly understood. In the current thesis proposal therefore particular attention has been paid to isolate, characterize and understand the role of antioxidant and polysaccharide fractions from selected dietary sources-ginger and mango ginger both belonging to the family Zingiberaceae. Attributes of selected antiulcer components towards anticancer property has also been investigated.

GINGER & MANGO GINGER

	Ginger	**Mango ginger**
Kingdom	Plantae	Plantae
Division	Magnoliophyta	Magnoliophyta
Class	Liliopsida	Liliopsida
Order	Zingiberales	Zingiberales
Family	Zingeberaceae	Zingeberaceae
Genus	Zingiber	Curcuma
Specis	*Zingiber officinale*	*Curcuma amada*

1.18. Ginger

In recent times there has been scientific research to test the validity of the medicinal claims made about ginger. Some exciting results with respect to the medicinal properties of ginger including, anti-emetic effect or control of nausea and vomiting (Ernst & Pittler, 2000), prevention of coronary artery disease (Bordia et al, 1997), healing and prevention of arthritic conditions and stomach ulcers (Marcus & Suarez, 2001) are reported. In addition, ginger has

been shown to be effective against tumor growth (Surh et al 1999), rheumatism (Srivastava & Mustafa, 1992) Ginger has also been known for its activity as an antioxidant (Kikuzaki & Nakatani, 1993; Kikuzaki et al, 1994; Lee & Ahn, 1985) in the body. Understanding of the bioactive compounds involved in the health beneficial properties displays varieties of biomolecules in ginger.

Ginger has been categorized under both medicinal plants and dietary sources (nutraceuticals). Phenolic compounds are being highlighted (Siddaraju & Shylaja, 2007; Stoilovaa et al, 2007) to exert varieties of biological actions such as free radical (FR) scavenging, metal chelation, modulation of enzymatic activity etc. (Siddaraju & Dharmesh, 2007a & 2007b, Koshihara et al, 1984). As a result of the putative role, they are receiving particular attention in the prevention of several human diseases (Table 1.3, Figure 1.28). Phenolic acids and their derivatives are widely spread in plants (Deshpande et al., 1984) and a number of phenolic acids are linked to various cell wall components such as arabinoxylans, proteins (Harris & Hartley, 1976) and pectic polysaccharides (Bunzel, et al., 2004; Srikanta et al., 2007). Free and bound phenolics are known to play a crucial role in the defense mechanism, offering protection against oxidative stress (OS) caused by both biotic and abiotic factors (Siddaraju & Dharmesh, 2007a & 2007b; Srikanta et al., 2007; Nara et al., 2006). Phenolics from grape seed (Saito et al., 1998) cacao liquor (Galati et al., 2003) and prickly pear (Osakabe et al., 1998) have been shown to possess antiulcer activity. The antioxidant activity of phenolics may be important in contributing to gastroprotective, anticancer and other activities since Reactive Oxygen Species (ROS) are related to the occurrence of chronic diseases including ulcers and cancers.

1.18.1. Historical Background

Ginger is a member of the Zingiberaceae family and it is originated from South-East Asia. It grows best in tropical areas that have high rainfall and hot & humid conditions. Dutch, Portuguese, Arab and Spanish explorers introduced Ginger to Europe or traders from about the 13th to the 16th century. The name 'ginger' is derived from the sanskrit word *'Srngaveram'* which means 'horn root'. In South East Asia the most popular form of ginger is raw ginger. It is grated or finely chopped then added to the meal not long before serving. Ginger adds to the flavor of a meal creating a fresh, spicy pungent taste that is now becoming a valued commodity all over the world.

Ginger grows best in tropical and sub tropical areas, which have good rainfall with hot and humid conditions during the summer season. Belief in the medicinal properties of ginger existed in ancient Indian and oriental cultures where ginger was used alone or as a component in herbal remedies. This practice continues today in many areas of the world, including India, Africa, Brazil, China, Fiji, Indonesia, Mexico, Peru, Sudan and Thailand.

The rhizome or "root" is the part of the plant that is harvested and is found entirely under the surface of the soil. The vast majority of the harvested ginger is consumed fresh or in dehydrated form, while some commercial ginger is sugar preserved.

1.18.2. Ginger in traditional medicine

Ginger has been used as one of the important components in Ayurveda Medicine. Ayurveda is the ancient (before 2500 b.c.) Indian system of health care and longevity. It involves a holistic view of man, his health, and illness. Ayurvedic treatment of a disease consists of salubrious (Healthy/hygienic) use of drugs, diets, and certain practices. Medicinal preparations are invariably complex mixtures, based mostly on plant products. Around 1,250 plants are currently used in various Ayurvedic preparations. Many Indian medicinal plants have come under scientific scrutiny since the middle of the nineteenth century, although in a sporadic fashion. The first significant contribution from Ayurvedic materia medica came with the isolation of the hypertensive alkaloid from the sarpagandha plant (*Raulfia serpentina*), valued in Ayurveda for the treatment of hypertension, insomnia, and insanity; later expanded with many medicinal plants including ginger.

1.18.3. Ginger as a medicinal spice

It is used since time immemorial as a food spice in daily life in Ayurveda. Besides, ginger is also considered as one of the important dietary sources - nutraceuticals particularly consumed as spice for medicinal value (Shukla & Singh, 2007). This spice is used for the treatment of nausea associated with chemotherapy and of ulcers. The active component, gingerol, has been shown to exhibit chemopreventive potential. Ginger suppresses prostaglandin synthesis through inhibition of cyclooxygenase-1 and cyclooxygenase-2. Ginger also suppresses leukotriene biosynthesis by inhibiting 5-lipoxygenase. This pharmacological property distinguishes ginger from nonsteroidal anti-inflammatory drugs. This discovery preceded the

observation that dual inhibitors of cyclooxygenase and 5-lipoxygenase may have a better therapeutic profile and have fewer side effects than non-steroidal anti-inflammatory drugs (Grzanna et al., 2005).

Almost 25 centuries ago, Hippocrates remarked, "Let food be thy medicine and medicine be thy food." This differs little later as - "You are what you eat." Vasco da Gama, a Portuguese sailor, left for India almost 500 years ago in search of spices, and the route he used is called "the spice route." Why were spices so precious that he was willing to make this arduous journey? People of Vscoda Gama's time revered spices not just for their brilliant colors and taste but also for their medicinal value. The true medicinal value of spices, however, is only now beginning to be unveiled.

1.18.4. Chemical composition of ginger

The powdered rhizome was found to contain 3-6% fatty oil, 8-9% protein, 60-70% carbohydrates, 3-8% crude fiber, about 7-8% ash, 9-12% water and 2-3% volatile oil. However, only volatile oils such as linalool, zingiberol, zingiberine and oleoresins-gingerol, shogaol have been reported to possess antioxidant activity (Masuda et al., 2004). Various other chemical components are also reported.

1.18.5. Ginger and its bioactivity

For over 5000 years ginger has been recognized as the “universal medicine” by the ancient Orientals of China and India (http://www.buderimginger.com). Today ginger remains a component of more than 50% of the traditional herbal remedies and has been used to treat nausea, indigestion, fever and infection and to promote vitality and longevity (http://www.buderimginger.com). Ginger is also effective in the treatment of many other illnesses and discomforts as depicted in Figure 1.28.

Table 1.3. Bioactive compounds from ginger

SL NO	Constituents	Activity	References
1	6-shogaol,	Gastrostimulant	Yamahara et al., 1990.
2	1,8-cineole;6 gingerol ; 6-shogaol benzaldehyde; borneol ; gingerol ; myrcene ; p-cymene ; quercetin ;	12-Lipoxygenase-inhibitor Cyclooxygenase inhibitor Analgesic	Isa et al., 2008.
3	10-dehydrogingerdione; 10-gingerdione ;6-dehydrogingerdione ; 6-gingerdione; alpha-curcumene ;alpha-linolenic-acid ; alpha-pinene ; beta-pinene ; borneol ; caryophyllene; chlorogenic-acid ; salicylates ;shogaol; vanillic-acid ; zingerone ; curcumin ; p-cymene	Antiinflammatory (Antiedemic) Antirheumatalgic	Grzanna et al., 2005. Chrubasik et al., 2007. Ynn et al., 1986.
4	6-shogaol ; 1,8-cineole ; benzaldehyde ; borneol ;bornyl-acetate; capsaicin ; caryophyllene geraniol; kaempferol ; limonene ; linalool ; myrcene ; p-coumaric-acid ; quercetin	Spasmolytic	Ghayur and Gilani, 2006.
5	6-gingerol ; 6-shogaol ; 8-gingerol 10-dehydrogingerdione ; 10-gingerdione; 6- ferulic-acid ; gingerol ; kaempferol ; myricetin ; quercetin ; salicylates; shogaol ; vanillic-acid ; zingerone 1,8-cineole ; 6-gingerol ; 6-shogaol ; alpha-pinene ; alpha-terpineol ; shogaol Alpha-pinene, zingerone;Curcumin ; Eugenol ; gingerol, p-cymene	Sedative Spasmolytic Vasodilator Antiemetic.	Lim et al., 2005. Ghayur et al., 2008. Nanthakomon and Pongrojpaw 2006.

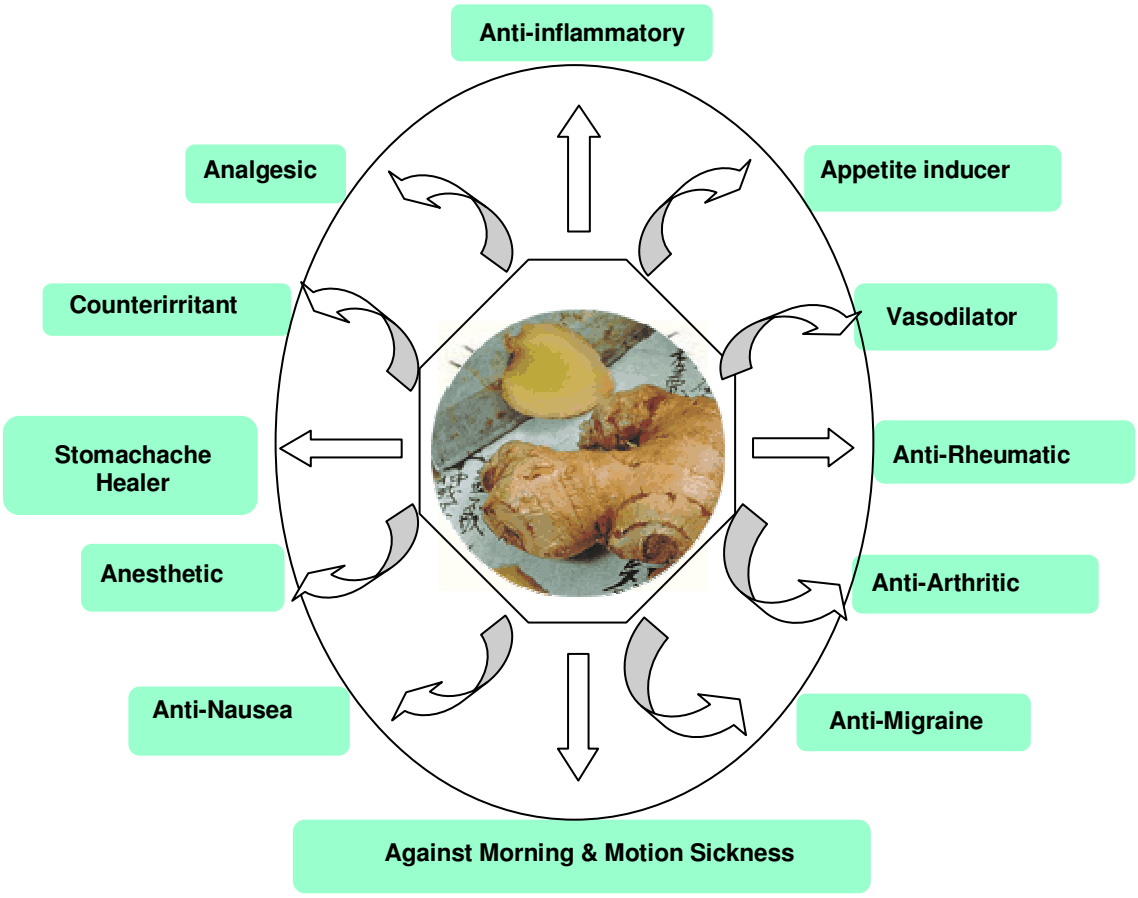


Figure 1.28. Bioactivities of ginger

1.19. Mango ginger

Curcuma amada belonging to the family of *Zingiberaceae,* popularly known as mango ginger has been known for its potent antioxidant activity also. Mango ginger cultivated mainly in the Indo-Malaysian regions is well known for culinary preparation like pickles due to its exotic mango aroma. Mango flavor is mainly attributed to carene and cis-ocimene among the 68 volatile aroma components present in the essential oil of mango ginger rhizome (Achut & Bandyopadhyaya 1984, Srinivas et al., 1989) (Figure 1.29).

A B C D

Fig 2.29. Mango ginger – various parts known to possess health beneficial its powder is used in Ayurveda and traditional medicine.

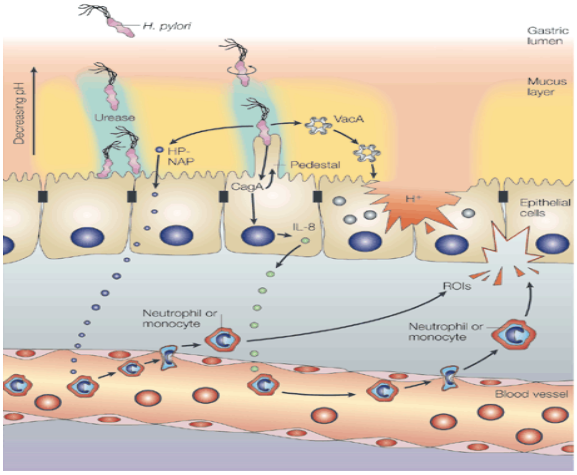
A – Leaf; B – Inflorescence; C & D – Rhizome.

Mango ginger has been used as stomachic, carminative, alexiteric, antipyretic, diuretic, emollient, expectorant, appetizer, aphrodisiac and laxative (Hussain et al., 1992). Antioxidant and antibacterial activity of mango ginger has been also reported recently (Policegoudra et al,. 2007). In traditional and ayurvedic medicine system, mango ginger was found to be a classic herb for digestive system because of its stomachic and carminative properties, which in turn help to prevent the major gastric problems like hyperacidity, gastritis, and ulcer.

A number of chemicals have been identified as major components of Curcuma amada. Among these are ocimene, dihydro-ocimene, α-pinene, α-curcumene, β-curcumene, linalool, cuminyl alcohol, keto-alcohol, camphor, turmerone, linalyl acetate, safrole, curcumin, myristic acid, car-3-ene, myrcene, 1,8-cineol, limonene, perillene, etc.

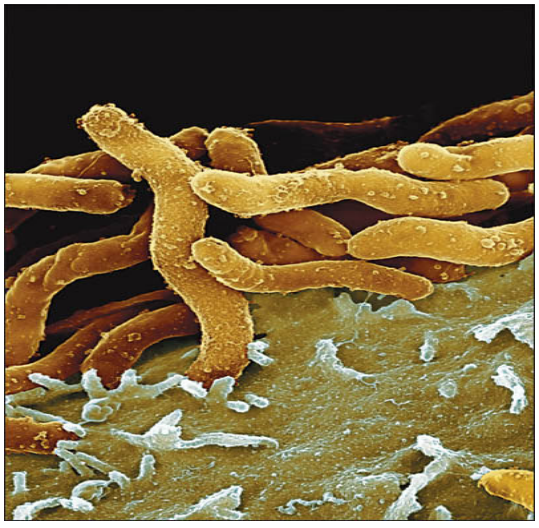
Chapter 1

General Introduction

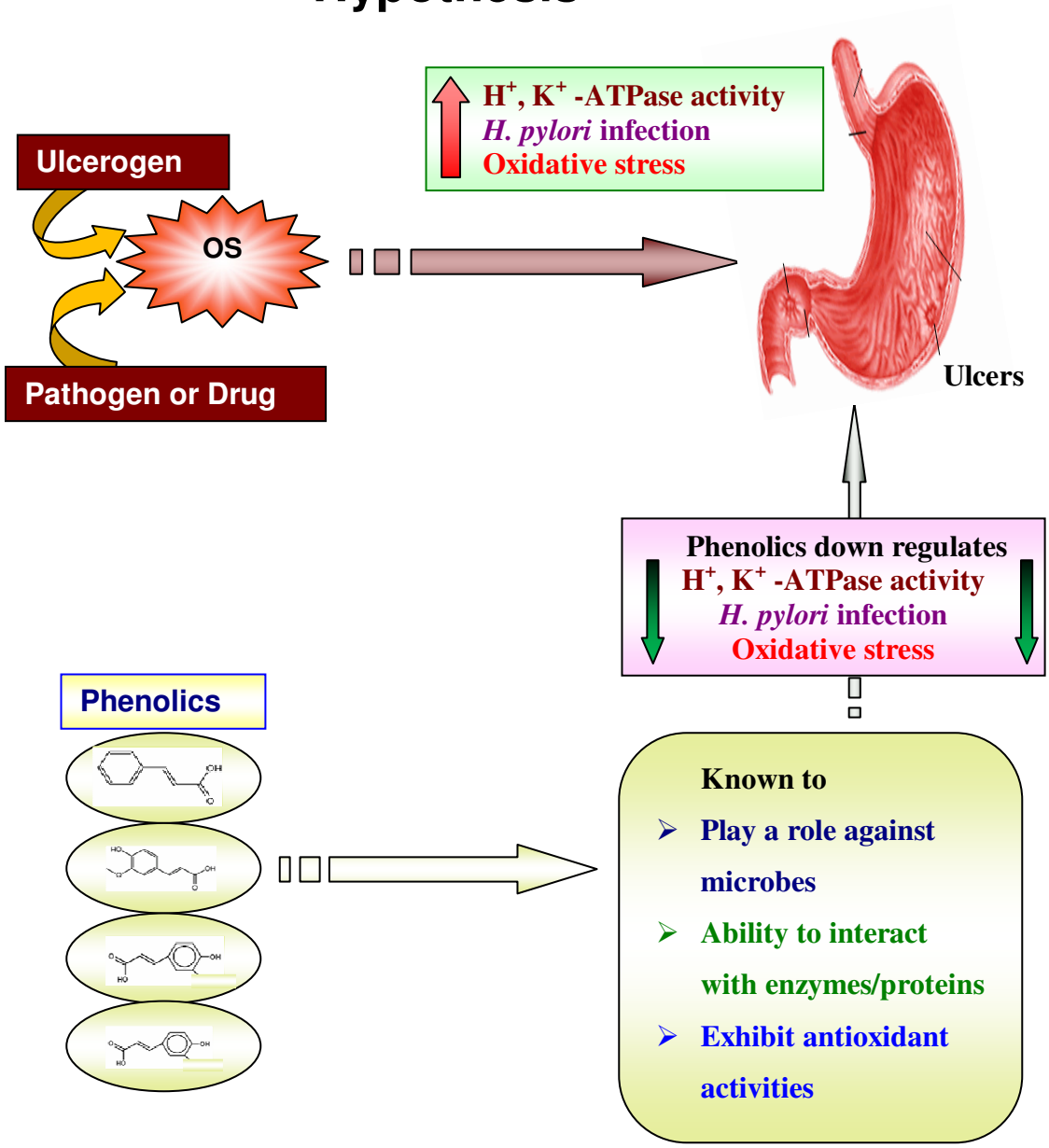


Chapter 2

Anti ulcerative Action of Antioxidant Fractions of Ginger (*Zingiber officinale*) and Mango ginger (*Curcuma amada*)



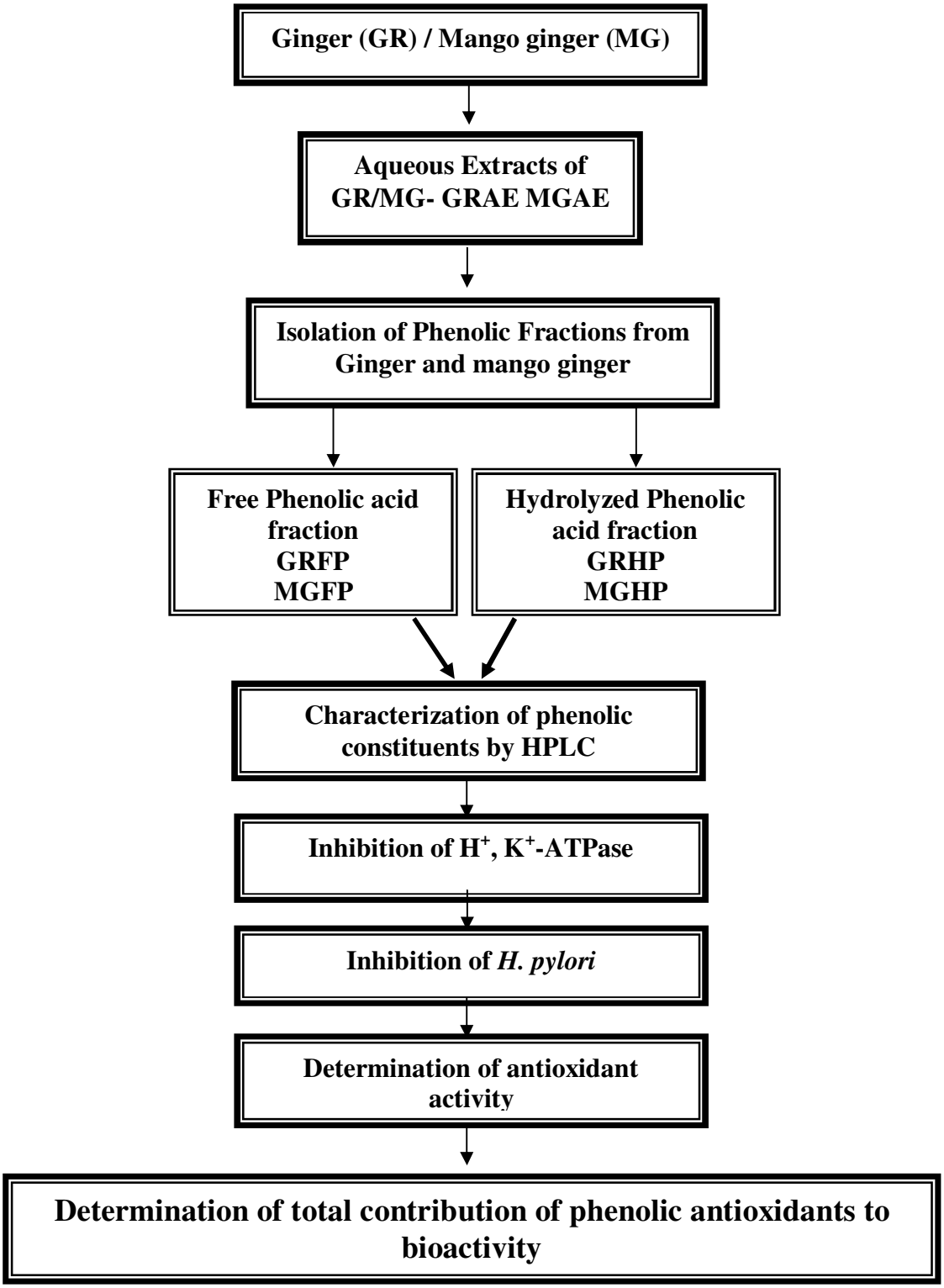
Hypothesis



Ulcerogens generate oxidative stress (OS) leading to susceptibility for ulcer formation by activating H^+, K^+ -ATPase, enabling *H. pylori* colonization and invasion, mucosal damage etc. question addressing is can phenolics of ginger and mango ginger downregulates these events? That can prevent ulcer formation.

Can antioxidant phenolics of Ginger and Mango ginger offer protection against gastric ulcer *in vitro* ?

Work plan


Ginger (GR) / Mango ginger (MG)
Aqueous Extracts of GR/MG- GRAE MGAE
Isolation of Phenolic Fractions from Ginger and mango ginger
Free Phenolic acid fraction GRFP MGFP
Hydrolyzed Phenolic acid fraction GRHP MGHP
Characterization of phenolic constituents by HPLC
Inhibition of H+, K+-ATPase
Inhibition of H. pylori
Determination of antioxidant activity
Determination of total contribution of phenolic antioxidants to bioactivity

2.1. Introduction

Gastric hyperacidity and ulcer are major recurrent diseases of gastro intestinal tract and sufferers of human population of all geographical regions. It is an imbalance between damaging factors with in the lumen and protective mechanism with in the gastro duodenal system. Hyperacidity is due to excess secretion of HCl from the mucosa, which is due to hyper activity of proton pumping by H^+, K^+-ATPase of parietal cells (Kishor et al., 2007).

Gastro duodenal ulcers on the other hand are caused by the loss of gastro protection by various factors like stress related gastric mucosal damage (Mitchell 2004), non-steroidal anti-inflammatory drugs induced gastric lesions (Ivey 1998) and *H. pylori* mediated ulcer lesions (Konturek & Konturek, 1994). Other than the damaging role of acid and mucosal damage, Reactive Oxygen Species (ROS) especially the hydroxyl radical [OH*] plays a major role in causing oxidative damage to mucosa which in turn leads to ulcer and offers susceptibility for the entry of *H. pylori* that aggravate the gastric conditions. Indeed, in the stomach there is a massive production of ROS and their concentration being 1000-fold higher than in other tissues or plasma, most susceptible for damage. ROS generates Oxidative Stress (OS) due to imbalance between ROS accumulation and defense mechanism in the body leading to diseases such as cancer, inflammation, ulcer and brain dysfunction (Middleton et al., 2000 & Aruoma 1998).

Dietary antioxidants play an important role against this OS and hence have been known to prevent many degenerative diseases (Gerber et al., 2002). Diets rich in phenolic acids particularly free and bound phenolics constituting different phenolic acids have been shown to possess antiulcer activity (Saito et al., 1998 & Galati et al., 2003). Phenolic acids such as caffeic, ferulic, cinnamic and protocatechuic acids have recently been shown from our laboratory to exert antioxidant and anti-microbial activity (Siddaraju & Shylaja 2007a). 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 for ulcer (Das et al., 1997).

The dietary antioxidants with the potent ability to scavenge oxygen and nitrogen free radicals, breaking lipid chain peroxidation reactions, therefore may behave as gastro protective factors. Phenolic compounds are one of the major classes of dietary antioxidants, where apart from

their action as radical scavengers, exhibit several indirect effects such as inhibition of lipoxygenase (Laughton et al., 1991), reduction of platelet aggregation, inflammation (Ferro-Luzzi & Ghiselli, 1993) and hence may potentially reduce the entry of human pathogens to guts.

Dietary plant phenolic compounds have been described to exert varieties of biological actions such as free radical scavenging, metal chelation, modulation of enzymatic activity *etc*, (Editorial 1994, Koshihara et al., 1984, Reddy et al., 1994). They receive particular attention due to their putative role in prevention of several human diseases. Phenolic acids and their derivatives are widely spread in plants (Deshpande 1984) and a number of phenolic acids are linked to various cell wall components such as arabinoxylans, proteins (Harris & Hartley 1976, Hartley 1990) and pectic polysaccharides (Bunnzel et al., 2004). Free and bound phenolics known to play a crucial role in defence mechanism, offering protection against OS caused by both biotic and abiotic factors. Phenolics from grape seed (Saito et al., 1998) cacao liquor (Galati et al., 2003) and prickly pear (Osakabe et al., 1998) have been shown to possess antiulcer activity. The antioxidant activity of phenolics may be an important contributing antiulcer factor since Free Radicals (FR) / Reactive Oxygen Species (ROS) are related to the occurrence of ulcers (Das et al., 1997).

Ginger (*Zingiber officinale*) belonging to the family Zingiberaceae is cultivated in various parts of the world especially in India, China and Mexico. It is used since time immemorial as a dietary component in daily life as a spicy material. Ayurvedic, Chinese and traditional medicine systems have recorded ginger as an important medicinal plant (Figure 2.1). Ginger has been reported to exert antioxidant, antiulcer, (Hirahara 1974, Lee et al., 1985) antiemetic (Sharma 1997), anti inflammatory, anti tumour (Katiyar et al., 1996), carminative, diaphrodic, digestive, expectorant, as well as gastro protective (Al-Yahya et al., 1989) activities. The powdered rhizome contains 3-6% fatty oil, 8-9% protein, 60-70% carbohydrates, 3-8% crude fibre, about 7-8% ash, 9-12% water and 2-3% volatile oil (Govindarajan 1982, Mustafa 1993). However only volatile oils such as linalool, zingiberol, zingiberene and oleoresins - gingerol, shogaol have been reported to possess antioxidant activity (Masuda et al., 2004, Yamahara et al., 1998) and very little information is available on the phenolics of ginger and their potential contribution to antiulcer activity. It is possible that some phenolics may be included in volatile oils and in turn may contribute to total antioxidant activity also.

Fig 2.1. Ginger rhizome – its powder is used in Ayurveda and traditional medicine

Mango ginger (*Curcuma amada)* belonging to the family of *Zingiberaceae,* has been known for its potent antioxidant activity also. Mango ginger cultivated mainly in the Indo-Malaysian regions is well known for culinary preparation like pickles due to its exotic mango aroma (Figure 2.2). Mango flavor is mainly attributed to carene and cis-ocimene among the 68 volatile aroma components present in the essential oil of mango ginger rhizome (Achut & Bandyopadhyaya, 1984, Srinivas et al., 1989).

Fig 2.2. Mango ginger – various parts known to possess health beneficial, its powder is used in Ayurveda and traditional medicine. A – Leaf; B – Inflorescence; C & D – Rhizome.

Mango ginger has been used as stomachic, carminative, alexiteric, antipyretic, diuretic, emollient, expectorant, appetizer, aphrodisiac and laxative (Hussain et al., 1992). Antioxidant and antibacterial activity of mango ginger has been also reported recently (Policegoudra et al., 2007). In traditional and ayurvedic medicine system, mango ginger was found to be a classic herb for digestive system because of its stomachic and carminative properties, which in turn help to prevent the major gastric problems like hyperacidity, gastritis, and ulcer.

The modern approach of the therapy for ulcer disease therefore includes the proton pump blockers for inhibition of gastric acid over secretion by H^+, K^+-ATPase, scavenging the free radicals and the eradication of *H. pylori* from dietary sources. Considering the side effects and disadvantages of available antiulcer drugs (Breenton 1991), the current study addressed the isolation of antioxidant fraction - free and hydrolyzed phenolics from ginger and mango ginger and evaluated their potential antiulcer effect by examining proton potassium ATPase blockade, ability to inhibit *H. pylori* growth and antioxidant properties *in vitro*.

To evaluate on these aspects, initially total phenolics and antioxidant activity were determined in aqueous extracts of ginger (GRAE & GRME) and mango ginger (MGAE & MGME). Since traditional knowledge indicate the effective use of aqueous extracts of ginger and mango ginger, in the current chapter emphasis has been put towards understanding the constituents present in aqueous extracts and their potential antiulcer activity *in vitro.*

Objectives

- **Isolation and characterization of free and hydrolyzed phenolics of ginger (GRFP & GRHP) and mango ginger (MGFP & MGHP).**
- **Determination of potential antiulcer properties *in vitro* – inhibition of H^+, K^+ -ATPase; inhibition of *H. pylori* growth and antioxidant properties.**

2.2. Materials and Methods

2.2.1. Chemicals

Adenosine triphosphate (ATP), Agarose, calf thymus DNA, omeprazole, lansoprazole, phenolic acid standards such as gallic, tannic, caffeic, p-coumaric, ferulic, gentisic, protocatechuic, syringic and vanillic acids, 1,1-Diphenyl-2-picryl hydrazyl (DPPH) and 2-thiobarbituric acid (TBA) were obtained from Sigma (St. Louis, MO). The other chemicals such as Folin-Ciocalteu reagent, ferric chloride, trichloroacetic acid, sodium carbonate, ferrous sulfate, ascorbic acid were of the highest quality purchased from Qualigens fine chemicals (Mumbai, India). HPLC column (Shimpak C_{18}) was obtained from Shimadzu Corp, Kyoto, Japan and HPLC grade solvents employed for HPLC analysis were purchased from Spectrochem biochemicals pvt. Limited (Mumbai, India).

2.2.2. Plant material

Ginger (*Zingiber officinale*)-GR and mango ginger (*Curcuma amada*)-MG rhizome were purchased from three different vendors (n = 3) from a local market (Devaraja market, Mysore, Karnataka, India), cleaned, washed under running tap water, cut into small pieces; air dried, powdered in a mixer (Gopi, C. Lal Electrical and Mechanicals Co. Ambala, India) for particle size of 20 mesh, and preserved in dry condition at 4 °C until further extraction.

2.2.3. Preparation of aqueous extract

Ten gram of defatted (refluxed with hexane) powdered sample was mixed with 100 mL of distilled water and boiled for 5 min, cooled and centrifuged at 5000 g for 10 min. The clear supernatant was separated, stored at 4 ºC till the completion of the experiment and referred as aqueous extract. Complete extraction was ensured by reextracting the residue 3 times and concentrated the sample by lyophilization.

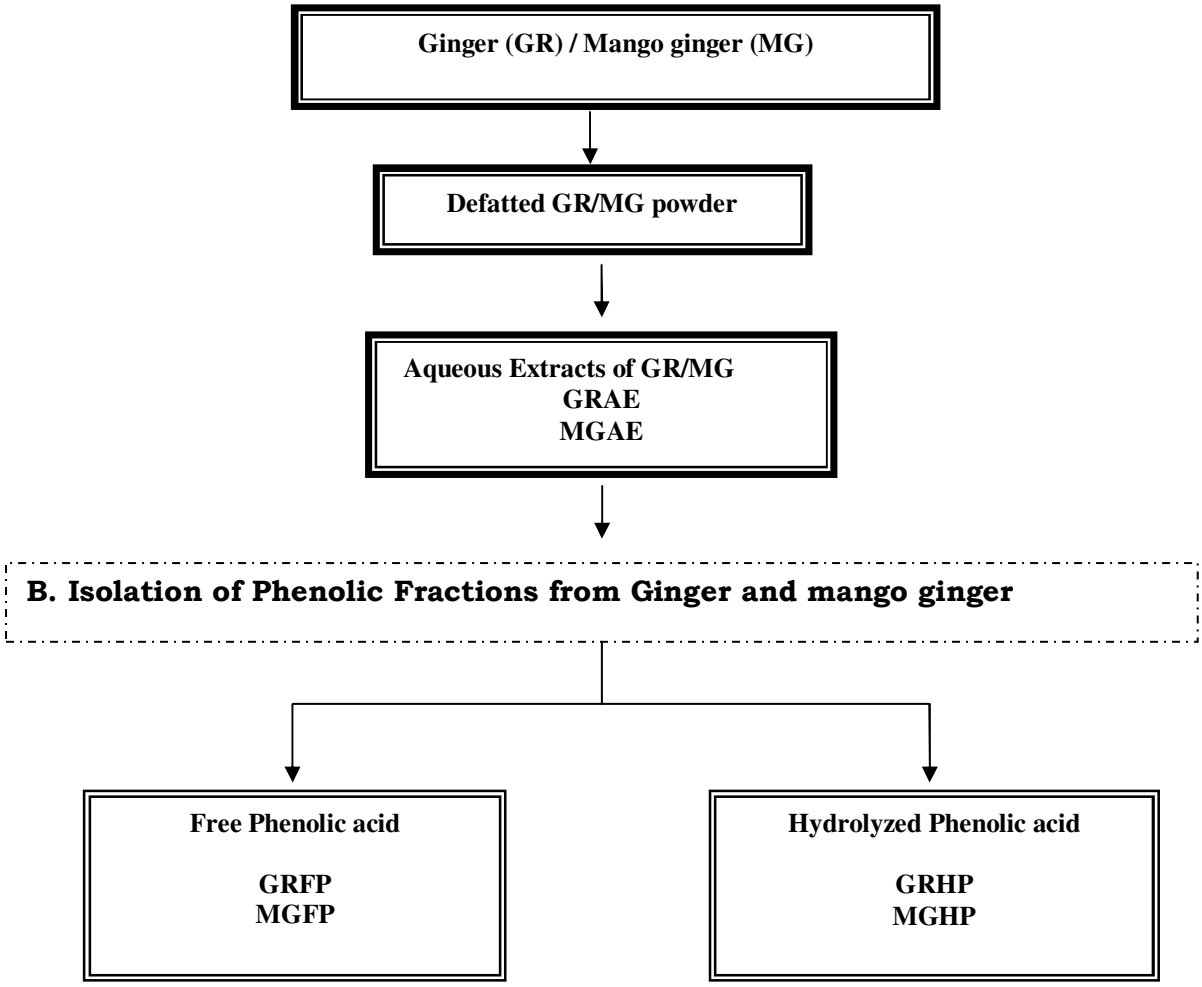
Preference has been given to aqueous extract since solvent soluble components have been reported earlier. Also in Ayurveda and traditional medicine use of water decoction is popular.

2.2.4. Extraction of free and bound/hydrolyzed phenolic acids of ginger and mango ginger.

Since phenolics were hydrolyzed from bound phenolic fraction they are termed as hydrolyzed phenolics. Free and hydrolyzed phenolics were extracted as per the scheme -2.1. Free phenolics were extracted according to the method of Ayumi *et al (*1999). Briefly, 2 g of GR/MG powder was extracted with 4X50 mL each of 70% ethanol at room temperature (25 ± 2 °C) for 2 h with constant stirring. The mixtures were then centrifuged at 3000 g for 10 min (Sigma 3-16K, USA) at room temperature and supernatants were collected and combined. The solvent was evaporated at 30 °C under vacuum in a flash evaporator (Buchi 011, Switzerland) to approximately 40 mL. Concentrated supernatant was acidified to pH 2 with 4 N hydrochloric acid. Phenolic acids were separated by ethyl acetate phase separation (5X50 mL) and the pooled fractions were treated with anhydrous disodium sulphate to remove moisture, filtered and evaporated to dryness and taken in 2 mL of methanol (w/v), designated as ginger/mango ginger free phenolic fraction (GRFP/MGFP).

Hydrolyzed phenolics were extracted according to the method of Nordkvist *et al* (1984). 2 g of ginger/mango ginger powder was extracted with 4X50 mL of 70% ethanol, followed by 4X50 mL of hexane to remove free phenolics and fat respectively. The dried samples were extracted with 2X100 mL of 1 M sodium hydroxide containing 0.5% sodium borohydride under nitrogen atmosphere for 2 h and the clear supernatant was collected followed by centrifugation at 3000 g for 10 min. The combined supernatants were acidified with 4 N HCl to pH 1.5 and phenolic acids were processed as mentioned in the case of free phenolic acid and it was designated as ginger/mango ginger hydrolyzed phenolic fraction (GRHP/MGHP). Extraction was performed in triplicates to evaluate the yield with statistical significance.

Free and hydrolyzed phenolic acids were dissolved separately in 2 mL of methanol and stored at –20 °C until used within 1 week.



Scheme 2.1 : Extraction of free and hydrolyzed phenolic acids of ginger and mango ginger

2.2.5. Determination of total phenol content

The Folin-Ciocalteu reagent assay was used to determine the total phenolic content (Singleton & Rossi, 1965). A sample aliquot of 100 µL was added to 900 µL of water, 1 mL of Folin-Ciocalteu reagent previously diluted with distilled water (1:2 v/v) and 2 mL of 10% sodium carbonate solution in distilled water, mixed in a cyclo mixer. The absorbance was measured at 765 nm with a Shimadzu UV-Visible spectrophotometer (Shimadzu UV-160 spectrophotometer, Kyoto, Japan) after incubation for 2 h at room temperature. Gallic acid was used as standard for the calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram *per* gram sample. The assay was performed in triplicates.

2.2.6. Identification of phenolic acids by HPLC

The potential active components in sample (GR & MG) extracts were characterized by HPLC (model LC-10A. Shimadzu Corporation, Tokyo, Japan) analysis on a reverse phase Shimpak C_{18} column (4.6 × 250 mm) using a diode array UV-detector (operating at 280 nm). A solvent system consisting of water : acetic acid : methanol (isocratic; 80 : 5 : 15, v/v/v) was used as mobile phase at a flow rate of 1 mL/min (Siddaraju & Shylaja, 2007a). Standard phenolic acids such as tannic, caffeic, *p*-coumaric, *p*-hydroxybenzoic, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids were used for identification of phenolic components present in the sample extract.

2.2.7. Determination of antiulcer activity *in vitro*

2.2.7.1. Determination H^+, K^+-ATPase inhibition (PPI)

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.

Fresh sheep stomach was obtained from local slaughterhouse at Mysore, India. The mucosa of gastric fundus was cut off and the inner layer was scraped for parietal cells (Siddaraju & Shylaja, 2007a), homogenized in 16 mM Tris buffer (pH 7.4) containing 10% 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.

The enzyme extract (350 µg/mL) was incubated with different fractions of ginger and mango ginger phenolics in a reaction mixture containing 16 mM Tris buffer (pH 6.5) and the reaction was initiated by adding substrate 2 mM ATP, in addition to 2 mM $MgCl_2$ and 10 mM KCl. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of assay 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. Results were compared with known antiulcer proton potassium ATPase inhibitor drug Lansoprazole and with standard phenolic acids, since the active fraction of ginger and mango ginger contained phenolic acids.

2.2.7.2. Anti *Helicobacter pylori* activity

Research studies have shown that most ulcers are caused by an infection by a bacteria called *Helicobacter pylori*, a rod shaped pathogenic bacteria, normally existing in human stomach (Kurata & Nogawa 1997).

H. pylori was obtained by endoscopic samples of ulcer patients from KCDC (Karnataka Cardio Diagnostic Centre, Mysore, India) and cultured on Ham's F-12 nutrient agar medium with 5% FBS at 37°C for 2-3 days in a microaerophelic condition (Traci et al 2001). *H. pylori* culture was characterised by specific tests such as urease, catalase, oxidase, gram staining, colony characteristics and morphological appearance under scanning electron microscope and also confirmed by growth of culture in presence of susceptible and resistant antibiotics.

2.2.7.3. Agar diffusion assay

H. pylori activity was tested by the standard agar diffusion method (Iris et al 2005). Briefly the petri plates were prepared with Ham's F-12 nutrient agar media containing 5% FBS inoculated with 100 µL of *H. pylori* culture (10^5 cells/mL). Sterile discs of high-grade cellulose of diameter 5.5 mm were impregnated with 20 µL of known extract at 5, 10 and 15 µg/disc and placed on the inoculated petri plates. Amoxicillin was used as positive reference standard and 0.9% saline as negative control. For comparative evaluation discs containing 10 µg of amoxicillin was performed in addition to the control. *H. pylori* growth inhibition was determined as the diameter of the inhibition zones around the discs. The growth inhibition diameter was an average of four measurements taken at four different directions and all tests were performed in triplicates.

2.2.7.4. Minimal inhibitory concentration (MIC)

MIC values were determined by conventional broth dilution method (Iris et al 2005). Serial dilutions (final volume of 1 mL) of test samples (0.05-50 µg/mL) were performed with 0.9% saline. Following this 9 mL of Ham's F-12 nutrient medium with 5% FBS was added. Broths were inoculated with 100 µL of *H. pylori* suspension ($5X10^4$ CFU) and incubated for 24 h at 37°C. Amoxicillin was used as a positive control since *H. pylori* is susceptible to amoxicillin and 0.9% saline as negative control. After 24 h *H. pylori* growth was assayed by measuring absorbance at 625 nm. MIC was defined as the lowest concentration in µg of GAE to restrict the growth to < 0.05 absorbance at 625 nm (No macroscopic visible growth).

2.2.8. Measurement of antioxidant activity

2.2.8.1. Scavenging effect of extracts on DPPH radical

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 (GR & MG) extract and standard antioxidants of various concentrations were mixed with 100 mM tris-HCl buffer (800 µL, pH 7.4) and then added to 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.

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

2.2.8.2. Measurement of reducing power

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-quanitative determination of dilute concentrations of antioxidants, which participate in the redox reaction.

The reducing power of samples and standard antioxidants were determined according to the method of Yen and Chen (1995). The sample (GR & MG) extracts and standard antioxidants of various concentrations were mixed with equal volume of 0.2 M phosphate buffer, pH 6.6 and 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An equal volume of 10 % trichloroacetic acid was added to the mixture and centrifuged at 1000 g for 10 min. The supernatant was mixed with distilled water and 0.1 % $FeCl_3$ at a ratio of 1: 1: 2 (v/v/v) and the absorbance were measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.2.8.3. Inhibition of lipid peroxidation of rat liver homogenate

Lipid peroxidation generates a number of degradation products, such as malondialdehyde (MDA), hexanol *etc.* and is found to be an important cause of cell membrane destruction and cell damage (Yoshikawa et al 1991). MDA is a highly reactive species and crosslinks DNA with protein and thus damages the cells (Kubow 1990).

In vitro lipid peroxidation levels in rat liver homogenate was measured as Thiobarbituric Acid Reactive Substances (TBARS). 10% fresh liver homogenate was prepared in 20 mM phosphate buffer saline (PBS), pH 7.4 (Ohkawa et al 1979). Briefly, 0.25 mL of liver homogenate was incubated with 2-10 μg/mL of GR and MG extracts in 20 mM PBS, pH 7.4. After 5 min of pretreatment, 0.5 mL each of ferric chloride (400 mM) and ascorbic acid (400 mM) was added and incubated at 37°C for 1 h. The reaction was terminated by addition of 2.0 mL of TBA reagent (15% TCA, 0.37% TBA in 0.25 N HCl) and tubes were boiled for 15 min at 95 °C, cooled, centrifuged and read at 532 nm. TBARS was measured by using a standard TMP (1,1,3,3 tetramethoxy propane) calibration curve (0.1-0.5 μg) and expressed as percent inhibition of lipid peroxidation by extracts.

2.2.8.4. DNA protection assay

Fenton's reagent generates hydroxyl radical, and when this reagent is added to DNA, it induces strand breaks due to oxidation reaction. When the DNA is electrophoresed on an agarose gel it will migrate faster compared to that of native DNA. Comparing the electrophoretic mobilities of oxidized, native and antioxidants treated DNA; hydroxyl radical scavenging abilities of antioxidants were evaluated.

The DNA protective effect of phenolic fractions was determined electrophoretically (Submarine electrophoresis system, Bangalore Genei, Bangalore, India) using calf thymus DNA (Rodriguez & Akman 1998). Calf thymus DNA (1 μg in 15 μL) was subjected to oxidation by Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$). Relative difference in the migration between the native and oxidized DNA was ensured on 1% agarose gel electrophoresis after staining with ethidium bromide. Gels were documented (Herolab, Germany) and the intensity of the bands were determined (Easywin software). Protection to

DNA was calculated based on the DNA band corresponding to that of native in the presence and absence of 2 - 4 µg of GR and MG extracts.

2.2.9. Determination of percent contribution to various ulcer preventive properties by constituent phenolic acids

Aqueous extracts of ginger (GRAE) and mango ginger (MGAE) and, phenolic fractions of ginger (GRFP/GRHP) and mango ginger (MGFP/MGHP) contained different phenolic acids. These phenolic acids in pure form possessed differential abilities for either H^+, K^+ -ATPase inhibition, *H. pylori* inhibition or antioxidant activities. Based on the abundance of each of these phenolic acids, total contribution to various bioactivities in ginger and mango ginger fractions were determined and the activity is expressed as relative percent.

2.2.10. HSA – phenolics interaction studies

Stock solution of human serum albumin (HSA) was prepared to a concentration of 1.0×10^{-4} M in Tris-HCl buffer of pH 7.4 containing 100 mM sodium chloride. All the phenolic compounds were prepared to a concentration of 10 mg/100 mL in ethanol (95%) because ethanol has no fluorescence and does not affect the determinations. All fluorescence measurements were made in a Shimadzu RF-5301PC spectrofluorophotometer.

A series of assay solutions were prepared by adding 10 µL of the stock solution of HSA and varied concentrations of phenolics (0.5-2.5 µg/mL) into each marked tube respectively, and diluted to the mark- 1.0 mL with Tris-HCl buffer of pH 7.4. The concentration of HSA was constant and the possible interaction was studied at different concentrations of phenolic acids. Tubes were mixed thoroughly and placed in the thermostat water-bath at 37 ˚C for 5 min, and transferred to the quartz cuvette 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 and were expressed as Sterner's constant.

2.2.11. Statistical Analysis

All the experiments were carried out in triplicates and the results were expressed as mean ± standard deviation (n=3). The significance of difference was calculated by Student's *t* test, and values *p < 0.05 and **p < 0.01 were considered to be significant. One way ANOVA followed by Duncan's multiple range test was also used to determine the difference in mean values between sample groups.

2.3. Results

2.3.1. Total phenolic content in ginger and mango ginger.

The total phenolic content in aqueous extracts of ginger (GRAE) and mango ginger (MGAE) - were found to be 7.6 ± 0.5 and 5.4 ± 0.4 mg gallic acid equivalent (GAE)/g respectively. (Table 2.1).

Table 2.1. Total phenolics in Ginger and Mango ginger

	Yield mg/g GAE	Percent yield (g/100 g)
Ginger	7.6 ± 0.5	0.76
Mango ginger	5.4 ± 0.4	0.54

All data are the mean ± SD of three replicates

The total phenolic content in GRFP and GRHP phenolic fractions was found to be 2.6 ± 0.24 mg GAE/g and 1.1 ± 0.07 mg GAE/g of dried, defatted ginger powder, respectively. Approximately 3-5% yield of phenolics was noticed in ginger phenolic fractions (Table 2.2). Where as in MGFP and MGHP phenolic fractions was found to be 2.1 ± 0.20 mg GAE/g and 1.9 ± 0.17 mg GAE/g of dried, defatted mango ginger powder, respectively. Approximately 2-3% yield of phenolics was noticed in mango ginger phenolic fractions (Table 2.2).

Table 2.2. Total phenolic content and yield of phenolics in free and hydrolysed fractions in ginger and mango ginger

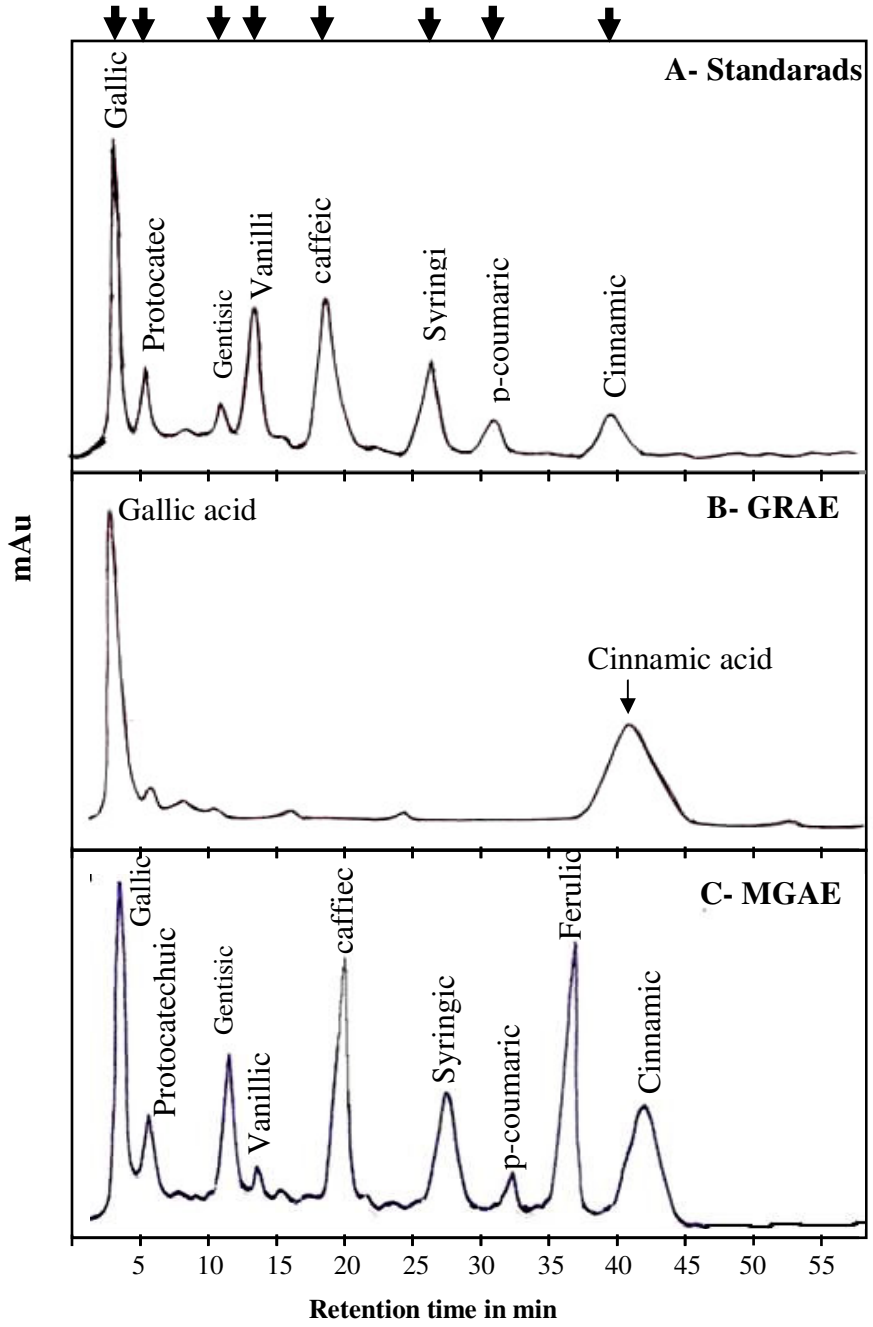
Phenolic Fraction	Yield mg/g dry wt.	Percent yield (g/100 g)	Phenolic content (mg GAE /g)
GRFP	$3.2^{d} \pm 0.30$	0.32	$2.6^{c} \pm 0.24$
GRHP	$1.8^{a} \pm 0.08$	0.18	$1.1^{a} \pm 0.07$
MGFP	$2.8^{c} \pm 0.22$	0.28	$2.1^{b} \pm 0.20$
MGHP	$2.3^{b} \pm 0.18$	0.23	$1.9^{b} \pm 0.17$

GRFP- Ginger free phenolic fraction; GRHP- Ginger hydrolysed phenolic fraction. MGFP- Mango ginger free phenolic fraction; MGHP- - Mango ginger hydrolysed phenolic fraction. All data are the mean ± SD of 3 replicates, mean value followed by different letters (a - d) in the same column are significantly different ($p \leq 0.05$)

2.3.2. Identification of phenolic acids in Ginger and Mango ginger

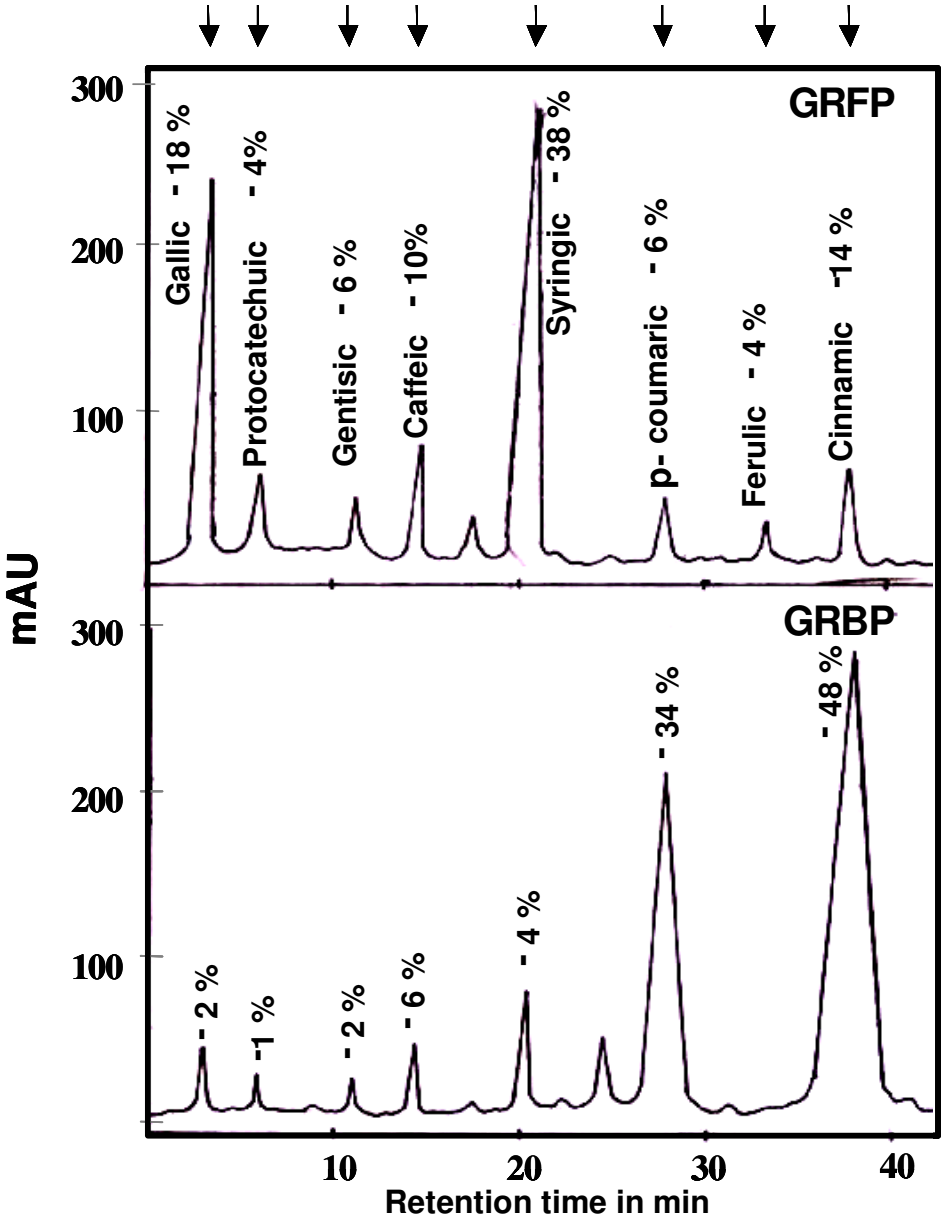
HPLC analysis of GRAE revealed that cinnamic acid (50%) and gallic acid (46%) were the major phenolic acids with small amounts of caffeic, ferulic, gentisic, protocatechuic, syringic and vannilic acids. Where as in mango ginger gallic (15%), caffeic (14%), ferulic (15%) and cinnamic (35%) with small amounts of other phenolic acids as depicted in Figure 2.3.

Figure 2.3. HPLC profile of aqueous extract of ginger and mango ginger on C_{18} column.



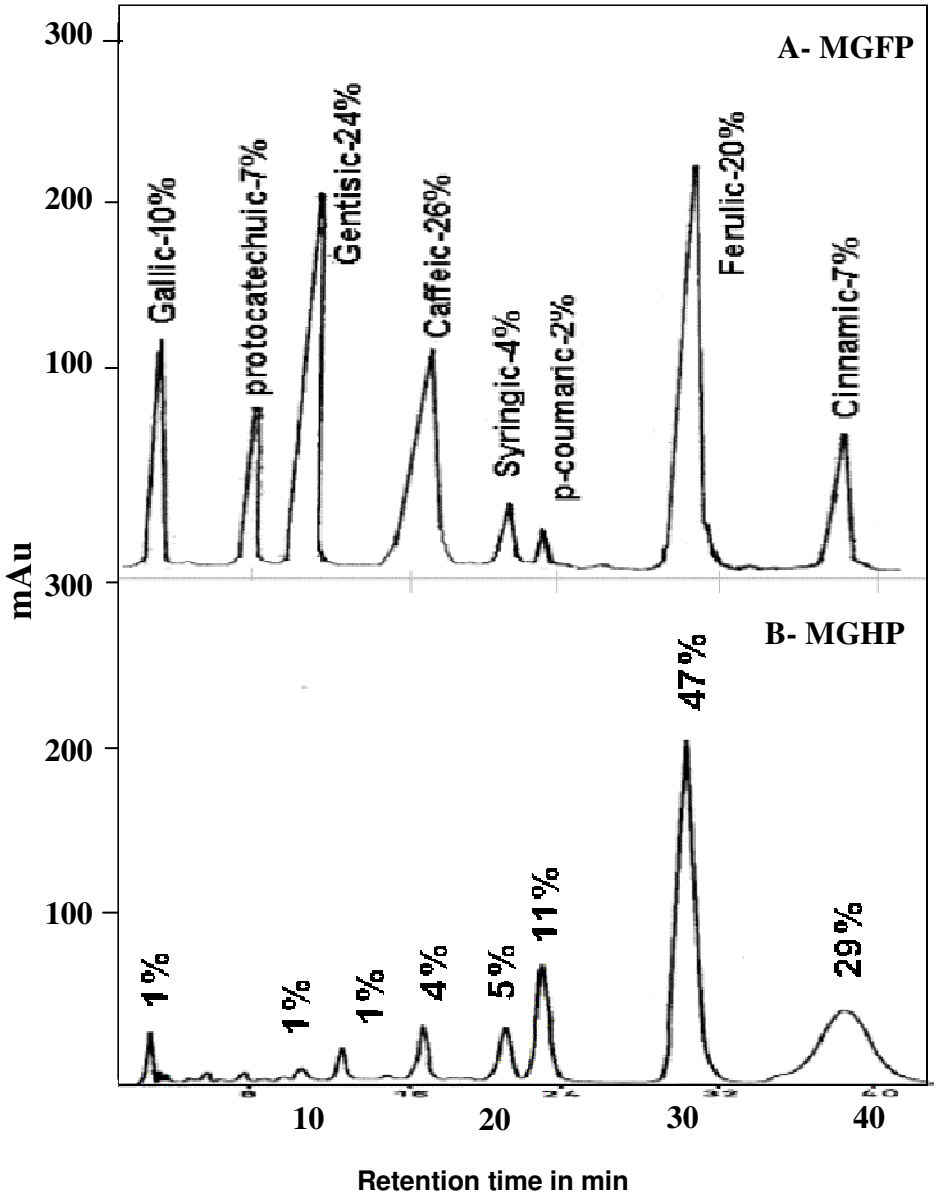
A- standard phenolic acids, Aqueous extract of ginger- GRAE (B) and mango ginger- MGAE (C)

Figure 2.4. HPLC profile of (A) ginger free phenolics -GRFP and (B) ginger hydrolyzed phenolics-GRHP on C-$_{18}$ column.



Abundancy of each phenolic acid is indicated above the respective peaks in terms of percentage.

Figure 2.5. HPLC profiles of mango ginger free phenolics –MGFP (A) and hydrolyzed phenolics-MGHP (B) on C-$_{18}$ column.



Abundancy of each phenolic acid is indicated above the respective peaks in terms of percentage.

The major phenolic acids present in GRFP were syringic acid (38%) followed by gallic acid (18%) and cinnamic acid (14%). Small amounts of caffeic (10%), gentisic (6%), protocatechuic (4%), and ferulic (4%) acids were also found to be present. GRHP fraction on the other hand contained cinnamic (48%) and p-coumaric (34%) acids as major phenolic acids. Caffeic (6 %), syringic (4 %), gallic (2%), gentisic (2%) and protocatechuic (1%) acids were present in small amounts (Figure 2.4 & Table 2.3).

Table 2.3. Percent of individual phenolic acids in ginger and mango ginger phenolic fractions.

Phenolic acids	Ginger			Mango ginger		
	GRAE	GRFP	GRHP	MGAE	MGFP	MGHP
Gallic	46	18	2	15	10	1
Protocatechuic	<1	4	1	4	7	-
Gentisic	<1	6	2	8	24	1
Caffeic	1	10	6	14	26	4
Vannilic	-	-	2	1	-	2
Syringic	2	38	4	6	4	5
p-coumaric	<1	6	34	2	2	11
Ferulic	<1	4	-	15	20	47
Cinnamic	50	14	48	35	7	29

Phenolic composition in ginger and mango ginger were analysed by HPLC on C_{18} column. % of individual phenolic acids was calculated. GRAE- ginger aqueous extract, GRFP- ginger free phenolics, GRHP- ginger hydrolysed phenolics, and MGAE- mango ginger aqueous extract, MGFP- mango ginger free phenolics, MGHP- mango ginger hydrolysed phenolics.

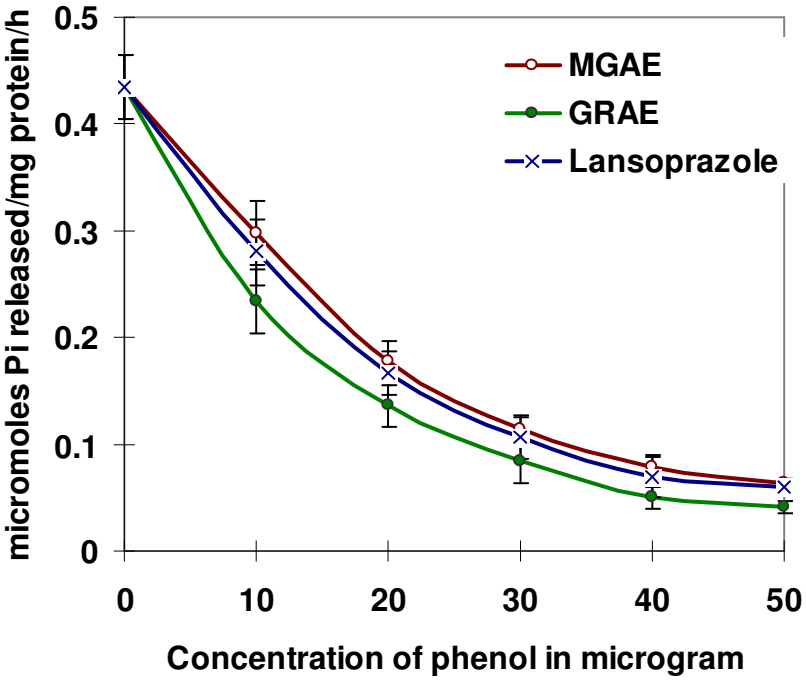
Similarly, the phenolic acids present in MGFP were caffeic (26%), gentisic (24%), ferulic (20%) followed by gallic (10%) cinnamic (7%), protocatechuic (7%), and small amounts of syringic (4%) and p-coumaric (2%) acids. MGHP fractions contained ferulic (47%) and cinnamic acid (29%) as major phenolic acid, p-coumaric (11%), syringic (5%), caffeic (4%), vannilic (2%), gallic (1%) and gentisic (1%) acids were also present in small amounts (Figure 2.5). The consolidated table has been given in Table 2.3.

2.3.3. Ability of Ginger and Mango ginger phenolic fractions in gastric H^+, K^+-ATPase inhibition

H^+, K^+-ATPase is an important component of proton pump responsible for acid secretion in the stomach. It is located in the gastric membrane vesicle and catalyzes the electro neutral exchange of intracellular H^+ and extra cellular K^+ coupled with the hydrolysis of the cytoplasmic ATP. Hyper secretion of this enzyme in stomach leads to acidity and ulcer. Therefore this regulatory enzyme was found to be a pharmacological target for many ulcer drugs. Hence in order to understand the potential antiulcer property of ginger and mango ginger, gastric H^+, K^+-ATPase inhibitory effects were studied.

Ginger/mango ginger aqueous extracts inhibited gastric H^+, K^+-ATPase activity in a concentration dependent manner (Figure 2.6). Concentrations required to inhibit 50% of H^+, K^+-ATPase activity is designated as IC_{50} and GRAE and MGAE showed an IC_{50} of 16.5 ± 1.2 μg/mL and 18.6 ± 1.9 μg/mL, respectively; when compared to IC_{50} of 19.3 ± 2.2 μg/mL of lansoprazole, a known proton pump inhibitor. Isolated ginger phenolic fractions- GRFP and GRHP also inhibited gastric H^+,K^+-ATPase activity in a concentration dependent manner (Figure 2.7A). GRFP and GRHP showed an IC_{50} of 2.9 ± 0.18 and 1.5 ± 0.12 μg/mL, a 6 and 11 fold better activity than GRAE respectively.

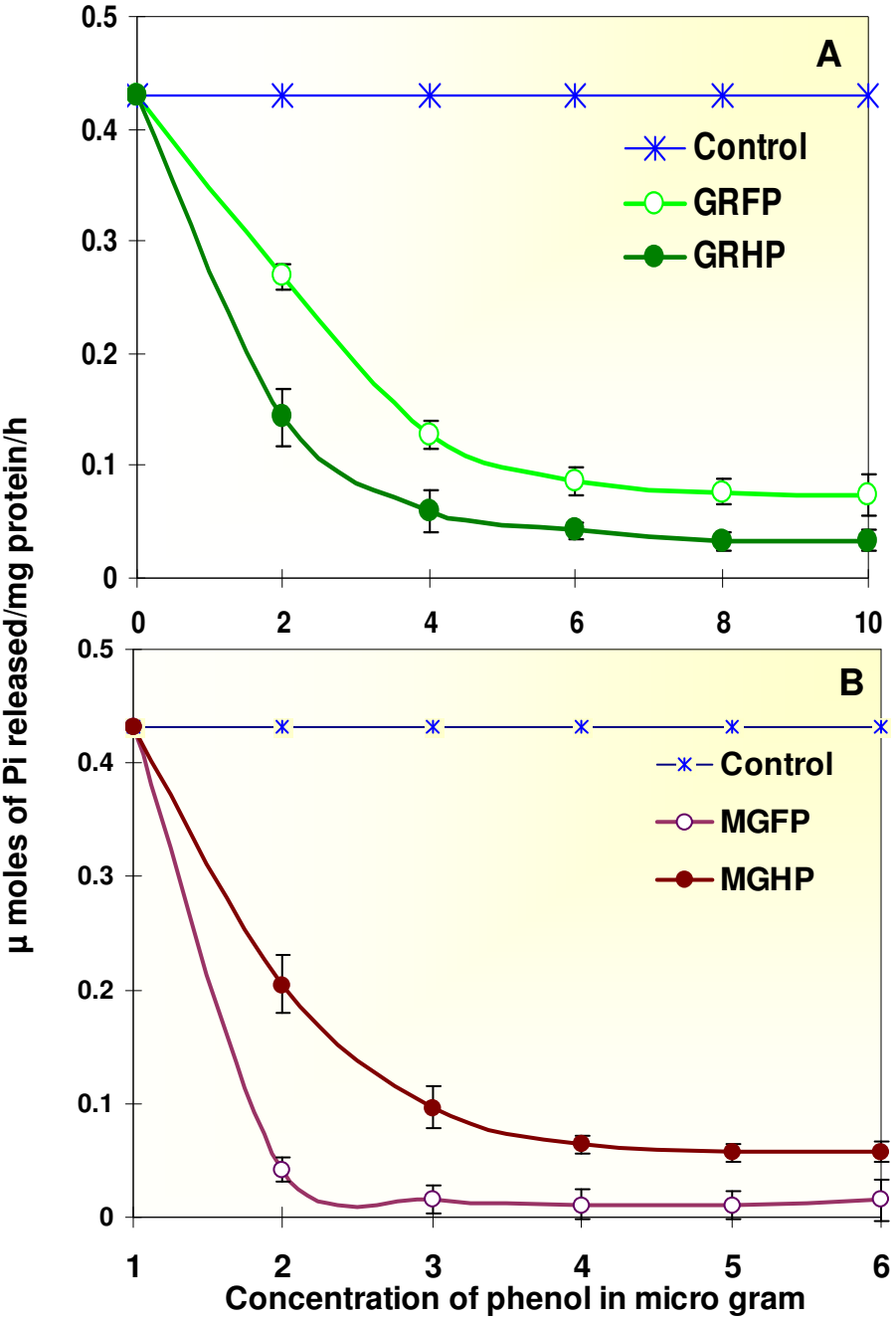
Figure 2.6. Inhibition of proton potassium ATPase enzyme activity by aqueous extract of ginger and mango ginger.



Sheep parietal cell extract was employed as gastric-H^+,K^+-ATPase source, and activity was determined employing the protocol described under materials and methods. 350 µg enzyme protein/mL of reaction volume was incubated with 10-50 µg of ginger/mango ginger aqueous extract- GRAE/MGAE. Enzyme activity is represented as µ moles of Pi released/mg enzyme protein/h. All data are the mean ± SD of triplicates.

When parallel experiments were conducted with mango ginger, phenolic fractions of MG also found to be good inhibitors as that of GR (Figure 2.7B). MGHP inhibited H^+, K^+-ATPase better (0.7 ± 0.08 µg/mL) than MGFP (2.2 ± 0.21 µg/mL). The consolidated IC_{50} values are given in the Table 2.4.

Figure 2.7. Inhibition of proton potassium ATPase enzyme activity by free and hydrolysed phenolics of ginger and mango ginger.



A. Free and hydrolysed phenolics of ginger. (B) Free and hydrolysed phenolics of mango ginger. All data are the mean ± SD of triplicates.

Table 2.4. IC_{50} value for proton potassium ATPase enzyme activity

Samples	IC $_{50}$ value (µg GAE/mL)
GRAE	16.5 ± 1.2
GRFP	2.9 ± 0.18
GRHP	1.5 ± 0.12
MGAE	18.6 ± 1.9
MGFP	2.2 ± 0.21
MGHP	0.7 ± 0.08
Lansoprazole	19.3 ± 2.2

All data are the mean ± SD of triplicates.

2.3.4. H^+, K^+-ATPase inhibitory effect of standard phenolic acids

Different extracts and fractions of GR and MG showed differences in the H^+, K^+-ATPase inhibitory effect. Since the fractions are specially enriched in phenolic acids as indicated in figures 2.3, 2.4, & 2.5 and also has differences in the nature of phenolic acids, attempt has been made to understand the ability of individual phenolic acids to inhibit H^+, K^+-ATPase inhibitory activity. In order to understand the probable ability of individual phenolic acids present in extracts in inhibiting gastric H^+,K^+-ATPase, pure phenolic acids were examined for their ability to inhibit the enzyme. As indicated in Table 2.5, cinnamic acid showed maximum inhibitory effect with an IC_{50} of 15.1 µg/mL, followed by caffeic (IC_{50} 27.1 µg/mL), ferulic (IC_{50} 33.6 µg/mL), syringic (IC_{50} 37.4 µg/mL), p-coumaric (IC_{50} 39.7 µg/mL), protocatechuic (IC_{50} 47.1 µg/mL), gentisic (IC_{50} 59.1 µg/mL) and gallic acid (IC_{50} 132.1 µg/mL).

Accordingly, the percentage contributed by individual phenolic acids in extracts and fractions of GR and MG are calculated and depicted in Figure 2.21. In both the cases cinnnamic acid contributed significantly (~ 48 and 63% respectively)

Table 2.5. H^+, K^+-ATPase inhibition in standard phenolic acids and known antiulcer drug

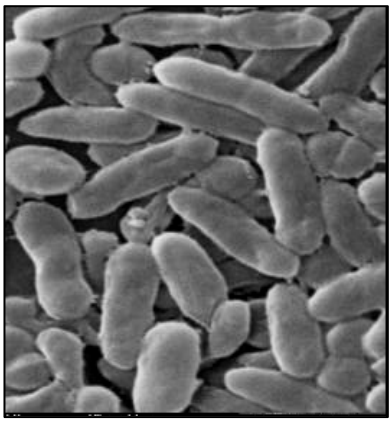
Sample/ Standard phenolic acids	PPA activity IC_{50} (µg)
Cinnamic acid	$15.1^{a} \pm 1.80$
Caffeic acid	$27.1^{cde} \pm 3.10$
Ferulic acid	$33.6^{de} \pm 3.80$
Syringic acid	$37.4^{de} \pm 4.10$
p-coumaric acid	$39.7^{e} \pm 3.20$
Protocatechuic acid	$47.1^{f} \pm 4.20$
Gentisic acid	$59.1^{g} \pm 6.10$
Gallic acid	$132.0^{h} \pm 14.0$
Lansoprazole	$19.3^{bc} \pm 2.20$

In order to understand the probable contribution of identified phenolic acids in ginger/mango ginger fractions, under similar experimental conditions H^+,K^+-ATPase activity was performed for standard pure phenolic acids. All data are the mean ± SD of 3 replicates, mean value followed by different letters (a, b, c, d, e, f, g, h) in the same column are significantly different ($p \leq 0.05$)

2.3.5. Inhibition of *Helicobacter pylori* by extracts of ginger and mango ginger

The bacteria were isolated from endoscopic samples from infected human patients from gastroenterology division of local hospitals in Mysore. Upon culturing bacteria from the specified media as described under materials and methods, they were subjected to biochemical tests to establish its specific identity. Results as shown in Table 2.6 suggest that it is a Gram-negative bacteria with motility by the flagella. Substantiating to this it is observed under scanning electron microscope (Figure 2.8). Culture also showed positive for urease, catalase and oxidase tests (Table 2.6) (Parsonnet 1998) as per its reactivity with that of standard *H. pylori* culture. Further the response to antibiotics as it was resistant to antibiotics like erythromycin, nalidixic acid, polymixin B, penicillin, and vancomycin and was susceptible to amoxicillin, clarithriomycin and metronidazole. Added to this the appearance of a characteristic white mucilaginous colony confirms the identity of bacteria as *H. pylori*. As mentioned here, series of tests confirms that the isolated bacteria from the endoscopic sample of an ulcer patient indeed is *H. pyori,* attempts were made to examine the anti-*H.pylori* activity in extracts and isolated - free and hydrolyzed phenolic fractions of ginger and mango ginger.

Figure 2.8. Scanning electron microscopic picture of *H. pylori*.



H. pylori obtained from endoscopic excision was subjected to various biochemical tests to confirm the identity of isolated bacteria as *H. pylori.* And electron microscopic observations was done at 15 K magnification.

Table 2.6. Characteristic Biochemical tests for *H. pylori*.

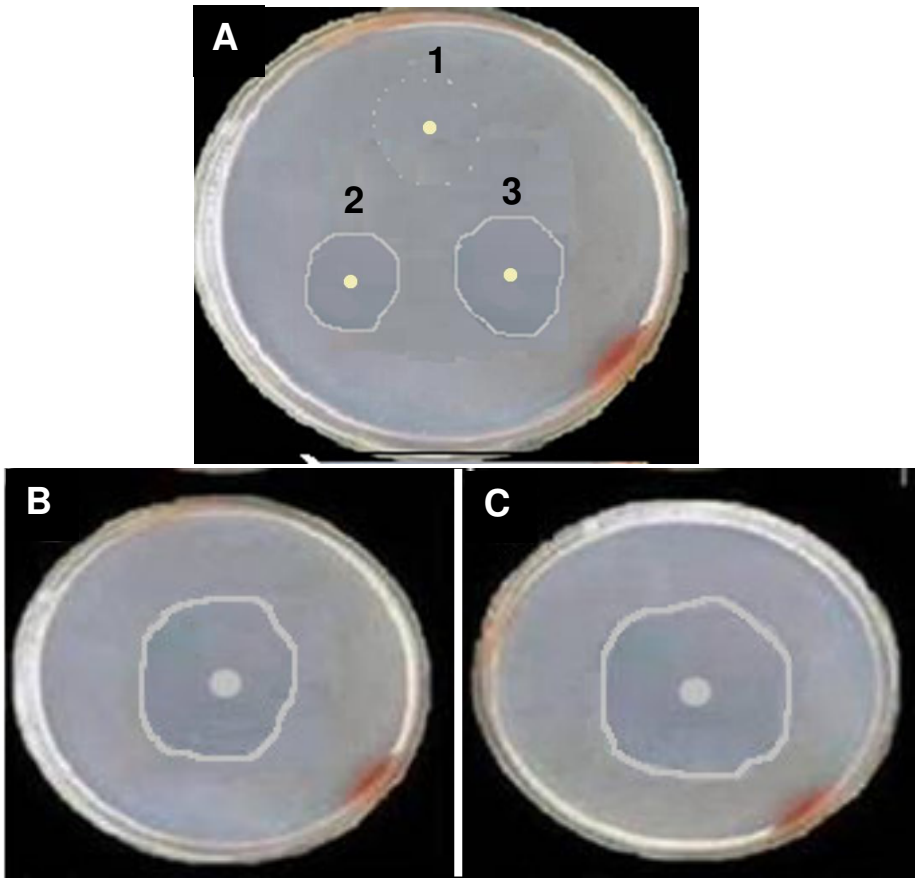
Tests	Results
Urease	+ ve
Catalase	+ ve
Oxidase	+ ve
Gram staining	Gram negative
Motility	Motile
Colony characteristic	White mucilage type
Antibiotics	
Erythromycin	Resistant
Nalidixic acid	Resistant
Polymixin	Resistant
Penicillin	Resistant
Vancomycin	Resistant
Amoxicillin	Susceptible
Clarithromycin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

H. pylori obtained from endoscopic excision was subjected to various biochemical tests to confirm the identity of isolated bacteria as *H. pylori.* Gram staining, enzyme analysis and morphological analysis as well as antibiotic resistance/susceptibility were included in the tests for characterization of *H. pylori*.

2.3.5.1. Inhibition of *Helicobacter pylori* by extracts of ginger

The initial antibacterial activity against *H. pylori* was assayed by agar diffusion method. GRAE showed a clear inhibition zone around the disc at 50 µg/mL concentration equivalent to that of a susceptible antibiotic amoxicillin at 10 µg/mL (Figure 2.9A1). Figure 2.9B & C shows the clear inhibition zone around the disc at 10 µg/mL concentration of GRFP and GRHP. To quantitate the inhibitory effect of *H. pylori,* the diameter of growth inhibition area was measured and expressed in milli meter (Figure 2.11)

Figure 2.9. Effect of ginger on *H. pylori*.



Anti *H. pylori* activity was tested by the standard agar diffusion method**.** 5.5 mm discs containing 10 µg/mL each of Amoxicillin-a known antibiotic **(A.2);** GRAE **(A.3);** GRFP **(B)** and GRHP **(C)** were impregnated with agar and **A.1** served as control with no inhibitor disc. Clear area around the disc represents the inhibition zone due to the effect of the test fraction.

2.3.5.2. Inhibition of *Helicobacter pylori* by extracts of mango ginger

MGAE also showed a clear inhibition zone around the disc at 50 µg/mL concentration almost equivalent to that of GRAE (Figure 2.10). The phenolic fractions of mango ginger- MGFP and MGHP showed a clear inhibition zone around the disc at 10 µg/mL concentrations. The diameter of growth inhibition area was measured and expressed in milli meter (Figure 2.11)

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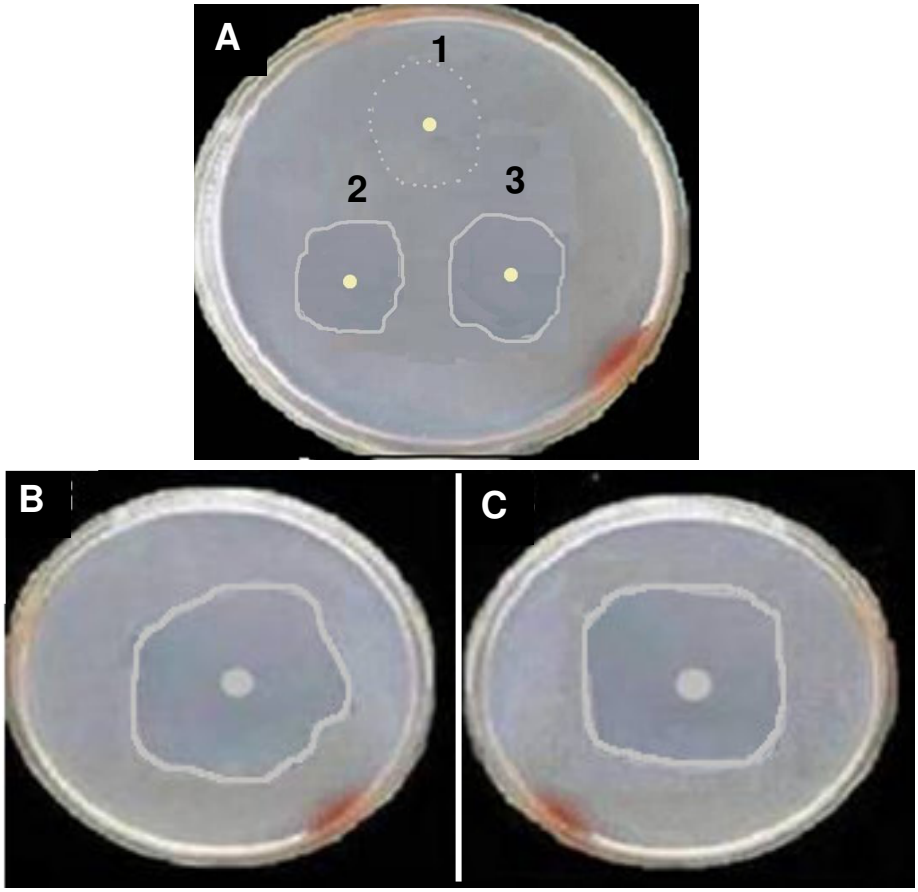
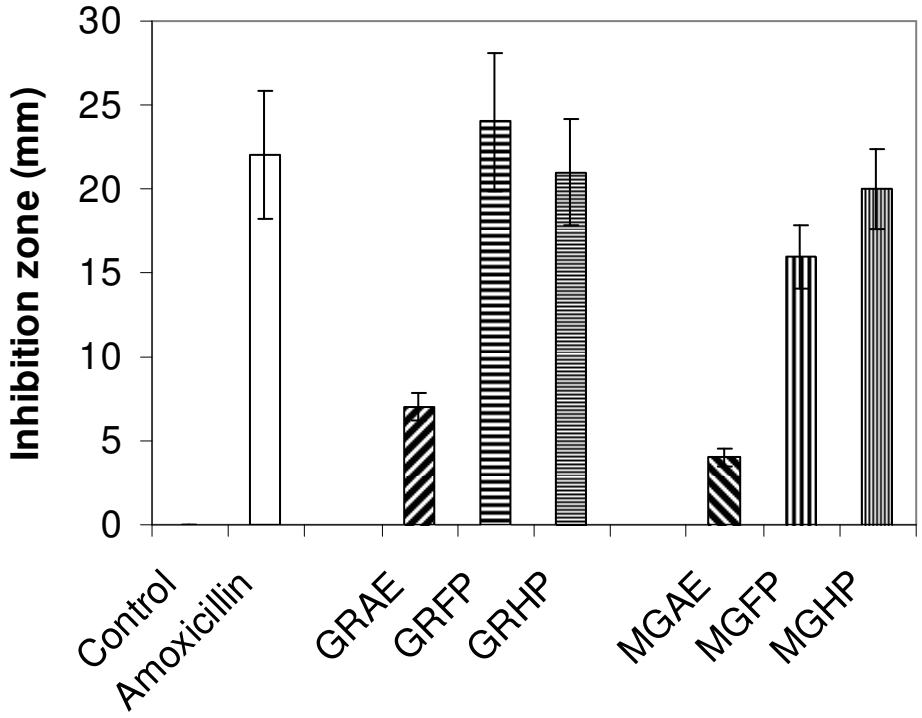
Figure 2.10. Effect of mango ginger on *H. pylori*.



Anti *H. pylori* activity was tested by the standard agar diffusion method. 5.5 mm discs containing 10 µg/mL each of Amoxicillin-a known antibiotic **(A.2);** MGAE **(A.3);** MGFP **(B)** and MGHP **(C)** were impregnated with agar and **A.1** served as control with no inhibitor disc. Clear area around the disc represents the inhibition zone due to the effect of the test fraction.

Figure 2.11. Inhibitory zones formed around the discs with ginger and mango ginger fractions.



Anti *H. pylori* activity was tested by the standard agar diffusion method**.** 5.5 mm discs containing 10 µg/mL each of Amoxicillin-a known antibiotic and ginger/mango ginger extract. The diameter of growth inhibition area was measured and expressed in milli meter. At 10 µg of each samples-Amoxicillin showed 22±3 mm. In ginger fractions, GRAE 7±0.5; GRFP 24±4; GRHP 21±3 and in mango ginger fractions, MGAE 4±0.3; MGFP 16±4; MGHP 20±3 mm. All data are the mean ± SD of 3 replicates.

In view of the result obtained by the disc diffusion method, the minimum inhibitory concenration (MIC) values were determined by broth dilution method. The MIC values obtained confirm the significant ($p = 0.003$) anti *H. pylori* activity, with MIC values – GRAE (300 ± 38 µg/mL), GRFP 49 ± 4.1 µg/mL and GRHP 38 ± 3.4 µg/mL and in mango ginger fractions – MGAE 356 ± 43, MGFP 64 ± 6.1 µg/mL and MGHP 38 ± 2.2 µg/mL (Table 2.7).

Table 2.7. MIC value of ginger and mango ginger phenolic fractions for *H. pylori* inhibition

Samples	MIC in µg / mL	Inhibition zone (mm) by agar diffusion method at 10 µg/mL
Control	No inhibition	No inhibition
Amoxicillin	26 ± 3.2	22± 3
Ginger		
GRAE	300 ± 38	7± 0.5
GRFP	49 ± 4.1	24± 4
GRHP	38 ± 3.4	21± 3
Mango ginger		
MGAE	356 ± 43	4 ± 0.3
MGFP	64 ± 6.1	16± 4
MGHP	38 ± 2.2	20± 3

MIC value was calculated by broth dilution method and expressed in µg/mL. All data are the mean ± SD of 3 replicates

Difference in inhibitory activity may be due to the nature of phenolic acids present in each fraction. In order to understand the probable ability of individual phenolic acids present in extracts in inhibiting *H. pylori*, pure phenolic acids were examined for their ability to inhibit the bacteria (Table 2.8). Although both ginger and mango ginger showed potent activity as that of Amoxicillin (antibiotic to which *H. pylori* is susceptible). Ginger however showed better activity than mango ginger.

Table 2.8. Effect of pure phenolic acids on *H. pylori*

Standard phenolic acids	H. pylori- inhibition zone (mm)
Cinnamic acid	34 ± 4.1
Caffeic acid	28 ± 3.4
Ferulic acid	25 ± 2.6
Syringic acid	18 ± 2.1
p-coumaric acid	18 ± 2.8
Protocatechuic acid	16 ± 1.8
Gentisic acid	16 ± 2.1
Gallic acid	14 ± 1.6
Amoxicillin	22 ± 3.2

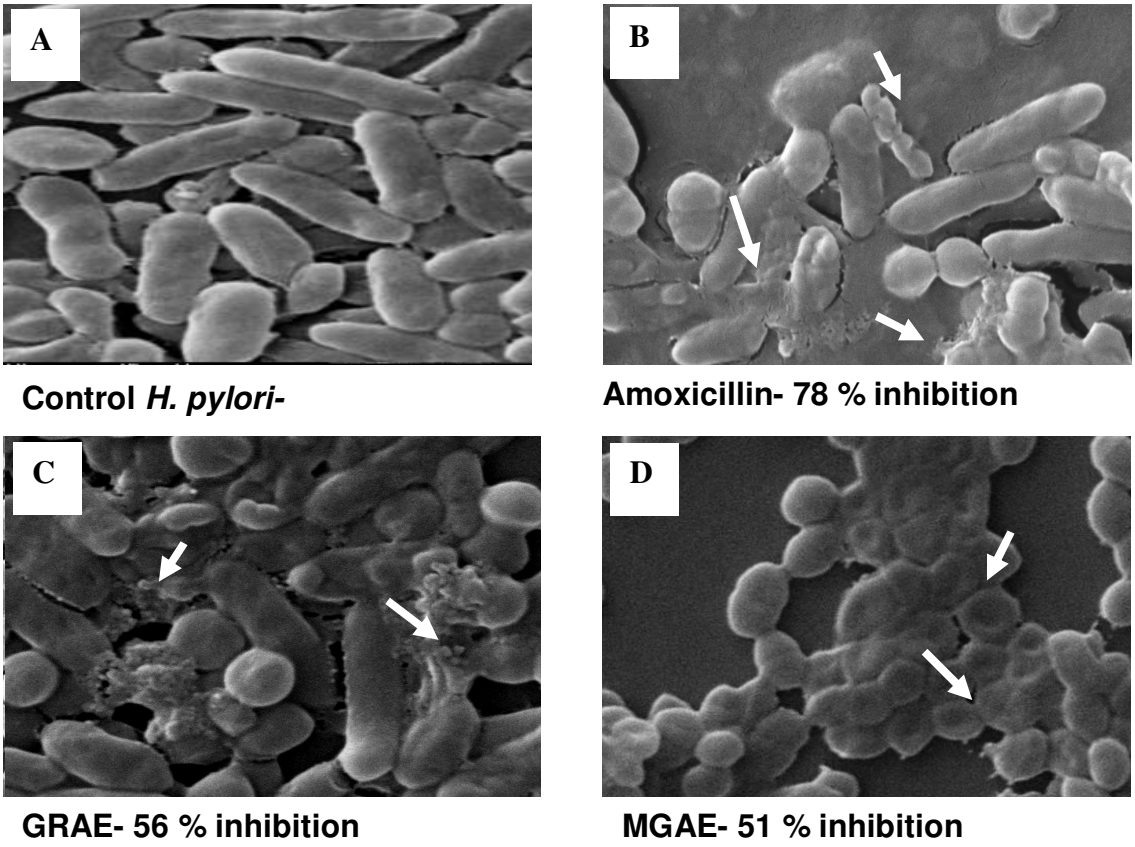
MIC value was calculated by broth dilution method and expressed in µg/mL. All data are the mean ± SD of 3 replicates

2.3.5.3. Scanning Electron Microscopy.

The inhibitory effect of *H. pylori* by phenolic fractions of ginger and mango ginger fractions and standard phenolic acids were confirmed by observing destruction of *H. pylori* by electron microscopic observations. Untreated *H. pylori* showed uniform rod shaped cells (Figure 2.12A), whereas the cells treated with ginger and mango ginger aqueous extracts changed from helical form to coccoid and became necrotic (Figure 2.12C&D). A similar coccoid and necrotic form was also observed with *H. pylori* treated with phenolic fractions of ginger and mango ginger (Figure 2.9A&B). Amoxicillin (Figure 2.12B) and standard phenolic acids such as gallic (Figure 2.14C), caffeic (Figure 2.14D), ferulic (Figure 2.14E) and cinnamic acids (Figure 2.14F) showing alteration in *H. pylori* structure. In all these treatments coccoid form

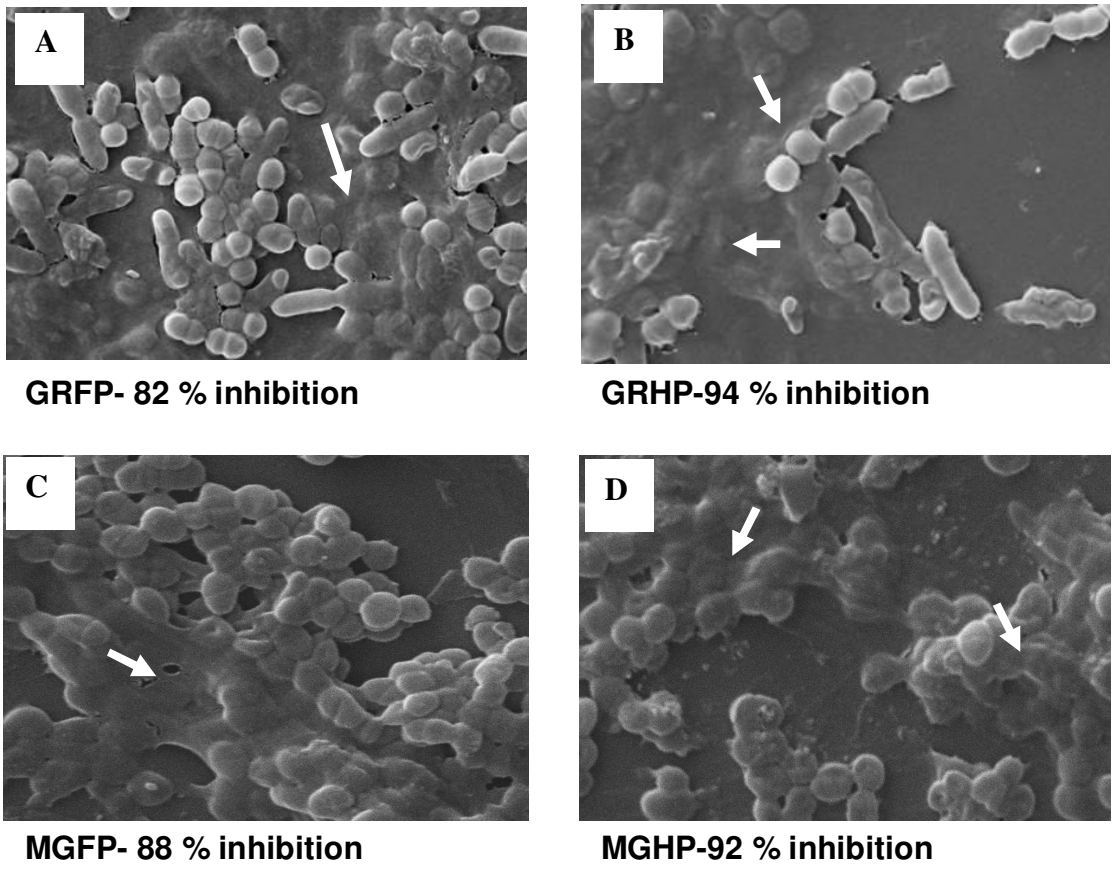
with blebs in the bacterial surface, appearance of vacuoles, granules and an area of low electron density in the cytoplasm were observed. These coccoid forms were known to result in the loss of infectivity (Kusters et al 1997). The lysis of *H. pylori* thus confirms the antimicrobial nature of ginger and mango ginger fractions.

Figure 2.12. Effect of aqueous extracts of ginger and mango ginger on *H. pylori*



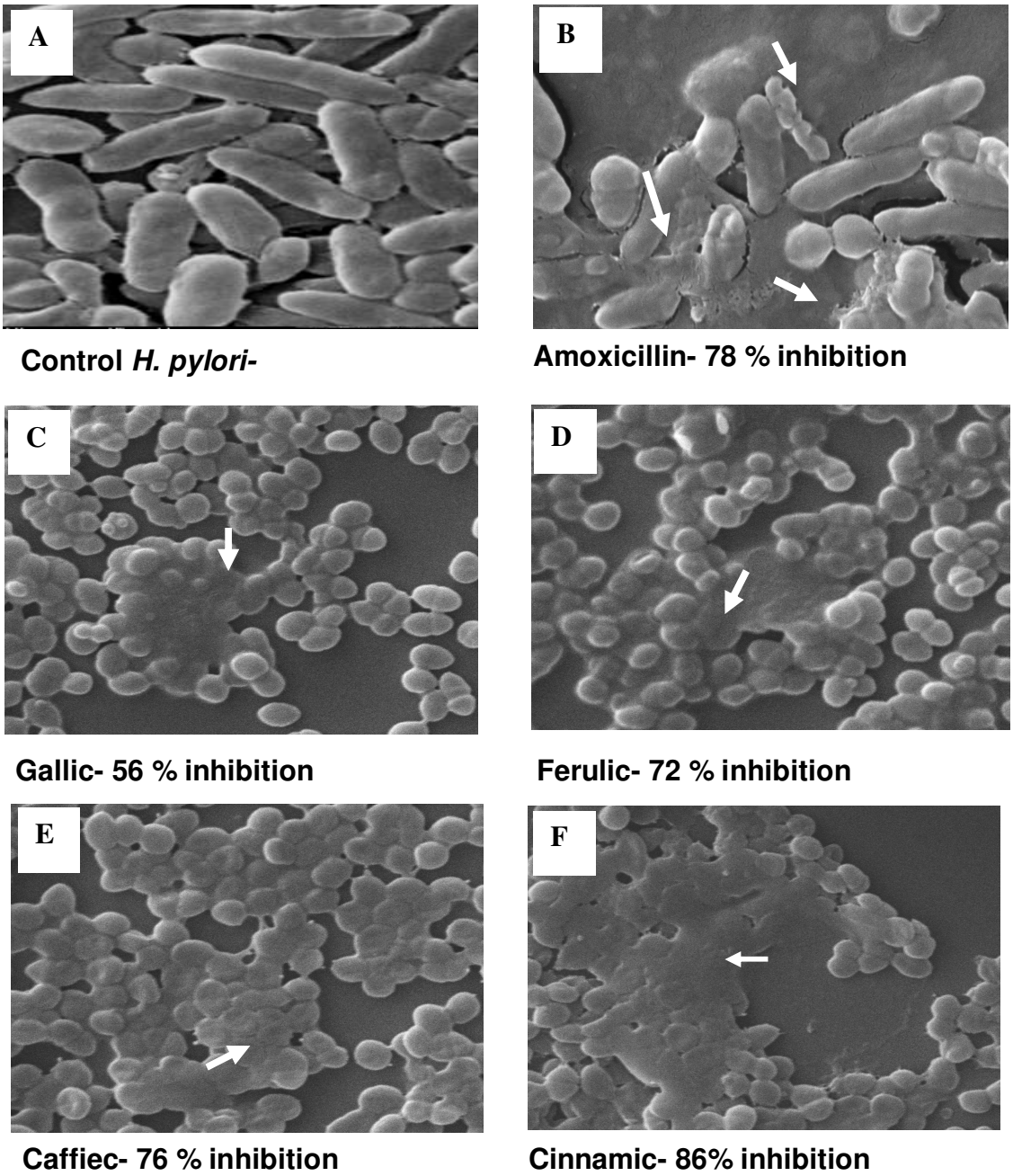
Scanning electron microscopic pictures at 15 k magnification of control (A), amoxicillin (B), GRAE (C), MGAE (D) treated *H. pylori*

Figure 2.13. Effect of free and hydrolysed phenolics of ginger and mango ginger on *H. pylori.*



Representative pictures of Scanning electron microscopy at 10 k magnification of *H. pylori* treated with GRFP (A), GRHP (B), MGFP (C) and MGHP (D).

Figure 2.14. Effect of standard phenolic acids on *H. pylori*



Representative pictures of Scanning electron microscopy at 10 k magnification of *H. pylori* treated with Sstandard phenolic acids: gallic (C), ferulic (D), caffeic (E) and cinnamic (F) acids.

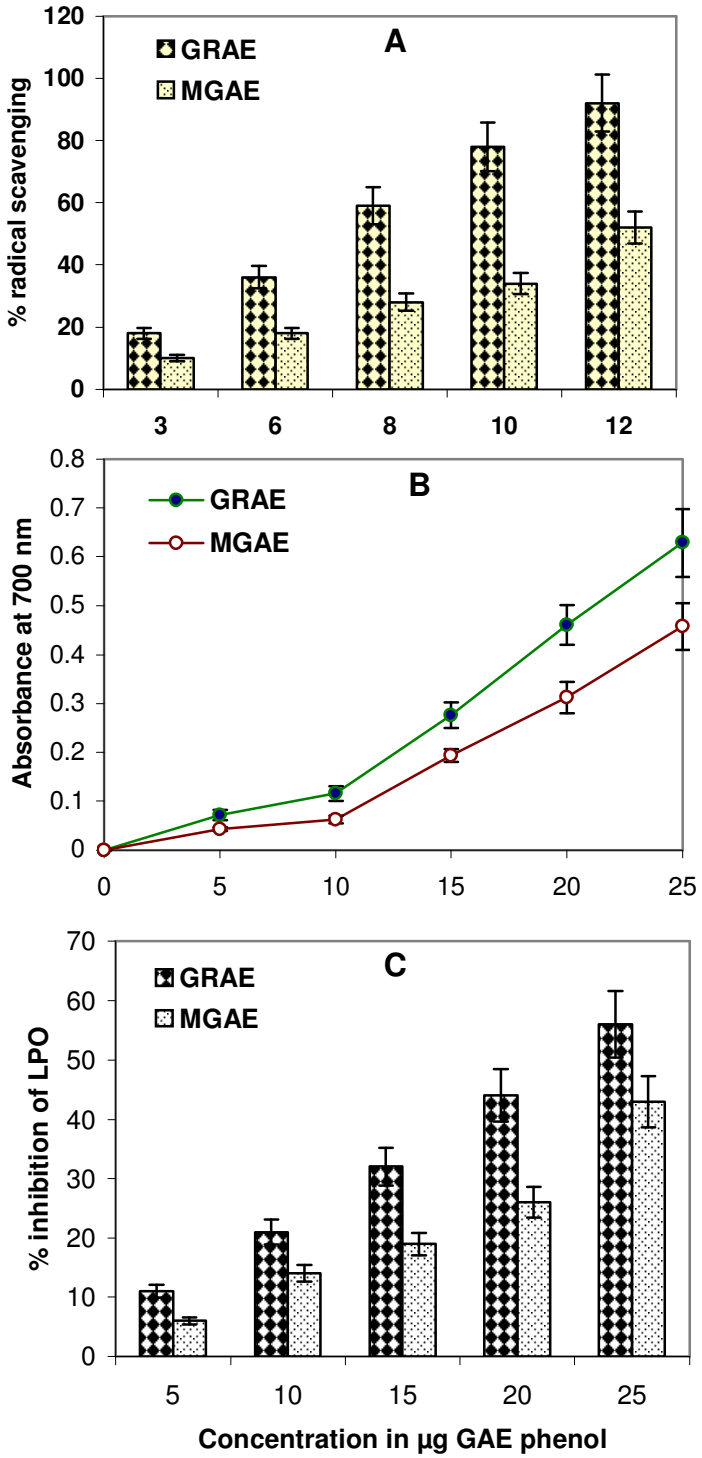
2.3.6. Multipotent antioxidant activity in aqueous extracts of ginger and mango ginger (GRAE and MGAE)

In order to provide evidence that phenolic fractions also possess antioxidant activity, which is essential to counteract oxidative stress induced ulcers, which potentiates *H. pylori* infection, antioxidant potencies were determined.

2.3.6.1 Free radical scavenging activity

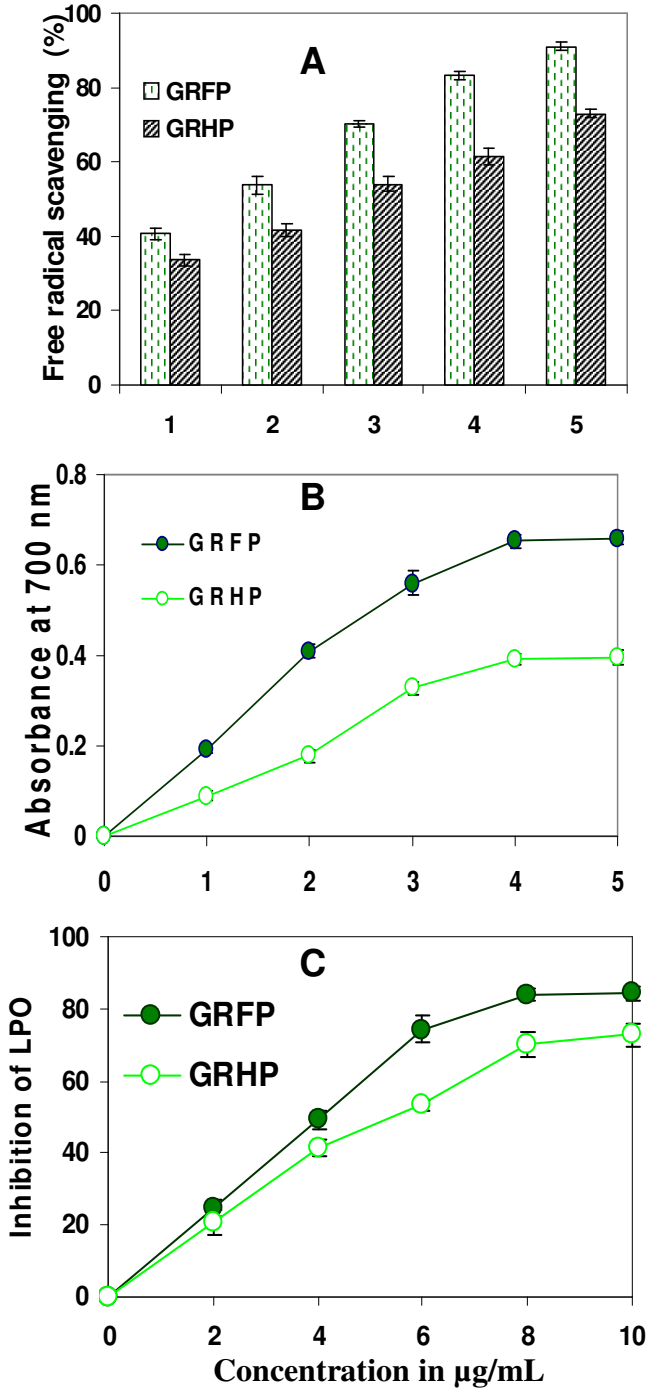
The scavenging activity of ginger and mango ginger fractions on DPPH free radical was determined which primarily evaluates the proton radical scavenging ability of the phenolic compounds. In the present study both ginger and mango ginger aqueous extracts- GRAE/MGAE showed concentration dependent radical scavenging activity (Figure 2.15A). The phenolic fractions of ginger -GRFP showed better radical scavenging activity with IC_{50} of 1.7 ± 0.07 µg/mL compared to GRHP - 2.5 ± 0.16 µg/mL (Figure 2.16A). Similarly MGFP also showed potential radical scavenging activity with IC_{50} of 2.2 ± 0.17 µg/mL and MGHP - 4.2 ± 0.36 µg/mL (Figure 2.17A). The scavenging activity was directly attributed to their phenolic content, since we have shown antioxidant potencies of pure phenolic acids (Table 2.9), which are the representative antioxidant components of ginger extracts.

Figure 2.15. Antioxidant potency of GRAE and MGAE.



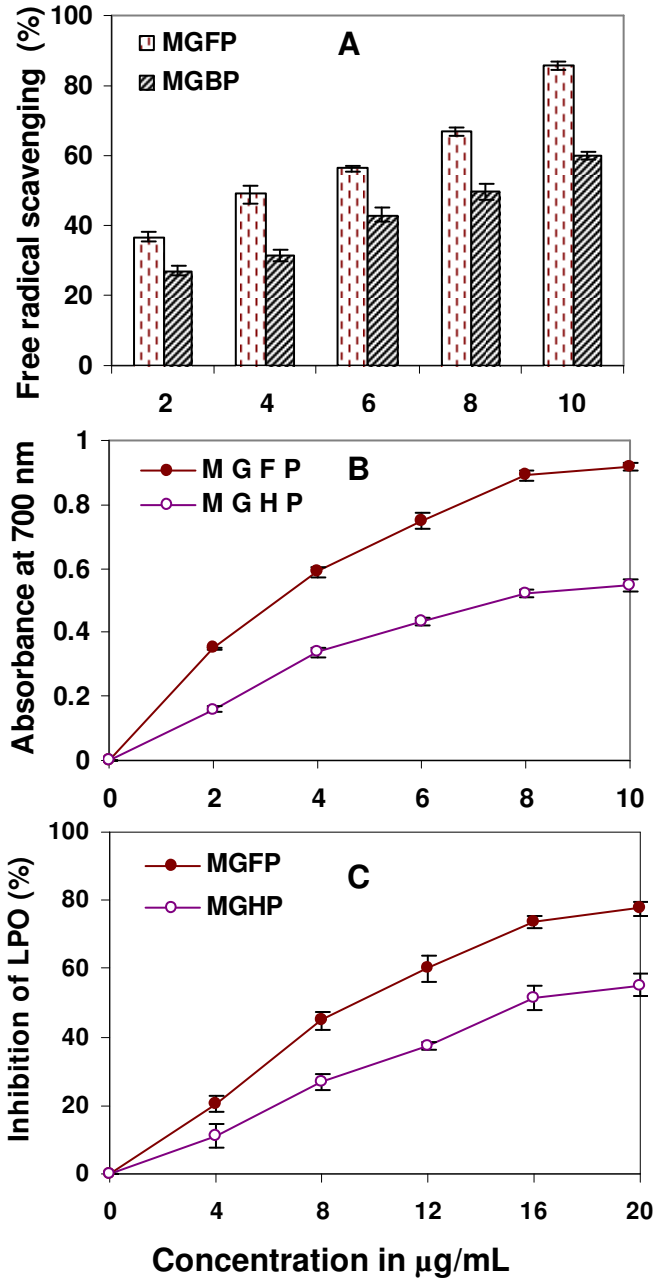
GRAE and MGAE were examined for free radical scavenging (A), reducing power (B) and inhibition of lipid peroxidation (C) as per the protocol described under materials and methods. All data are the mean ± S D of 3 replicates.

Figure 2.16. Antioxidant potency of GRFP and GRHP.



GRFP and GRHP were examined for free radical scavenging (A), reducing power (B) and inhibition of lipid peroxidation (C) as per the protocol described under materials and methods. All data are the mean ± S D of 3 replicates.

Figure 2.17. Antioxidant potency of MGFP and MGHP.



MGFP and MGHP were examined for free radical scavenging (A), reducing power (B) and inhibition of lipid peroxidation (C) as per the protocol described under materials and methods. All data are the mean ± S D of 3 replicates.

2.3.6.2. Reducing Power Assay

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Chen & Ho 1995*).* The reducing power of the sample increased with increase in concentration of phenolics. Both ginger and mango ginger aqueous extracts-GRAE/MGAE showed concentration dependent reducing power ability (Figure 2.15B). Approximately 4 fold increase in the total reducing power was observed in GRFP than GRHP with 338.15 ± 34 U/g and 80.9 ± 11 U/g respectivel (Figure 2.16B). Similarly MGFP showed approximately two fold increase in the total reducing power than MGHP (Figure 2.17B). MGFP and MGHP exhibited 193 ± 21 U/g and 104 ± 8.6 U/g reducing ability respectively. This data may indicate that the phenolic fractions tested may act as reductones by donating electrons to free radicals and there by converting free radicals to more stable product and terminates free radical chain reaction.

2.3.6.3. Inhibition of lipid peroxidation

Lipid peroxidation generates a number of degradation products, such as malondialdehyde (MDA), hexanol *etc.* and is found to be an important cause of cell membrane destruction and cell damage (Yoshikawa et al 1991). MDA is a highly reactive species and crosslinks DNA with protein and thus damages the cells (Kubow 1990), disrupts its activity leading to chronic diseases. In the present study we measured the lipid peroxidation inhibitory potential of ginger and mango ginger aqueous extracts (Figure 2.15C) as well as phenolic fractions of GR and MG. All the tested samples inhibited lipid peroxidation products (TBARS) but to the varying degrees. The hydroxy radical generated through the Fenton reaction was scavenged by co-incubation of rat liver homogenate with varying concentration (2 µg - 10 µg GAE/g sample) of GRFP/GRHP and MGFP/MGHP. GRFP showed maximum inhibition of lipid peroxidation with an IC_{50} 3.6 ± 0.21 µg GAE/g sample compared to GRHP (5.2 ± 0.46 µg GAE/g sample) (Figure 2.16C). And in mango ginger phenolic fractions MGFP showed maximum inhibition of lipid peroxidation with an IC_{50} of 10.3 ± 0.91 µg GAE/g sample compared to the IC_{50} of MGHP (15.6 ± 1.6 µg GAE/g) (Figure 2.17C).

Table 2.9. Anti-ulcer potency of ginger and mango ginger fractions

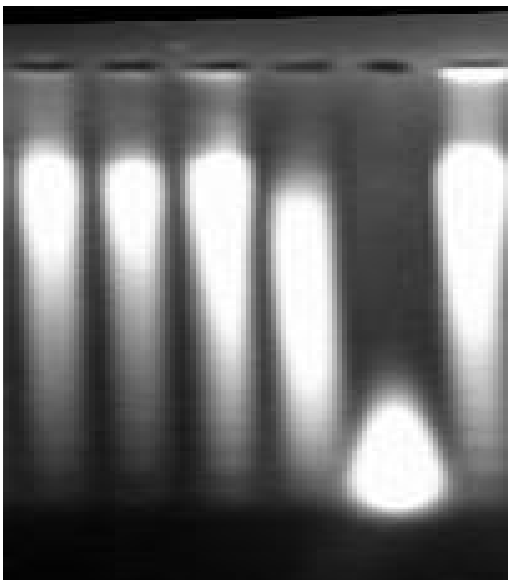
Samples	H^+, K^+-ATPase inhibition (µg GAE/mL)	*H. pylori* Inhibition (MIC) (µg GAE/mL)	Free radical scavenging (IC_{50} value) (µg GAE/mL)	Reducing power ability (U/g GAE)	Lipid Peroxidation (IC_{50} value) (µg GAE/mL)
GRAE	16.5	300	6.8 ± 0.4	116 ± 12	16.8 ± 1.2
GRFP	2.9	49	1.7 ± 0.07	338 ± 34	3.6 ± 0.21
GRHP	1.5	38	2.5 ± 0.16	80.9 ± 11	5.2 ± 0.46
MGAE	18.6	56	14.3 ± 1.1	102 ± 10	19.2 ± 1.4
MGFP	2.2	64	2.2 ± 0.17	193 ± 21	10.3 ± 0.91
MGHP	0.7	38	4.2 ± 0.36	104 ± 8.6	15.6 ± 1.6

In order to provide evidence that phenolic fractions also possess antioxidant activity, which is essential to counteract oxidative stress induced ulcers, antioxidant potencies were determined using the assays of a) H^+, K^+-ATPase inhibition b) *H. pylori* Inhibition (MIC) c) free radical scavenging activity d) reducing power ability and e) lipid Peroxidation. All data are the mean ± SD of 3 replicates.

2.3.6.4. DNA Protection activity

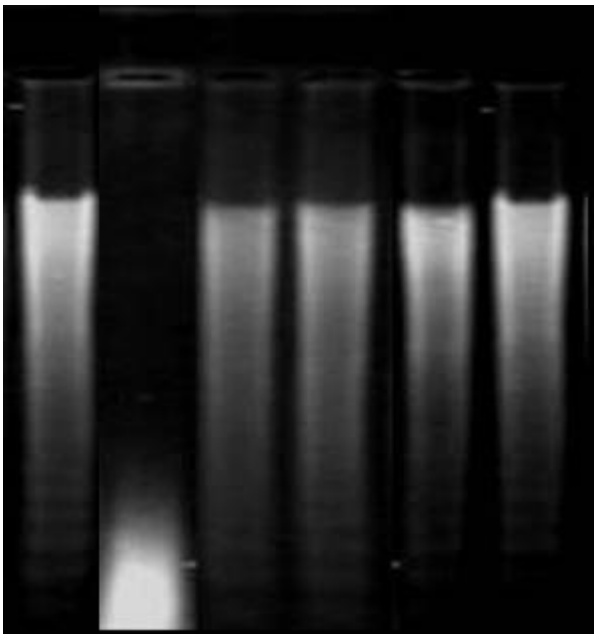
DNA fragmentation by Fenton's reagent was recovered at varying degrees with the treatment of ginger/mango ginger aqueous extract and phenolic fractions prior to oxidative stress (as visualized by increased electrophoretic mobility of DNA). A dose-dependent protection was observed by both free and hydrolyzed phenolics of ginger and mango ginger at 2 to 4 µg GAE respectively (Figure 2.18 & 2.19). A significant (>80 %, $p<0.005$) protection to native DNA during oxidation in the presence of these fractions was observed. These results indicate that free and bound phenolics of mango ginger can quench free radicals and thereby may protect the DNA against oxidative stress-induced damage.

Figure 2.18. DNA protective ability of free and hydrolyzed phenolics of ginger



1 µg of native calf thymus DNA in (Lane 6); 1 µg DNA treated with Fenton's reagent (Lane 5); DNA pretreated with 2-4 µg of GRFP (Lane 1, 2) and GRHP (Lane 3, 4) followed by Fenton's reagent were loaded on to the 1% agarose gel. Bands were visualized by staining with Ethidium bromide and in the transilluminator increased mobility represents DNA damage.

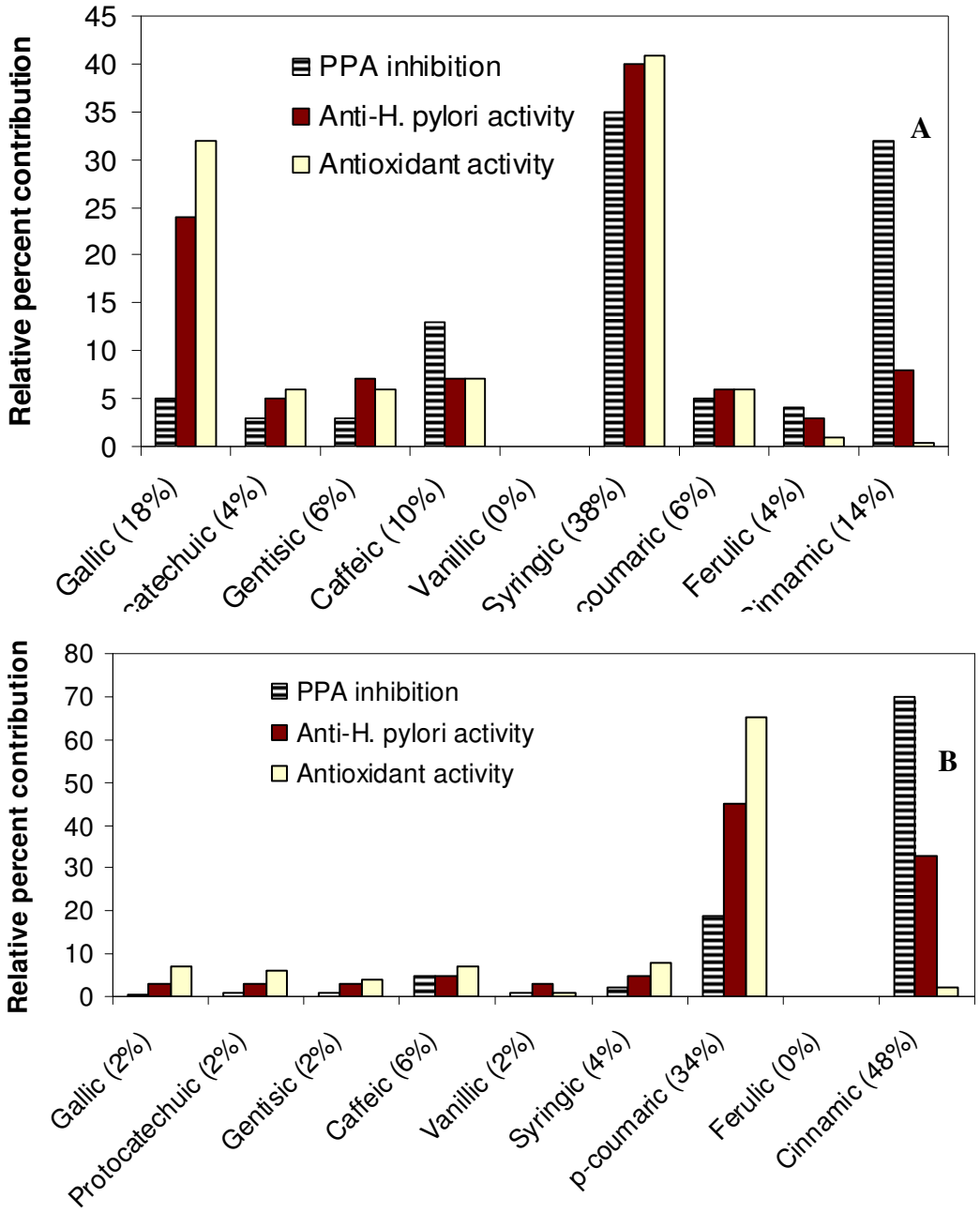
Figure 2.19. DNA protective ability of free and hydrolyzed phenolics of mango ginger



Native DNA	+	+	+	+	+	+
Fenton's reagent	-	+	+	+	+	+
MGHP	-	-	+	+	-	-
MGFP	-	-			+	+

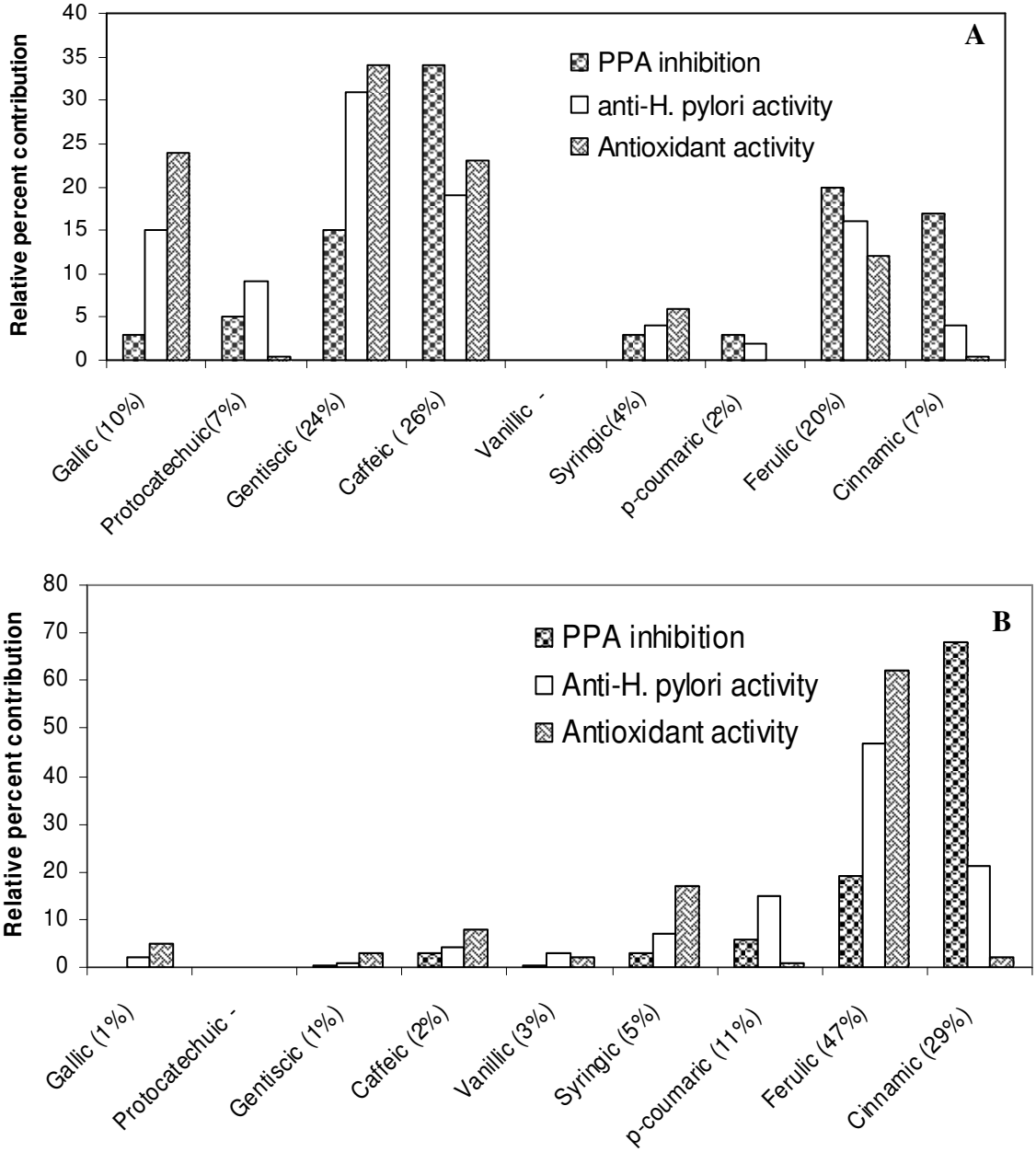
1 µg of native calf thymus DNA in (Lane 1); DNA treated with Fenton's reagent (Lane 2); DNA pretreated with 2-4 µg of MGFP (Lane 2, 3) and MGHP (Lane 3, 4) were loaded on to the 1 % agarose gel. Bands were visualized by staining with Ethidium bromide and in the transilluminator increased mobility represents DNA damage.

Figure 2.20. Relative percentage contribution of individual phenolic acids of ginger towards PPA inhibition, anti-*H. pylori* and antioxidant activity.



Relative percentage contribution of individual phenolic acids toward antioxidant, anti *H. pylori*, and PPA inhibition. This graph depicts the relative percent contribution of each phenolic acid found in GRFP (A) and GRHP (B) against H^+, K^+-ATPase activity (red bars), anti *H. pylori* (brown bars), and antioxidant activity (yellow bars). Percentage indicated in parentheses under each phenolic acid depicts the actual percent of them as revealed by HPLC.

Figure 2.21. Relative percentage contribution of individual phenolic acids of mango ginger towards PPA inhibition, anti-*H. pylori* and antioxidant activity.



Relative percentage contribution of individual phenolic acids toward antioxidant, anti *H. pylori*, and PPA inhibition. This graph depicts the relative percent contribution of each phenolic acid found in MGFP and MGBP against H^+, K^+-ATPase activity (red bars bars), anti *H. pylori* (brown bars), and antioxidant activity (yellow bars). Percentage indicated in parentheses under each phenolic acid depicts the actual percent of them as revealed by HPLC.

Maximum H^+, K^+-ATPase inhibition is contributed by cinnamic acid both in ginger (63%) and mango ginger(48%) followed by 12 % by syringic acid in ginger and 17 % each by caffeic acid and ferulic acids in mango ginger respectively. Contribution by cinnamic acid is attributed to increased potency of cinnamic acid in inhibiting H^+, K^+-ATPase compared to other phenolic acids.

2.3.7. Total percent contribution to various bioactivities by ginger and mango ginger

As depicted under materials and methods total percent contribution by individual phenolic acids were determined in both free and hydrolyzed phenolic fractions (Eg. % contribution in ginger = % contribution from GRFP + % contribution from GRHP) and the data represented in Table 2.10 & 2.11. Different phenolic acids present in both free and hydrolysed phenolics were considered in ginger/mango ginger. Average of the total sum of each of these phenolic acids has been considered for total abundance. Relative percent contribution was calculated based on the potency of individual phenolic acids to inhibit H^+, K^+ -ATPase activity, anti- *H. pylori,* antioxidant activity and the total abundance as described in our paper (Siddaraju & Shylaja 2007b).

Table 2.10. Total percent contribution of individual phenolic acid of ginger

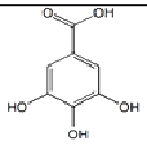
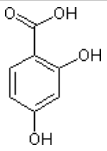
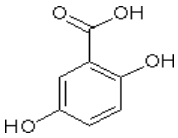
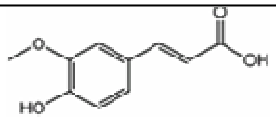
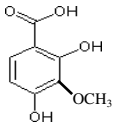
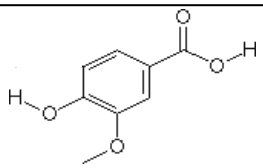
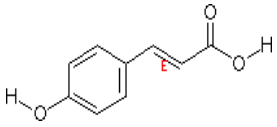
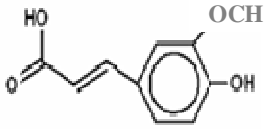
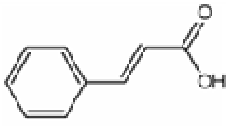
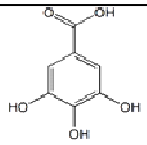
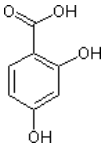
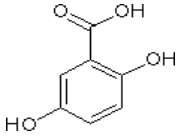
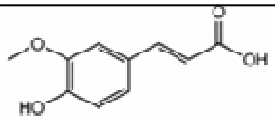
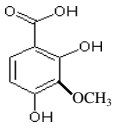
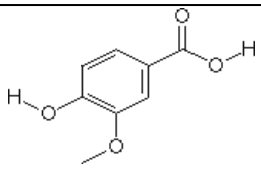
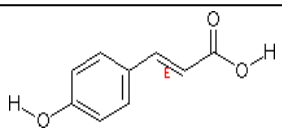
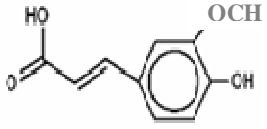
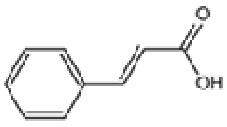
Phenolic acids	PPA inhibition (%)	*H. pylori*- inhibition (%)	AOX Activity (%)
Gallic acid (3,4,5-Trihydroxy benzoic acid) 	**5**	**31**	**44**
Protocatechuic acid (3,4-Dihydroxybenzoic acid) 	**2**	**3**	**5**
Gentisic acid (2,5-Dihydroxy benzoic acid) 	**1**	**4**	**4**
Caffeic acid (3,4-Dihydroxy cinnamic acid) 	**6**	**4**	**5**
Vanillic acid (4-Hydroxy-3-dimethoxybenzoic acid 	**1**	**1**	**1**
Syringic acid (4-Hydroxy-3,5-dimethoxybenzoic acid) 	**12**	**15**	**16**
p-coumaric acid (p-Hydroxy cinnamic acid) 	**8**	**17**	**24**
Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) 	**1**	**1**	**1**
Cinnamic acid (3-Phenyl-2-propenoic acid) 	**63**	**23**	**1**

Table 2.11. Total percent contribution of individual phenolic acid of mango ginger

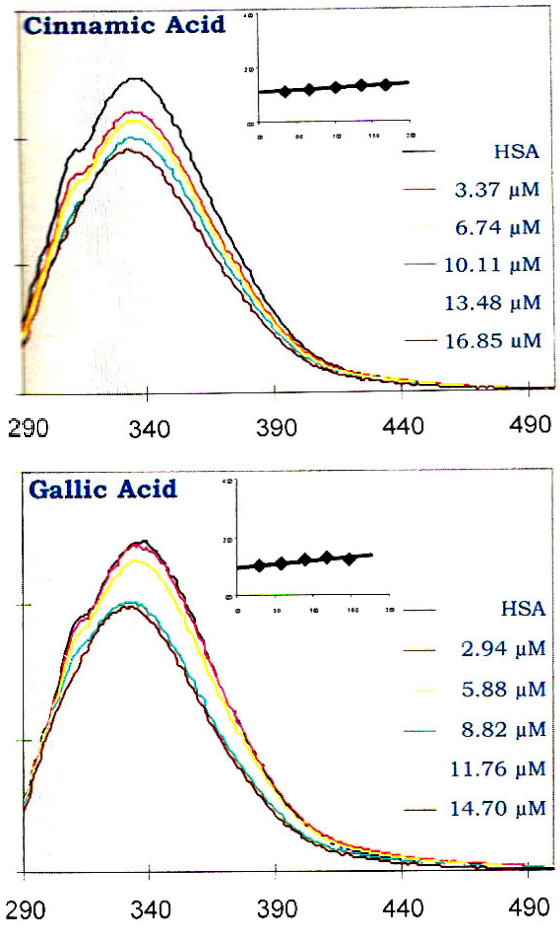
Phenolic acids	PPA inhibition (%)	*H. pylori*- inhibition (%)	AOX Activity (%)
Gallic acid (3,4,5-Trihydroxy benzoic acid) 	**2**	**14**	**24**
Protocatechuic acid (3,4-Dihydroxybenzoic acid) 	**2**	**5**	**3**
Gentisic acid (2,5-Dihydroxy benzoic acid) 	**6**	**14**	**17**
Caffeic acid (3,4-Dihydroxy cinnamic acid) 	**17**	**11**	**15**
Vanillic acid (4-Hydroxy-3-dimethoxybenzoic acid 	**1**	**1**	**1**
Syringic acid (4-Hydroxy-3,5-dimethoxybenzoic acid) 	**3**	**6**	**11**
p-coumaric acid (p-Hydroxy cinnamic acid) 	**3**	**7**	**1**
Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) 	**17**	**25**	**27**
Cinnamic acid (3-Phenyl-2-propenoic acid) 	**48**	**16**	**1**

2.3.8. HSA - phenolics interaction studies

Since there was a significant reduction in H^+, K^+-ATPase which could be attributed to phenolics as clearly demonstrated in the previous paper6, current study attempts to explore the possible binding of phenolics to the enzyme by virtue of phenolic acids. For comparative purpose two phenolic acids – gallic acid and cinnamic acids that showed poorer and potent H^+, K^+ -ATPase inhibitory activity respectively were examined in presence and absence of gallic/cinnamic acids and expressed as Sterner's constant.

Results from HSA interaction studies indicated that the changes occurred in the environment of tryptophan residues in HSA and was dependent on the applied phenolic acids. As shown in Fig. 6 both gallic and cinnamic acids showed HSA binding, but to varying extent. Ksv of cinnamic and gallic acid were found to be 0.024 x 106 M-1 and 0.016 x 106 M-1 respectively. ~ 1.5 fold increase in binding was observed with cinnamic acid than gallic acid. However, 5 fold better H^+, K^+-ATPase inhibition with cinnamic acid than gallic acid suggests that parameters other than binding may also influence H^+, K^+-ATPase inhibitory activity in case of cinnamic acid. Cinnamic acid being hydrophobic, may access the membrane domain of H^+, K^+-ATPase which is lacking in HSA, may possibly accounted for enhanced inhibitory activity.

Figure 2.22. Fluorescence emission spectra of HSA in the presence of phenolic acids



Phenolic acid	Stern-Volmer constant, Ksv(M^{-1})	Regression coefficient (r)
Gallic acid	0.016 x 10^6 M^{-1}	0.912
Cinnamic acid	0.024 x 10^6 M^{-1}	0.983

For all solutions, the concentrations of HSA were constant: 0.5µM. The excitation wave length was 280 nm. Both excitation and emission slit widths were 5 nm. Inlaid figure- stern-volmer plots (x-axis: concentration in µM; y-axis: F°/F)

2.4. Discussion

The phenolic acids and their antiulcer, antioxidant activities in ginger and mango ginger - free and hydrolzed phenolic fractions warranted a thorough investigation. Generally phenolics have the tendency to bind to various biomolecules particularly to polysaccharides and hence are possible to exist in bound form in natural resources. Varieties of dietary sources studied previously in our laboratory indicated the presence of various health beneficial phenolic compounds (Suresh Kumar et al 2006 & Leela srinivas et al 1992). In this study, we report the potential ulcer preventive ability of free and hydrolyzed phenolics of ginger by evaluating antioxidant, anti *H. pylori*, proton pump inhibition and DNA protective ability.

Both free and hydrolyzed phenolics of ginger and mango ginger exhibited potential H^+, K^+-ATPase inhibition property. 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. Ginger and mango ginger phenolic fractions GRFP/GRHP and MGFP/MGHP inhibited gastric H^+,K^+-ATPase activity in a concentration dependent manner (Fig. 2.7). Concentrations required to inhibit 50% of H^+,K^+-ATPase activity is designated as IC_{50} and GRFP and GRHP showed an IC_{50} of 2.9 ± 0.18 and 1.5 ± 0.12 µg/mL, respectively; when compared to IC_{50} of 19.3 ± 2.2 µg/mL of lansoprazole (Table 2.4), a known proton pump inhibitor. Approximately 7 and 13 fold increase in activity in GRFP and GRHP respectively was observed over Lansoprazple. MGFP and MGHP also showed 9 and 7 fold better H^+, K^+-ATPase inhibitory activity than that of the lansoprazole. Thus phenolic fractions were found to be good inhibitors of the enzyme and the inhibition could be due to the binding of phenolic acids to H^+,K^+-ATPase enzyme similar to those of other phenolics (Ricardo Reyes-Chilpa et al., 2006). In order to understand the probable ability of individual phenolic acids present in extracts in inhibiting gastric H^+,K^+-ATPase, pure phenolic acids were examined for their ability to inhibit the enzyme. As indicated in Table 2.5, cinnamic acid showed maximum inhibitory effect with an IC_{50} of 15.1 µg/mL, followed by caffeic (IC_{50} 27.1 µg/mL), ferulic (IC_{50} 33.6 µg/mL), syringic (IC_{50} 37.4 µg/mL), p-coumaric (IC_{50} 39.7 µg/mL), protocatechuic (IC_{50} 47.1 µg/mL), gentisic (IC_{50} 59.1 µg/mL) and gallic acid (IC_{50} 132.1 µg/mL).

The fact that GRHP inhibited H^+, K^+ -ATPase better (IC_{50} of 1.5 ± 0.12 µg/mL) than GRFP (IC_{50} of 2.9 ± 0.18 µg/mL), corroborates with the presence of increased levels of cinnamic acid in

GRHP (48 μg/g) than in GRFP (14 μg/g). In mango ginger also, MGHP inhibited H^+, K^+-ATPase 3 fold better (0.7 ± 0.08 μg/mL) than MGFP (2.2 ± 0.21 μg/mL) and this correlates with the presence of increased levels of cinnamic (237 mg/g) and ferulic acids (391.5 mg/g).

As indicated in Figure 2.21 total of 86% of the H^+, K^+-ATPase inhibition in MGFP is contributed by caffeic, gentisic, ferulic and cinnamic acids and in MGHP 70% of the PPA inhibition is contributed by cinnamic acid alone followed by -17% ferulic acid. The differences in the activity could be attributed to differences in phenolic acid composition in each source, based on the results obtained with standard phenolic acids (Table 2.5). Current study therefore reveals that GRFP/MGFP and GRHP/MGHP have stronger ability to inhibit H^+, K^+-ATPase by phenolic acids present in them. It is indicated both in the literature and from our study that H^+, K^+-ATPase is upregulated in ulcer condition (Sachs et al 1995), inhibition of the same therefore would result in gastric protection or antiulcer property.

Another potential property to demonstrate in test extracts is the determination of *H pylori* inhibitory effects of the extracts, since emerging trends in ulcer research suggest that *H pylori* is a major causative factor for ulcer. Studies therefore addressed the inhibition of *H pylori* growth.

H. pylori obtained is a Gram-negative, acid tolerant, microaerophelic bacterium that lives in the stomach and duodenum (Marshall & Warren, 1984). *H. pylori* have co-evolved with human and these species are indigenous to stomach of more than 5% population (Mitchel & Mégraud, 2002). Most chronic infection of *H. pylori* is asymptomatic and if colonization of bacteria persists, symptoms appear in 15-20% of the infected population and are associated with gastric ulcers. In the current study we have investigated the ability of phenolic fractions of ginger and mango ginger to inhibit the growth of *H. pylori*. Relative percentage contribution of each phenolic acid in inhibiting *H. pylori* by GRFP/MGFP and GRHP/MGHP was calculated and depicted in Figure 2.20 & 2.21 and Table 2.10. In MGFP, gentisic, caffeic and ferulic acids contributed ~66% towards the inhibition of *H. pylori* and although gallic acid is poorer in activity, due to its higher abundance contributed also significantly to *H. pylori* inhibition in MGFP. In MGHP, ferulic, cinnamic and p-coumaric acids contributed ~83%.

Results clearly suggest that phenolics of ginger and mango ginger are very potent in inhibiting *H. pylori* activity. Resutls are further supported by observation of Tabak (2005) and Vattem et al. (2005) where, phenolic phytochemicals such as cinnamic acid, cinnamaldehyde, coumarins and flavonoids have been suggested to exhibit high anti *H. pylori* activity. As shown in Figure 2.21, in MGFP, gentisic, caffeic and ferulic acids contributed ~ 66% toward the inhibiton of *H. pylori* and although gallic acid is poorer in activity, due to its higher abundance, it contributed also significantly to *H. pylori* inhibition in MGFP. In MGHP, ferulic, cinnamic, and *p*-coumaric acids contributed 83%.

Relative percent contribution towards potential ulcer preventive properties such as H^+, K^+ -ATPase inhibition, *H. pylori* inhibition and antioxidant activity was calculated per total gram weight of ginger and mango ginger. Data presented in Table 2.10 indicate that cinnamic acid is a major contributor both in ginger (63%) and mango ginger (48%). This is due to increase in specific activity as well as abundance. In case of *H. pylori* growth inhibition, activity is distributed between gallic acid (31%) followed by cinnamic (23%), p-coumaric (17%) and syringic acid in ginger. While in mango ginger, maximum activity is contributed by ferulic acid (27%) followed by

Data thus points out that although gallic acid is poorer in activity, contributing maximally to *H. pylori* inhibition in ginger (31%) and mango ginger (14%) due to increased abundance. Similarly increased antioxidant activity and abundance results in 44% contribution to antioxidant activity in ginger. In mango ginger however highest activity 27% was from cinnamic acid followed by gallic acid (34%). H^+, K^+ -ATPase and antioxidant activity (8-9 fold / 6 fold increase) in isolated phenolic fractions of ginger and mango ginger when compared to that of crude aqueous extracts – GRAE and MGAE suggest that phenolic acids play a critical role in these activities. In *H. pylori* inhibition however in mango ginger, activity was the same in MGAE and MGFP / MGHP suggesting that other than phenolic present in MGAE may also play significantly in inhibiting *H. pylori*. Detailed analysis are underway currently in the laboratory.

Phenolics were thought to exert their antimicrobial effect by causing a) hyper acidification at the plasma membrane interface of the microorganism (Shetty et al., 1998) or b) intracellular acidification, resulting in disruption of H^+, K^+-ATPase required for ATP synthesis of microbes

(Vattem et al 2004) or c) may be related to inactivation of cellular enzymes causing membrane permeability changes (Shahidi et al 2004). The rate of inactivation of microbial cellular enzymes is dependent on the rate of penetration of phenolic antioxidants into the cell. In case of *H. pylori*, phenolics may be inactivating the urease enzyme, which is specifically expressed at its surface to neutralize hyper acidification to survive in the gastric environment of the stomach (Catherine et al 1990).

Ginger has been known to contain gingerols and other bioactive compounds such as zingiberene, zingiberol etc. Gingerol appear to be a major constituent (1-3%) contributing to antiulcer, anti *H. pylori* (Mahady et al 2003) and antioxidant (Hirahara, 1974) activities. Similarly in mango ginger, curcumin has been shown to exhibit antioxidant activity. In the current study however phenolics extraction excludes such reported bioactive compounds since they are volatile and hydrophobic in nature. Current study thus may emphasise the role of phenolics as they offer ~ 1.6 fold better contribution to anti-ulcer/anti-*H. pylori*/antioxidant activity besides their stability under extraction/physiological conditions. In the current study we have isolated the phenolic acids, which are devoid of curcumin and other bioactive compounds in order to understand the contribution by phenolic acids in phenolic fractions of mango ginger.

The scavenging activity of GRFP/MGFP and GRHP/MGHP on DPPH free radical was determined which primarily evaluates the proton radical scavenging ability of the phenolic compounds. DPPH is a stable, free radical compound that possesses a proton free radical with a characteristic absorption at 517 nm which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al 1998). It is well established that DPPH free radical scavenging by antioxidants is mainly due to their hydrogen donating ability (Chen & Ho, 1995). In the present study GRFP showed better radical scavenging activity with IC_{50} of 1.7 ± 0.07 µg/mL compared to GRHP - 2.5 ± 0.16 µg/mL**.** The scavenging activity was directly attributed to their phenolic content, since we have shown antioxidant potencies of pure phenolic acids, which are the representative antioxidant components of ginger extracts. MGFP and MGHP also showed concentration dependent radical scavenging activity (Figure 2.17A). MGFP showed better radical scavenging activity with IC_{50} of 2.2 ± 0.17 µg/mL compared to MGHP - 4.2 ± 0.36 µg/mL. The scavenging activity was directly attributed to their phenolic content, since we have shown antioxidant potencies of pure phenolic acids, which are the representative antioxidant components of mango ginger extracts.

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant property by breaking the free radical chain by donating hydrogen atom (Siddhuraju et al 2002). The reducing power of the sample increased with increase in concentration of phenolics (Fig. 2.16B & 2.17B). Approximately 4 fold increase in the total reducing power was observed in GRFP than GRHP with 338.15 ± 34 U/g and 80.9 ± 11 U/g respectively. The reducing power of MGFP showed approximately two fold increase in the total reducing power than MGHP (Figure 2.17B). MGFP and MGHP exhibited 193 ± 21 U/g and 104 ± 8.6 U/g reducing ability respectively. Thus, the data presented here may indicate that the phenolic fractions tested may act as reductones by donating electrons to free radicals and there by convert free radicals to more stable product and terminates free radical chain reaction.

Lipid peroxidation generates a number of degradation products, such as malondialdehyde (MDA), hexanol *etc.* and is found to be an important cause of cell membrane destruction and cell damage (Yoshikawa et al 1991). MDA is a highly reactive species and crosslinks DNA with protein and thus damages the cells (Kubow 1990), disrupts its activity leading to chronic diseases. In the present study we measured the potential of GRFP/GRHP and MGFP/MGHP to inhibit lipid peroxidation products (TBARS). GRFP showed maximum inhibition of lipid peroxidation with an IC_{50} 3.6 ± 0.21 µg GAE/g sample compared to GRHP (5.2 ± 0.46 µg GAE/g sample). MGFP showed maximum inhibition of lipid peroxidation with an IC_{50} of 10.3 ± 0.91 µg GAE/g sample compared to the IC_{50} of MGHP (15.6 ± 1.6 µg GAE/g) (Figure 2.17C).

DNA protection ability is also an important test to consider the test compounds as antiulcer. Cellular damage encountered during ulcer condition has been known to also damage DNA. DNA protective ability therefore has been addressed. The Fenton's reagent causing DNA fragmentation (as visualized by increased electrophoretic mobility of DNA) was recovered with the treatment of GRFP/MGFP and GRHP/MGHP extracts prior to oxidative stress. A dose-dependent protection was observed by both free and hydrolysed phenolics of ginger at 2 to 4 µg GAE respectively (Fig. 2.18). A significant (>80%, $p<0.005$) protection to native DNA during oxidation in the presence of these fractions was observed. These results indicate that free and bound/hydrolyzed phenolics of ginger can quench free radicals and thereby may protect the DNA against oxidative stress-induced damage.

Since reduction in H^+, K^+ -ATPase and *H. pylori* inhibitory properties were attributed to mainly cinnamic acid and gallic acid, probable mechanism of inhibition was studied by interaction studies. They were believed to bind to the protein or *H. pylori* protein hence the inhibition. However results of binding studies as given in Figure 2.22 did not show differences in the binding between cinnamic acid a potent inhibitor of H^+, K^+ -ATPase and *H. pylori* and gallic acid, a poor inhibitor. However it is possible that cinnamic acid being hydrophobic in nature than gallic acid may bind effectively particularly to the membarane domain of H^+, K^+ -ATPase enzyme and *H. pylori*. Results can be substantiated by verifying the differential interaction ability with protein carrying membrane domain as that of H^+, K^+ -ATPase or *H. pylori* than with stable protein HSA.

Thus phenolic acids in both ginger and mango ginger by virtue of exhibiting the ability to inhibit H^+, K^+-ATPase, which causes acid secretion leading to gastric acidity, inhibit *H. pylori*, which otherwise may aggravate gastric ulcers and antioxidant potencies which puts a check on the continuous damage on mucosal epithelium may be potential candidate for prevention or management of gastric ulcer. This is the first report on the role of phenolic acids in exhibiting potential antiulcer property in both ginger and mango ginger.

2.5. Summary and Conclusions

- Ulcer is a common global problem characterized by acute gastric irritability, bleeding etc, due to either increased gastric cell H^+, K^+ -ATPase activity (PPA) or perturbation of mucosal defense. *H. pylori* has been identified as a major ulcerogen.

- In this chapter we report in aqueous extracts of ginger (GRAE), Mango ginger (MGAE) and their free and bound or hydrolyzed phenolic fractions (GRFP/GRHP and MGFP/MGHP) as potent inhibitors of PPA and *H.pylori* growth besides exhibiting antioxidant properties.

- GRFP/GRHP and MGFP/MGHP inhibited PPA at an IC_{50} of 2.9 ± 0.18/1.5 ± 0.8 µg/mL and 2.2 ± 0.21/0.7± 0.08 µg/mL respectively, exhibiting ~ 6 to 27 fold potent activity than lansoprazole, a known proton pump inhibitor suggesting that phenolics may play a crucial role in inhibiting PPA.

- Potent *H.pylori* and antioxidant activity exhibited by both, extracts and phenolic fractions of GR and MG, more or less to a similar extent as that of standard *H.pylori* inhibitor - amoxicillin and antioxidant - gallic acid respectively, suggest the potentiality of the selected sources to employ against ulcers.

- Determination of precise composition and their contribution to various assays suggest that cinnamic, ferulic and caffeic acids possessed significant PPA and H.pylori inhibitory while gallic, protocatechuic, gentisic and syringic acid possessed antioxidant activity. Increased specific activity and abundance of cinnamic and gallic acid respectively has been attributed to potential antiulcer property in GR and MG.

- Cinnamic acid and ferulic acid that contained less free hydroxyl groups are believed to contribute significantly to PPA and *H.pylori* inhibitory activity; while gallic acid rich in hydroxyl groups have been found to contribute to antioxidant activity.

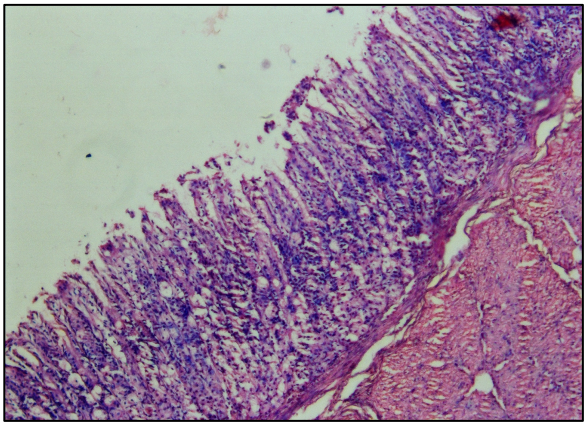
- Phenolic acids may exert their effect in inhibiting PPA and *H.pylori* preferably by binding to them. Results of the binding of various phenolic acids to BSA suggest that cinnamic acid may possess potent inhibition of PPA and *H.pylori,* probably by binding to their membrane domains.

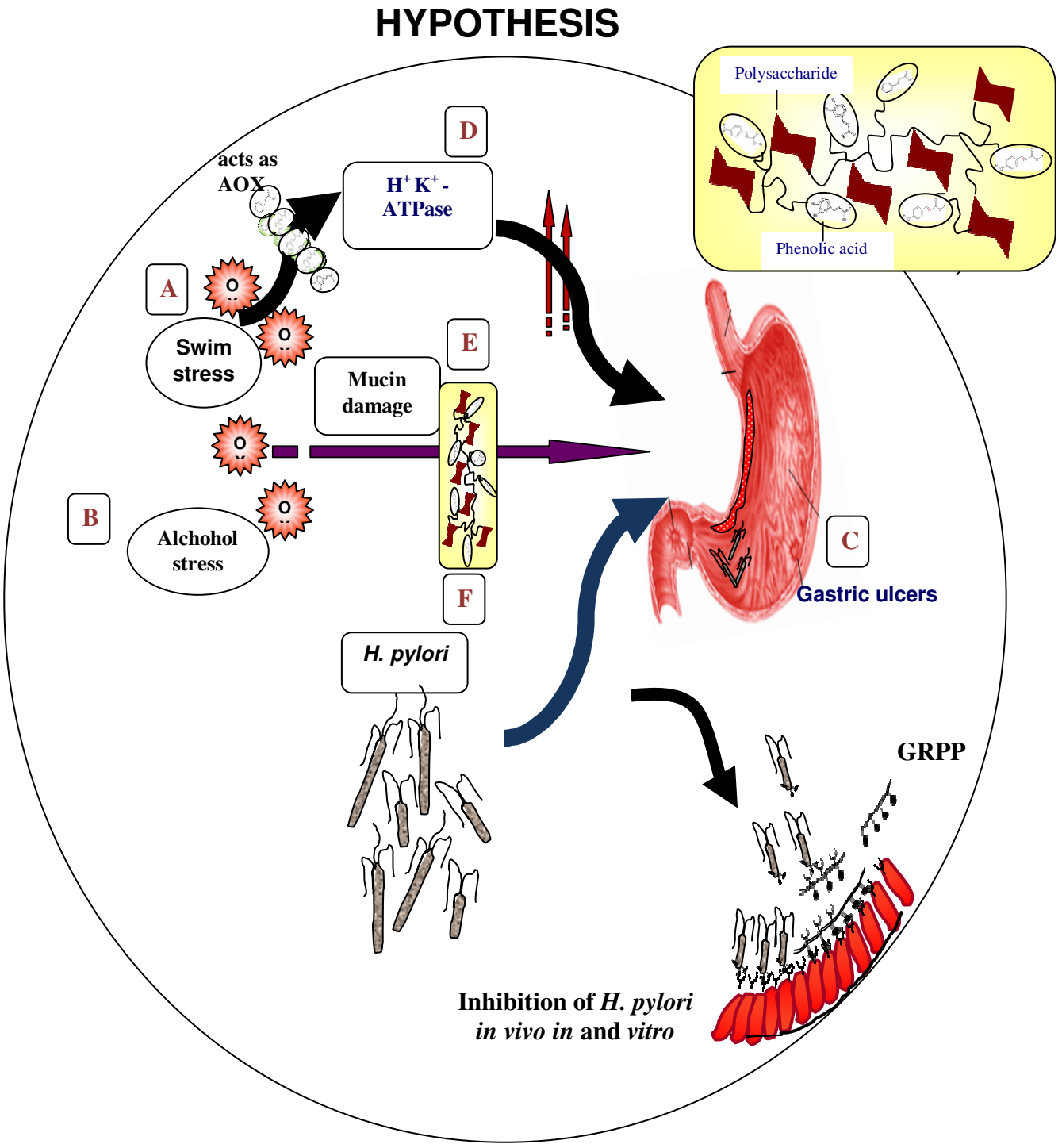
- Significant levels of phenolics found in GRAE (7.6 mg/g); MGAE (5.4mg/g), GRFP (2.6 mg/g); MGFP (2.1 mg/g), GRHP (1.1 mg/g) and MGHP (1.9 mg/g) together with multi-potent antioxidant activity including free radical scavenging, reducing power, anti-lipid peroxidation and DNA protection abilities and PPA and *H pylori* inhibition adds to the scope of the use of GR and MG as potential alternatives against gastric ulcer.

- This is the first report on the role of phenolics in exhibiting antiulcer potentials in ginger and mango ginger.

Chapter 3

Studies on the Mechanism and Anti-Ulcerative Action of Antioxidant and Polysaccharide Fractions *In Vitro* and *In Vivo* ; Individually and in Combination

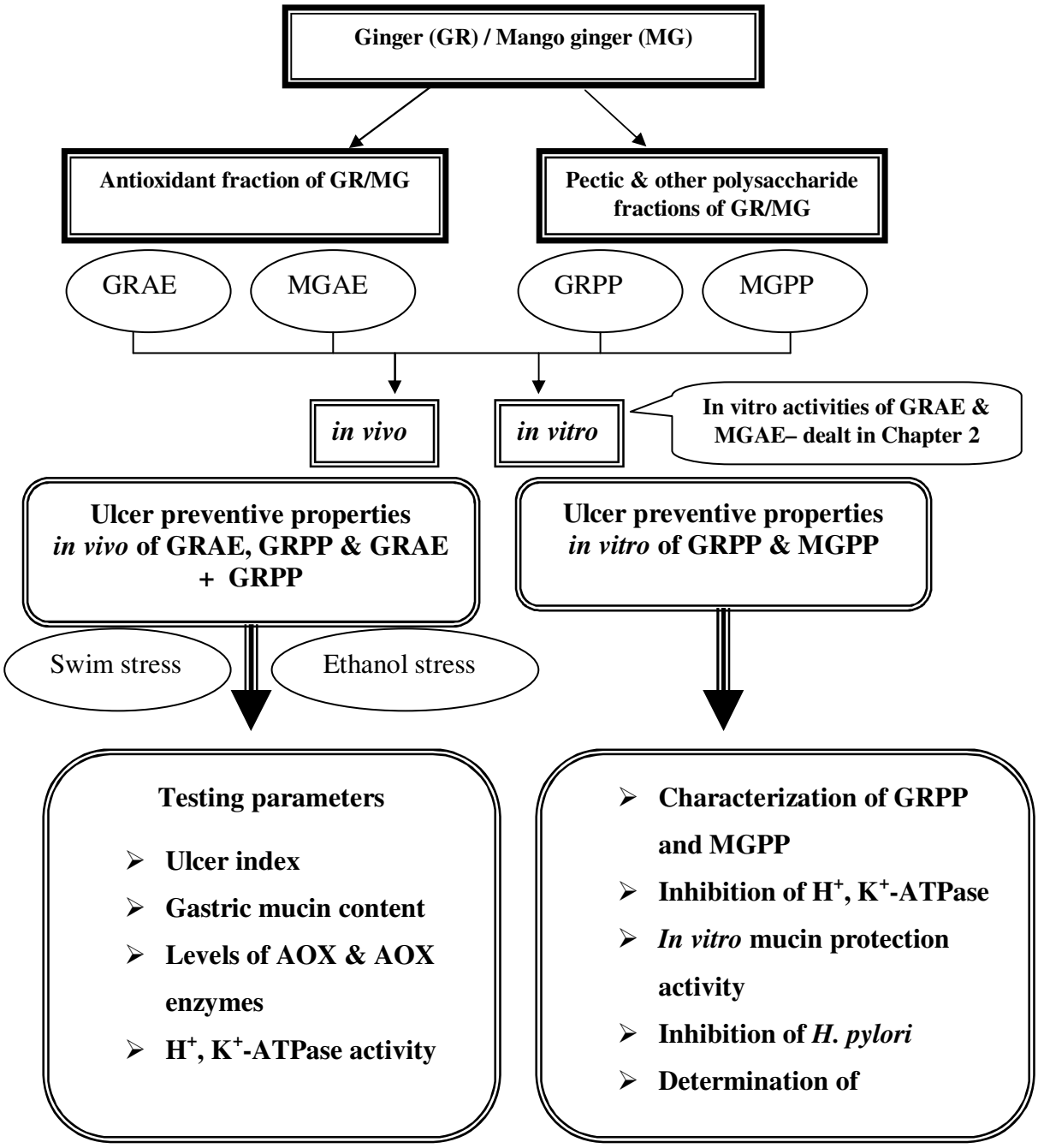




Swim [A] and alcohol [B] stress results in oxidative stress and gastric ulcers [C] via activation of H^+, K^+ -ATPase [D] and mucosal damage [E]. Damaged mucin is known to be susceptible for infection and hence severity [F]

Question addressed is….. Can ginger and mango ginger constituents prevent ulcers ? If so at what steps ?

WORK PLAN


Ginger (GR) / Mango ginger (MG)
Antioxidant fraction of GR/MG
Pectic & other polysaccharide fractions of GR/MG
GRAE
MGAE
GRPP
MGPP
in vivo
in vitro
In vitro activities of GRAE & MGAE– dealt in Chapter 2
Ulcer preventive properties in vivo of GRAE, GRPP & GRAE + GRPP
Ulcer preventive properties in vitro of GRPP & MGPP
Swim stress
Ethanol stress
Testing parameters
Ulcer index
Gastric mucin content
Levels of AOX & AOX enzymes
H+, K+-ATPase activity
Characterization of GRPP and MGPP
Inhibition of H+, K+-ATPase
In vitro mucin protection activity
Inhibition of H. pylori
Determination of

3.1. Introduction

During the past several decades, there has been a global trend for the revival of interest in the traditional system of medicine. Simultaneously the need for basic scientific investigation of medicinal plants using indigenous medical systems has become ever more interesting and relevant. A recent review of references indicates that the antiulcerogenic effects of many taxa of medicinal plants have been assessed worldwide (Gürbüz et al 2003. Watanabe et al, 1986). It is also reported that many plant species have been used to alleviate gastric symptoms such as gastric pain, gastric distribances, ingestion etc., in Turkish folk medicine (Gürbüz et al 2002).

The gastrointestinal tract is subjected to a wide variety of mucosal challenges such as *Helicobacter pylori* associated ulcer disease, non-steroidal anti-inflammatory drug (NSAID) associated ulcers, alcohol induced mucosal injury, and a variety of inflammatory conditions, including ulcerative colitis and Crohn's disease. No matter what the cause of the ulceration, the mucosa usually responds rapidly by triggering a cascade of repair mechanisms to stimulate repair mechanism to restore mucosal integrity.

H. pylori is recognized as a cause of chronic active gastritis, gastric and duodenal ulcers (Marshall & Warren, 1984) finally leading to gastric cancer. Infection with *H. pylori* causes gastritis, and may be associated with gastric and duodenal ulcers and also with such malignant diseases as MALT lymphoma and gastric carcinoma (Alexander et al. 1997). However the mechanisms of pathogenesis for *H. pylori*-associated diseases are not yet well understood. The ecological niche to which *H. pylori* is well adapted is the mucous layer of the human gastric antrum, which has mucin glycoproteins as major constituents. Mucins, high-molecular weight carbohydrate-rich glycoproteins that coat the surface of the stomach are secreted into the lumen and function to protect the stomach. It could also be important in *H. pylori* colonization. For further understanding of the pathogenesis of *H. pylori* related diseases, it is important to consider whether *H. pylori* colonization of the surface epithelium is associated, as cause or effect, with changes in the gastric mucin synthesized by surface mucous cells.

Mucins are thought to protect the surface of the gastrointestinal tract from mechanical damage, from dessication and from chemical irritants (Young et al, 1995). Gastric mucins are the major components of an unstirred mucous-bicarbonate layer that protects the gastric epithelium from the high concentrations of acid in the stomach lumen and from autodigestion by pepsin. The protective functions of the gastric mucous layer imposes rigid requirements on the structure of gastric mucins (Figure 1). They are very high in molecular weight and are heavily substituted with O-linked oligosaccharides. Human mucins are encoded by at least nine distinct mucin genes, of which three, MUC1, MUC5AC, and MUC6, are expressed at high levels in the normal stomach (Table 1).

Unlike most other mucins, the MUC1-encoded protein has a transmembrane segment and a cytoplasmic tail and therefore is important to interact with the irritant or an ulcerogen. The O-linked carbohydrates on MUC1 mucin are heterogeneous.

Array of literature is also building up to overcome the damage of mucin caused by gastric irritants. Currently available anti-ulcer drugs pose side effects and limitations in the use. Long term use of anti-ulcer drugs and drugs for other diseases are known to cause several health problems including causation of gastric cancers (Waldum et al, 2005). Also, it is documented that irrespective of the type of health complications, emergency patients have been reported to become susceptible for gastritis and gastric ulcers, which is attributed to trauma and stress (Miller, 1987). In this perspective now a day's natural product from dietary and phytal sources are in demand. Therefore use of products from natural resources are gaining attention for their less toxic but effective implications. In developing countries, including India the use of phytal sources especially from the traditional plants for the treatment of gastric disorders has been popular, partly because of their low cost and minimal side effects.

Plant and plant products are being used as a source of diet and medicine from time immemorial. Diet invariably contains carbohydrates of various size, concentrations and chemical sequences strating from simple monosacchrides to complex polysacchrides. Among them pectic polysaccharides in particular have been shown to play critical therapeutic roles against ulcer (Matsumoto et al, 2002, Ye et al, 2003, Gao et al, 2004), cancer (Wei et al, 2006) etc. Recently, polysaccharides have gained importance due to their role played in controlling ulcer (Kiyohara et al, 1994).

In our previous work, chapter II we have discussed the role of phenolics of ginger and mango ginger in contributing to potential antiulcerative action via inhibition H^+, K^+-ATPase and *Helicobacter pylori*, a major ulcerative pathogen in addition to the antioxidative properties (Siddaraju & Shylaja 2007a, 2007b). Besides this we have also demonstrated the multi-potent anti-ulcerative ability of a novel phenol bound pectic polysaccharide from Decalepis hamiltonii[8] (Srikanta et al. 2007).

In this chapter we have undertaken a detailed investigation on the antiulcer potentials particularly; a) In vivo efficacy of aqueous extracts of ginger (GRAE); b) Both in vitro and in vivo effect of pectic polysaccharide fractions of ginger (GRPP) and mango ginger (MGPP).

Objectives:

1. **The *in vitro* ulcer preventive effect of GRPP & MGPP.**
2. **Ulcer preventive & curative potential of GRPP (the most potent antiulcer component of ginger).**
3. **Combinational effect of GRPP and GRAE in ulcer preventive ability *in vivo*.**

.

3.2. Materials and Methods

3.2.1. Chemicals

Bovine serum albumin (BSA), lansoprazole, adenosine triphosphate (ATP), ammonium molybdate, (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) HEPES, monoclonal anti-gastric mucin, ethylenediaminetetraacetic acid (EDTA), triton X 100, tween 20, skimmed milk powder, paranitrophenyl phosphate (PNPP), diethanolamine, Alsever's medium, trypsin, hematoxylin, eosin, acridine orange, carbohydrate standards such as rhamnose, arabinose, xylose, mannose, galactose and glucose, protease, termamylase, glucoamylase, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sepharose CL-4B (4 % cross-linked, fractionation range for dextrans 30,000-50,000 Da), DEAE-cellulose (0.99 meq/g), Amberlite IR-120-P (8% cross-linked, 16-50 mesh), Dextran standards, T-Series viz., T-10, T-20, T-40, T-70, T-150, T-500, T-2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Alkaline phosphatase conjugated – rabbit anti mouse IgG secondary antibody was procured from GENEI, Bangalore, India. HPLC column (Shimpak C_{18}) was obtained from Shimadzu Corp. Tokyo, Japan. 3 % OV-225 (1/8"×6') on Chromosorb W (80-100 mesh) was from Pierce Chemical Company, Rockford, USA. Other chemicals such as hexane, ammonium oxalate, iodine solution, sodium phosphate buffer, perchloric acid ($HClO_4$), acetic acid, sodium acetate glutaraldehyde, glycine, sodium chloride, sulphuric acid and solvents used were of the analytical grade purchased from local chemical company. All chemicals and solvents used for HPLC and GLC were HPLC grade.

3.2.2. Isolation of aqueous ginger extract – GRAE

GRAE was prepared for *in vivo* studies as described in Chapter 2. Chapter dealt with the determination of *in vitro* antiulcer potentials in extracts of ginger and mango ginger. Although both methanol and aqueous extracts were prepared from ginger and mango ginger, aqueous extracts of ginger and mango ginger showed better activity. Of the two sources – Ginger and Mango ginger studied, ginger was selected since it showed better activity in all assays. Also, in traditional medicine, aqueous extract of ginger is reportedly given to alleviate the disease symptoms.

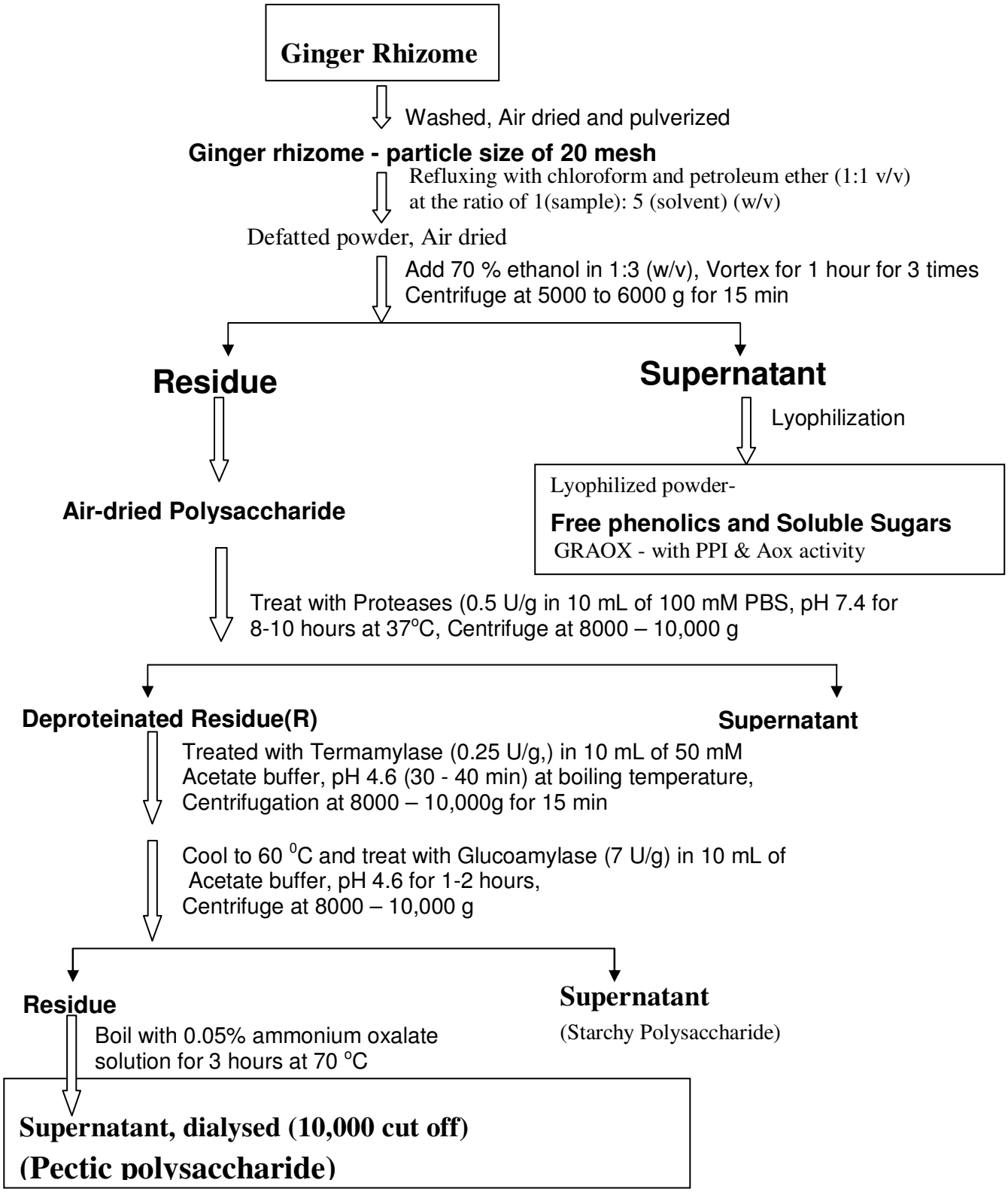
In this chapter therefore, particular attention has been emphasized to determine the potential antiucler property *in vivo* of the aqueous extracts of ginger as one of the objectives, in addition to the other objectives, as indicated earlier.

3.2.3 Isolation of pectic polysaccharide

Fresh ginger and mango ginger, were purchased from a local market (Devaraja Market, Mysore, Karnataka, India) and rhizomes were chopped into small pieces and air dried in the dark in a ventilated hood. The air dried samples of ginger and mango ginger rhizomes were ground and defatted in a soxhlet apparatus using hexane (200 mL/g, w/v). The defatted powder was air dried and preserved in dry condition until further extraction of pectic polysaccharides.

Pectic polysaccharides were isolated from defatted powder of ginger rhizome following the ammonium oxalate extraction method (Phatak et al, 1988) - **Scheme 3.1**. Briefly 100 g of defatted powder were depleted with proteins by protease at its optimum reaction condition. The residue was treated with termamylase to digest the starch and was digested till it showed negative to the iodine solution. The contents were cooled to 60 ^{0}C and then subjected to glucoamylase digestion for 1-2 h, centrifuged at 3000 g for 15 min, and the supernatant was dialyzed and lyophilized to get Water soluble polysaccharide (GWSP). The residue was precipitated with 0.05 % Ammonium oxalate and boiled for 3 hours; the obtained supernatant was precipitated with ethanol to get pectic polysaccharide (GRPP). Residue was processed further to get Hemi cellulose A (GHem A), Hemi cellulose B (GHem B) and Alkali insoluble residue (GAIR) fractions. As GRPP showed potent anti-ulcer property by inhibiting H^{+}, K^{+}-ATPase, H. pylori and also protection to mucin damage, it was further subjected to fractionation on DEAE cellulose Ion exchange column chromatography (Sathisha et al, 2007) and resolved fractions were analyzed for sugar composition analysis to understand structural implications.

Scheme 3.1: Preparation of Pectic Polysaccharide from Ginger / Mango ginger



3.2.4. Total phenol estimation

Total phenol was estimated as per the protocol (Singleton & Rossi, 1965) described in **Chapter 2.**

3.2.5. Total carbohydrate estimation

Total carbohydrate content was estimated (Dubois et al, 1956) in 0.5 mL of the sample (10 mg/100 mL of water) in a test tube. 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 (4-20 µg/mL).

3.2.6. Uronic acid estimation

Uronic acid was estimated (Bitter & Muir, 1962) in 0.5 mL of the sample solution (10 mg/100 mL) in a test tube and kept in ice cold water bath for 10 min. To this was added concentrated sulphuric acid (3 mL) slowly, contents were mixed thoroughly and kept in boiling water bath for 20 min. Contents were cooled to which carbazole solution (0.1 mL, 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 (10-50 µg/mL).

3.2.7. Fractionation of pectic polysaccharides; 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 M). After each treatment, the pH was adjusted to neutrality by washing thoroughly with water. 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.

The pectic polysaccharide (1 g) was dissolved in 2.0 mL of water and loaded on to DEAE-cellulose column and the elution was carried out with water, followed by ammonium carbonate (0.05 to 0.20 M) and sodium hydroxide (0.1 and 0.2 M) solutions. The flow rate was

maintained at 60 mL/h and fractions (10 mL) were collected, assayed for total sugar by phenol-sulfuric acid method as described earlier. Carbohydrate positive fractions were pooled, dialyzed and lyophilized. Fractions – 0.01 M, 0.1 M, 0.15 M, and 0.2 M, designated as GRPP - 0.05 M, GRPP – 0.1M, GRPP – 0.15 M and GRPP - 0.2M respectively were examined for potential antiulcer activity *in vitro* including inhibition of H^+, K^+ -ATPase activity, inhibition of *H.pylori* growth and determining antioxidant potencies in comparison with that of crude GRPP. Active fractions were further characterized by determining their sugar composition and comparison of structure – function relationship of each fraction.

3.2.8. Sugar composition analysis

3.2.8.1 Sulphuric acid hydrolysis

GRPP and GRPP fractions - 10 mg each was suspended in water and was hydrolyzed by prior solubilization with 72 % sulphuric acid at ice cold temperature followed by dilution to 8% acid and heating in a boiling water bath at 100 °C for 10-12 h. The above mixture was neutralized with barium carbonate (solid), filtered, deionized with Amberlite IR 120 H^+ resin and concentrated using a flash evaporator.

3.2.8.2 Regeneration of amberlite IR-120 H^+ resin

The Amberlite resin was washed with water to remove the fines, colour and other impurities. The water was drained by filtering it through a nylon cloth. The resin was then regenerated by suspending in HCl (2 N) for 1 h at room temperature with intermittent shaking. The resin was then filtered through nylon cloth and washed thoroughly with water till the filtrate gave neutral pH.

3.2.8.3. Preparation of alditol acetates

The neutralized and deionised sample was concentrated to about 0.5 mL. Sodium carbonate was added to a concentration of about 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 (2N). The excess borate and other salts were

removed by co-distilling with methanol (1mL, x4) and then evaporated to dryness. Dry and distilled acetic anhydride and pyridine (0.5 mL each) were added and kept in an oven at 100 °C for 2 h after tightly stoppering the tubes. Excess reagents were removed by co-distilling with water (1 mL, x3) and toluene (1 mL, x3). After thorough drying, the contents were taken in chloroform and filtered through glass wool and dried by passing nitrogen gas. They were taken in chloroform for Gas liquid chromatographic (GLC) analysis.

3.2.8.4. Gas liquid chromatography operating conditions

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.

3.2.9. Characterization of GRPP by Fourier Transform Infra-Red Spectroscopy (FTIR):

FTIR spectra were obtained using a FTIR spectrometer (Perkin – Elmer 2000 spectrophotometer) equipped with TGS detector with solid samples at a concentration of 1- 10 mg. The samples were prepared in the form of pellets by mixing with dry KBr. Potassium bromide discs containing 1% w/w of film material were scanned at 4 mm s^{-1} with a resolution of 4 cm^{-1} over 400 - 4000 cm^{-1}, averaging over 128 scans for each type of film.

3.2.10. Determination of the phenolic content and composition in GRPP

Since phenolics are generally found associated with polysaccharides, we have evaluated the phenolic content in GRPP using Folin-Ciocalteu reagent as described earlier (Singleton & Rossi, 1965). Gallic acid was used as standard for the generation of calibration curve. Total phenolic content was expressed as Gallic Acid Equivalents (GAE) in mg/g of GRPP.

Phenolics were extracted from GRPP by alkaline hydrolysis according to the method of Eric-Nordkvist *et al*. (1984). 2 g of GRPP were extracted with 2X100 mL of 1 M sodium hydroxide containing 0.5% sodium borohydride under nitrogen atmosphere for 2 h and the clear supernatant was collected followed by centrifugation at 3000Xg for 10 min. The combined

supernatants were acidified with 4 N HCl to pH 1.5 and phenolic acids were separated by ethyl acetate phase separation (5X50 mL) and the pooled fractions were treated with anhydrous di sodium sulphate to remove moisture, filtered and evaporated to dryness and taken in 2 mL of methanol (w/v) and analysed by HPLC.

Phenolic acids isolated from GRPP were analyzed by HPLC (model LC-10A. Shimadzu Corp. Kyoto, Japan) on a reversed phase Shimpak C_{18} column (4.6X250 mm, Shimadzu Corp, Kyoto, Japan) using a diode array UV- detector (operating at λmax 280 nm). A solvent system consisting of water/acetic acid/methanol (isocratic, 80: 5: 15 v/v/v) was used as mobile phase at a flow rate of 1 mL/min (Siddaraju & Shylaja, 2007a). Phenolic acid standards such as caffeic, coumaric, cinnamic, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids were employed for identification of phenolic acids present in GRPP by comparing the retention time under similar experimental conditions.

3.2.11. Measurement of potential antiulcer preventive ability of GRPP and MGPP

Potential antiulcer preventive ability for GRPP and MGPP were determined as described earlier in Chapter – 2 for GRAE. Free radical scavenging activity, reducing power ability, inhibition of H^+, K^+-ATPase activity and inhibition *H. pylori* growth was performed employing the protocol provided in Chapter – 2.

3.2.12. Effect of GRPP and GRAE against mucosal defense of parietal cells:

The parietal cells in the fundic part of the stomach produce mucus to protect themselves in addition to protect inner stomach lining. Any damage to this mucus layer hence leads to ulcer. In the fallowing assay, mucin was subjected for oxidative stress condition since it mimics the *in vivo* situation in ulcer. Fundic part of the sheep stomach membranes were pretreated with and without GRPP and GRAE for 15 min, and subjected to oxidation with 4 mM $FeSO_4$ and 0.1mM Ascorbic acid for 1h. After incubation, centrifuged and washed with 0.02 M phosphate buffer, pH 7.4 and mucin content was estimated by alcian blue binding assay (Bandyopadhyay et al, 2002). Extracts were soaked for 2 h, in 10 mL of a solution containing 0.1% Alcian blue, 0.16M sucrose and 50 mM sodium acetate buffer, pH 5.8. Excess dye was removed by two

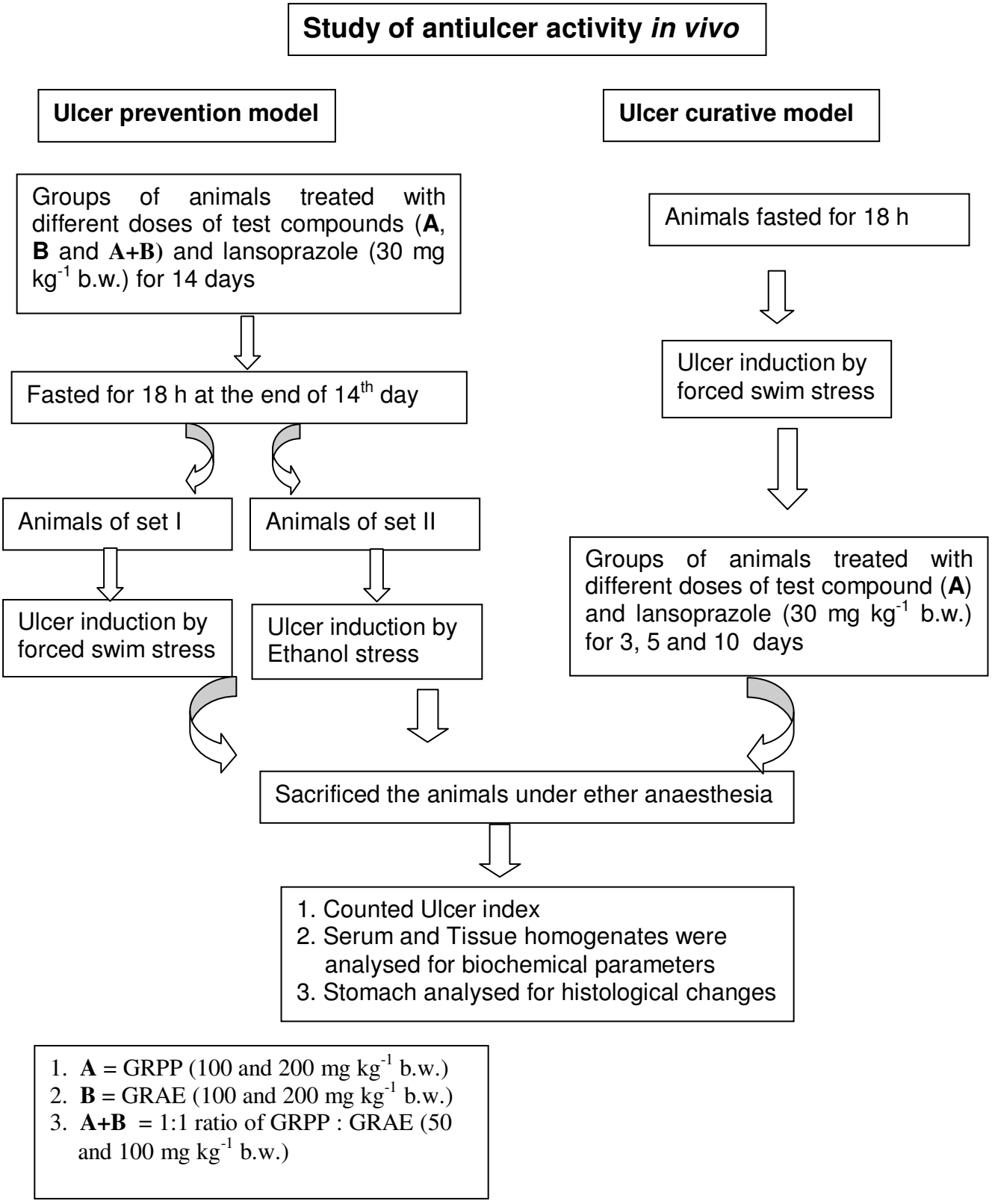
successive washings in 10 mL of 0.25 M sucrose for 15 min followed the same for 45 min. Dye complexed with adhered mucus was extracted with 10 mL of 50 mM $MgCl_2$ by shaking intermediately for 2 h. Four mL of the extract was then shaken with equal volume of ether until an emulsion is formed. After low speed centrifugation for 10 min, the ether layer was removed and the concentration of the Alcian blue was determined in the aqueous layer by measuring the absorbance at 598 nm.

3.2.13. Antiulcerative action of GRAE and GRPP *in vivo.*

3.2.13.1. Animals and treatments

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. Ulcer preventive and curative potentials were studied as per scheme-2.

SCHEME 3. 2


Study of antiulcer activity *in vivo*
Ulcer prevention model
Ulcer curative model
Groups of animals treated with different doses of test compounds (A, B and A+B) and lansoprazole (30 mg kg-1 b.w.) for 14 days
Fasted for 18 h at the end of 14th day
Animals of set I
Animals of set II
Ulcer induction by forced swim stress
Ulcer induction by Ethanol stress
Animals fasted for 18 h
Ulcer induction by forced swim stress
Groups of animals treated with different doses of test compound (A) and lansoprazole (30 mg kg-1 b.w.) for 3, 5 and 10 days
Sacrificed the animals under ether anaesthesia
1. Counted Ulcer index
2. Serum and Tissue homogenates were analysed for biochemical parameters
3. Stomach analysed for histological changes
1. A = GRPP (100 and 200 mg kg-1 b.w.)
2. B = GRAE (100 and 200 mg kg-1 b.w.)
3. A+B = 1:1 ratio of GRPP : GRAE (50 and 100 mg kg-1 b.w.)

3.2.14. Ulcer preventive study

The study was planned as depicted in scheme 3.2. All the animals were categorized into 2 sets of different groups of 6 numbers each (n=6). GRPP and Lansoprazole were administered orally twice daily for 14 days. At the end of 14th day all the animals were deprived of food, but not water for about 18 h, before inducing ulcer.

In the first set (swim stress induced ulcer) groups consisting of following specifications were set up. Group-I: Normal healthy, Group-II: Swim stress induced ulcer, Group-III: GRPP (100 mg kg^{-1} b.w.), Group-IV: GRPP (200 mg kg^{-1} b.w.), Group-V: Lansoprazole (30 mg kg^{-1} b.w.) treatment. On day 15, after 30 min. of GRPP and Lansoprazole treatment to respective groups of rats, the rats of group II, III, IV, and V were subjected to forced swim stress individually by making them to swim in a jar of 30 cm height and 10 cm diameter containing water up to 15 cm height and maintained at 22 °C ± 2 °C for 3 h (Brady et al, 1979).

In the second set (Ethanol induced ulcer) Group-I: Normal healthy, Group-II: Ethanol stress induced ulcer, Group-III: GRPP (100 mg kg-1 b.w.), Group-IV: GRPP (200 mg kg-1 b.w.), Group-V: Lansoprazole (30 mg kg-1 b.w.) treatment was included. On day 15, after 30 min. of GRPP and Lansoprazole treatment to respective rats, the gastric ulcers were induced to the rats of group II, III, IV, and V by administering 95 % ethanol at a dose of 5 ml kg-1 b.w. for one h (Jainu & Devi, 2006) and were sacrificed under deep ether anesthesia and stomach was removed, opened along greater curvature to count ulcer index (Kulkarni & Goel, 1996). Group VI and VII were treated with GRPP (200 mg kg-1 b.w) and lansoprazole (30 mg kg-1 b.w.), without inducing ulcer, serve as GRPP and lansoprazole controls respectively.

Animals were sacrificed under deep ether anesthesia; stomach/liver was removed and used for enzyme assays. Serum was collected from the blood of all animals and analyzed for various parameters as described.

The ulcer preventive effect of GRAE was also studied at 100 and 200 mg kg-1 b.w. concentrations as explained above using swim and ethanol stress induced ulcer models.

In order to examine whether there is any syngeristic effect between antioxidant rich GRAE and GRPP, ulcer preventive effect was studied using 1:1 ratio combination of GRAE and GRPP at a dose of 50 and 100 mg kg-1 b.w. doses as explained above.

3.2.15. Ulcer curative study

Since GRPP showed more potent ulcer preventive ability compared to GRAE, it was further studied for ulcer curative ability. Here, ulcers were induced by swim stress as explained above and then animals were orally fed with GRPP at 200 mg kg-1 b.w. for 3, 5 and 10 days. At the end of the treatment animals were fasted for 18 h, sacrificed under ether anesthesia and examined for macroscopic, microscopic and biochemical changes as described below.

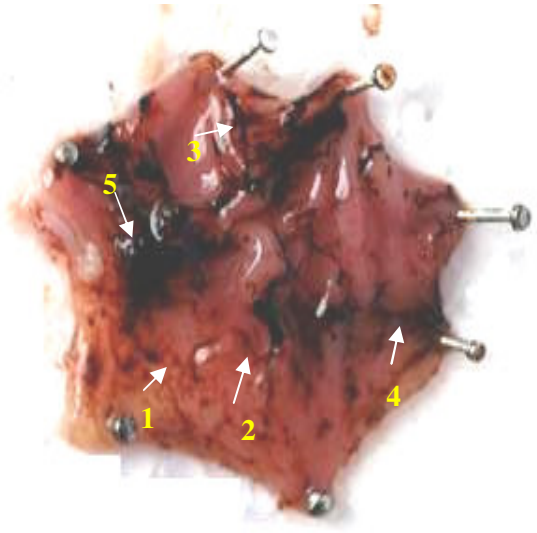
3.2.15. Determination of Ulcer Index (UI)

Stomachs from animals subjected to ulcer induction and those pretreated with test samples were opened along the greater curvature, washed with saline and ulcers were scored according to the protocol (17) in comparison with that of healthy control. Ulcer score was recorded as follows, 0- normal, 1- isolated haemorrhagic spot, 2- dense haemorrhagic spot, 3- small ulcer, 4- large ulcer, 5- perforation. Mean ulcer score for each experimental group was calculated and expressed as ulcer index (UI). Number of lesions X ulcer score = Ulcer incidence (Ui); sum of (Ui1 + Ui2 + Ui3 +Ui4 + Ui5 + Ui6) = Ulcer Index (UI) (Fig. 1).

3.2.16. Effect of GRPP and GRAE on biochemical parameters

3.2.16.1. Preparation of serum and tissue homogenates

All the animals after the treatment were anaesthetized with diethyl ether. Serum was collected from blood samples. Stomach, kidney and liver tissue were removed and five percent homogenates were prepared using cold 0.15 M potassium chloride 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).



- Ulcerous red spots
- Light streaks
- Dark reddish streaks
- Rupture streaks
- Severe hemorrhage

No. lesions X U Score = Ulcer incidence (Ui)

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

3.2.16.2. 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 5 % liver homogenate in 0.2 M sucrose in phosphate buffer (pH 7.4) 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 % autoxidation. The enzyme activity was expressed as unit/mg protein.

3.2.16.3. Estimation of Catalase (CAT, EC 1.11.1.6)

The activity of catalase was assayed according to the method described previously (Aebi, 1984). Briefly, 0.1 mL of liver 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 after 1 min at 240 nm using phosphate buffer as blank solution. The activity of catalase was expressed as units/mg protein (1 unit is the amount of enzyme that utilizes 1 µ moles of hydrogen peroxide/min).

3.2.16.4. Glutathione peroxidase (POX, EC.1.11.1.9)

The activity of glutathione peroxidase was determined according to the method described (Flohe and Gunzler, 1984). The mixture containing 0.1 mL liver 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 n moles of NADPH oxidized/min/mg protein.

3.2.16.5. Measurement of thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (as malondialdehyde) in serum and liver homogenate were analyzed according to the method of Buge and Aust (1978). Briefly, 0.25 mL of liver homogenate or serum was mixed with 2 mL of TCA- TBA-HCl reagent (15 % TCA, 0.375 % TBA in 0.25 N HCl) containing 0.05 % BHT and heated for 15 min in boiling water bath. The solution was cooled to room temperature. The precipitate was removed by centrifugation at 1000 *g* for 10 min at RT and the absorbance of the supernatant was measured at 532 nm. The amount of malondialdehyde was quantified using 1,1,3,3 tetramethoxypropane as standard.

3.2.16.6. 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 as described in **Chapter 2.**

3.2.16.7. Determination of gastric mucin

The glandular segments of stomachs were removed, weighed and incubated with 1% Alcian blue solution (in sucrose solution, buffered with sodium acetate pH 5) for 2 hrs, the Alcian blue

binding extract was centrifuged at 3000 g for 10 min. and the absorbance of supernatant was measured at 498 nm. The amount of Alcian blue was then calculated to know the amount of Alcian blue binding to mucus (Corne et al, 1974). Results were also substantiated by estimating gastric mucin by ELISA using a monoclonal antibody to gastric mucin (Phull et al, 1995).

3.2.16.8. Histological and Immunohistological Evaluation of Gastric Mucin

Histological and immunohistological evaluation was done as described previously (Yougender et al, 2007). The formalin (10%) fixed gastric tissue samples were embedded in paraffin, sectioned (3-5 µm) and stained with haematoxylin and eosin reagent. Another set of slides were immunostained with 1:100 diluted monoclonal anti-human gastric mucin antibody (MAb-GM) followed by goat anti-mouse IgG peroxidase conjugate (GENEI, Bangalore, India) at 1:1000 dilution and the peroxidase substrate TMB/H_2O_2. Slides were examined under light microscope for pathomorphological changes like, damaged mucosal epithelium, glands, inflammatory exudates, proliferated fibroblasts, mixed leukocyte infiltrate and cellular debris.

3.2.16.9. SDS-PAGE Analysis of Gastric Mucin

Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis (SDS- PAGE) was carried out using 4% stacking gel and 7.5% separating gel (Mall et al, 2002). Samples were boiled for 2 min (with or with out β-mercaptoethanol) and approximately 50 µg of mucin and mucin treated with GRPP was loaded on the gel. The gel was stained for glycoprotein with the PAS stain.

3.2.17. Toxicity studies

Toxicity studies were carried out in Albino Wistar rats, kept at controlled environment and acclimatized to laboratory conditions for one week before study. Rats (180–220 g) were orally fed once daily with GRPP (1 g/kg b.w.) for 14 days. The control group received the vehicle (distilled water) only. Twenty-four hours after the last dose, number of animals survived were noted and sacrificed by cervical dislocation, blood was collected and serum was used for estimation of TBARS, total protein and enzymes related to liver function tests- serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), and alkaline phosphatase (ALP)] using standard protocols previously (Yougender et al, 2007).

3.2.18. Hemagglutination assay

Microtiter agglutination was performed for the evaluation of interaction of *H. pylori* with GRPP and mucin. The bacterial suspension with a density of 10^8 CFU/mL was preincubated with different concentrations (10- 50µg) of mucin, GRPP and mucin + GRPP for 30 min, in U-shaped 96 micro well plate. Following to this, 100 µL of 1% erythrocytes suspension in PBS was added, mixed gently, incubated at room temperature for 1 h before scoring agglutinations. Percent agglutination was determined by counting free red blood cells and percent agglutination was calculated as

3.2.19. Interaction studies; UV spectrophotometric analysis:

Gastric mucin and *H. pylori* were known to exhibit a characteristic spectral profile with a λ_{max} at A_{280} nm. Binding of interactive substances are known to alter the profile. In this study we examined the possible interaction of GRPP with gastric mucin and *H. pylori*. Spectral profiles were generated between $A_{400 - 200\ nm}$ in a UV spectrophotometer (Shimadzu UV-160, Tokyo). Alterations in absorbance at 280 nm was measured. Fold variations between observed Vs theoretical values were calculated and this directly suggests the extent and the nature of interaction.

$$\left[\frac{\text{Total number of cells - Number of free cells}}{\text{Total number of cells}} \right] \text{X } 100$$

3.2.20. Statistical Analysis

All the experiments were carried out in triplicates and the results are expressed as mean value ± SD. Correlation between the activity and sugar composition was calculated as coefficient of determination –R^2 using linear regression model to understand the strong, moderate or weak linear trend employing the statistical programme SPSS for windows; erion 10.0. P value was calculated by the Mann-whitney test. Duncan's New Multiple Range Test (DMRT) was performed to understand the degree of significance between controls and treated samples.

3.3. Results

Antiulcer property of ginger (GRAE) and mango ginger (MGAE) was evaluated *in vitro* in chapter II. Further GRAE was evaluated for its *in vivo* efficacy.

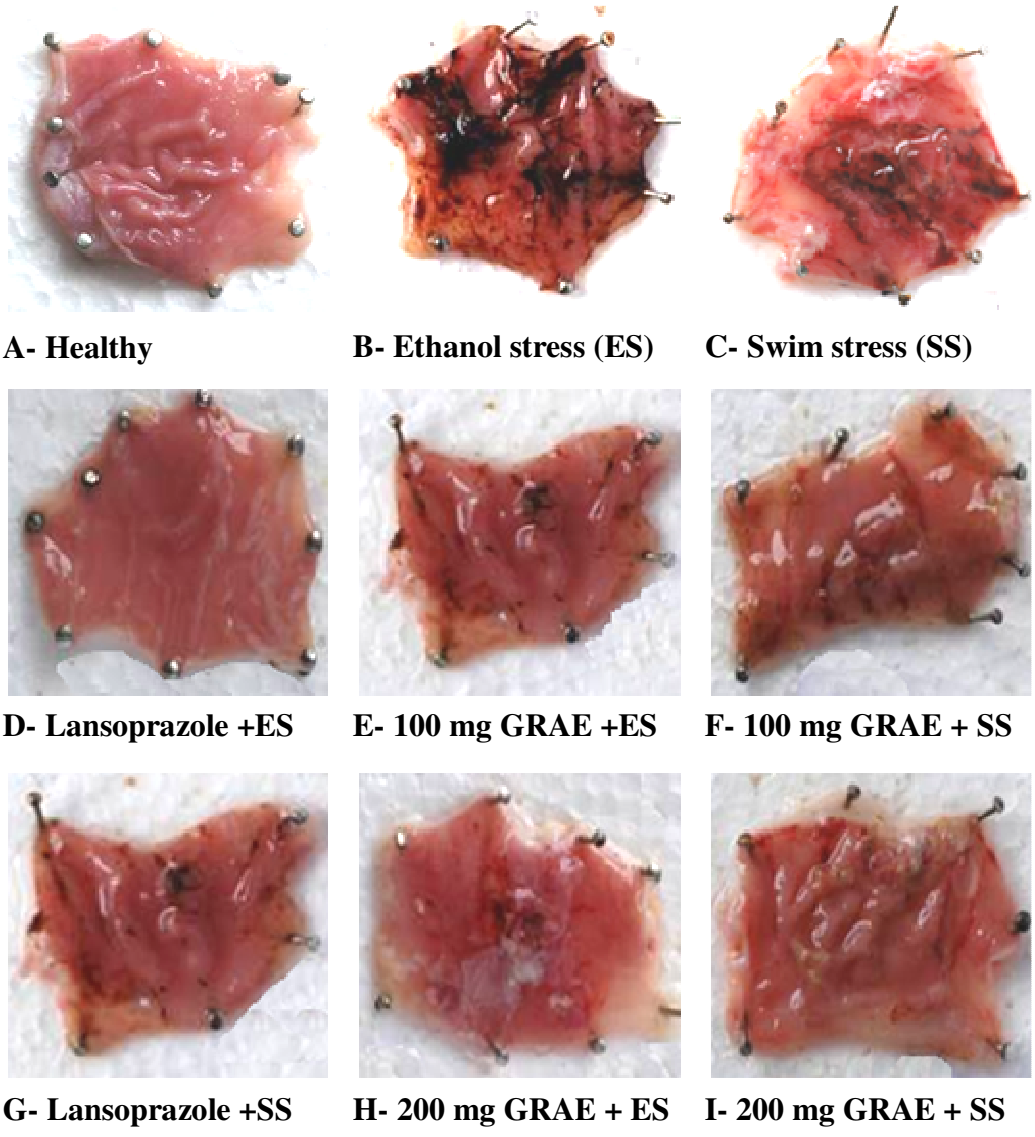
3.3.1. Ulcer Preventive Effect of GRAE in Swim-Stress/Ethanol Induced Ulcer Animal Model

Anti-ulcer effect of GRAE was evaluated by using swim/ethanol stress induced ulcers. Figure 3.1A depicts the stomach of healthy rat which showed no damage or lesions. In swim/ethanol stress induced ulcers the lesions were characterized by multiple hemorrhagic red bands of different size along the long axis of the glandular stomach. (Figure 3.1-B, C). Oral treatment of GRAE at 100 and 200 mg/kg b.w. as well as Lansoprazole at 30 mg/kg b.w. showed protection in a dose dependent manner with no intraluminal bleeding and insignificant number of gastric lesions (Figure 3.1-E, F, H and I). Quantitative reduction in ulcer index in treated rats compared to either ulcer induced or healthy is calculated and depicted in Figure 3.2. Data indicated that GRAE protected dose dependently up to 66 - 86% at 200 mg/kg b.w.

3.3.2. H^+, K^+-ATPase Inhibition and Mucin Protection by GRAE

The elevated levels of H^+, K^+-ATPase in swim stress and ethanol stress was normalized upon treatment with GRAE in a dose dependent manner. Oral pre-treatment of GRAE inhibited the H^+, K^+-ATPase activity and showed 3.1 and 2.9 fold reduction at100 and 200 mg / kg b.w. In case of lansoprazole slightly decreased level of H^+, K^+-ATPase activity was observed (Table 3.1) and the results were also validated by *in vitro* assay- inhibition of H^+, K^+-ATPase enzyme from sheep stomach parietal cells. GRAE inhibited H^+, K^+-ATPase activity with an IC_{50} of 16.5 ± 1.2 µg GAE µg as opposed to that of Lansoprazole (19.3 ± 2.2 µg w/w) indicating increased potency of GRAE. Further the damaged mucin in ulcerous condition was protected up to 68-72 % upon treatment with GRAE at 200 mg/kg b.w (Table 3.1).

Figure 3.1. Macroscopic observation of Ulcers in ulcer induced/protected



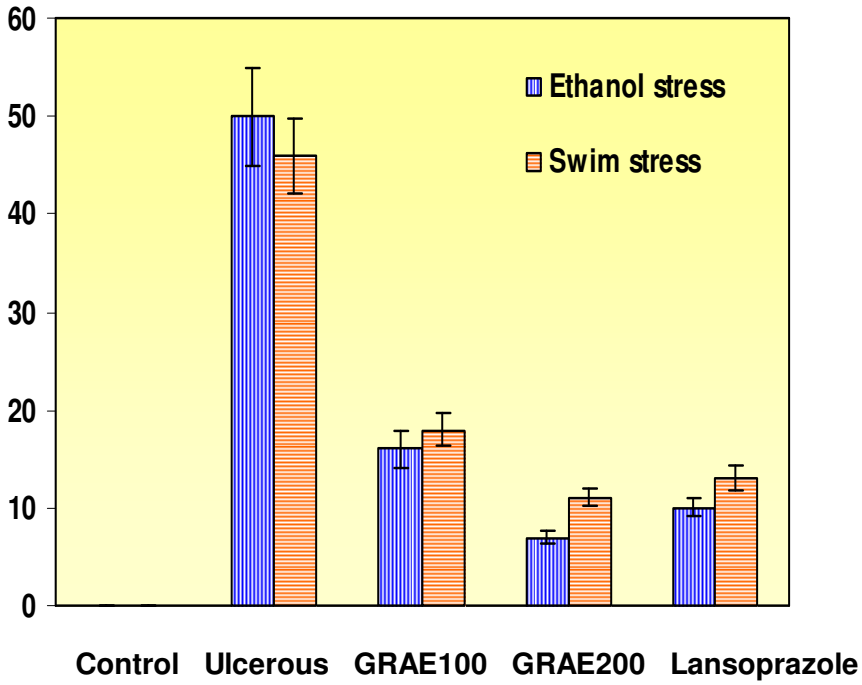
A- Healthy **B- Ethanol stress (ES)** **C- Swim stress (SS)**

D- Lansoprazole +ES **E- 100 mg GRAE +ES** **F- 100 mg GRAE + SS**

G- Lansoprazole +SS **H- 200 mg GRAE + ES** **I- 200 mg GRAE + SS**

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 control (A)-no ulcer lesions or damage in the stomach tissue was observed. In ethanol stress (B) and swim stress (C) induced animals ulcers score were very high. Lansoprazole (D and G) and GRAE at 100 and 200 mg/kg treated animals showed dose dependent reduction in stomach lesions (E, F, H and I).

Figure 3.2. Ulcer index in ulcer induced and treated animals



ES- ulcer index	**0**	**46±4**	**18±1.7**	**11±1**	**13±1**
% protection	**-**	**0**	**61**	**77**	**74**
SS- ulcer index	**0**	**50±6**	**16±2**	**7±0.5**	**10±1**
% protection	**-**	**0**	**68**	**86**	**80**

Maximum ulcer index observed during stress induction was controlled in a concentration dependent manner. Reduction in ulcer index and percent protection is depicted.

3.3.3. Evaluation of GRAE Potential on Oxidant and Antioxidant Status in Ulcerous and Treated Animals

A 2 – 2.4 fold increase in SOD and GPX levels in stomach tissue were observed in swim/ethanol stress induced animals and were normalized upon treatment with GRAE in a dose dependent manner. Where as, CAT and GSH decreased to 1.6 fold during stress induced ulcerous conditions were normalized upon treatment with GRAE as shown in Table 3.2 and 3.3. Approximately 2.6 fold increase in TBARS levels indicated the lipid peroxidation or damage of stomach tissue in ulcerous animals; and was recovered up to 91% upon treatment with GRAE. A 2- 2.3 fold increase in TBARS levels observed in serum and liver homogenate of stress induced ulcerous groups was recovered also up to 75% upon GRAE treatment at 200 mg/kg b.w.

Table 3.1. Gastric mucin and H^+, K^+-ATPase levels in healthy, ulcerated and protected rats. (n = 6) mean ± SD.

Group, n=6	Mucin content (mg/g)	H^+,K^+-ATPase μ moles Pi released/mg/h
Healthy	62.05^{d} ±5.1	0.721^{a}± 0.02
Swim stress induced ulcer model		
Swim stress induced	18.42^{a} ± 3.4	2.610^{d} ± 0.21
GRAE 100 mg kg^{-1} b.w.	43.36^{b} ±3.6	1.316^{c} ± 0.18
GRAE 200 mg kg^{-1} b.w.	48.41^{bc} ±3.4	0.831^{b} ± 0.14
Lansoprazole 30 mg kg^{-1} b.w.	35.14^{b} ±2.4	1.22^{b} ± 0.124
Ethanol stress induced ulcer model		
Ethanol stress induced	22.37^{a} ±2.3	2.318c ± 0.24
GRAE 100 mg kg^{-1} b.w.	36.32^{b} ±3.6	1.213^{b} ± 0.26
GRAE 200 mg kg^{-1} b.w.	46.54^{c} ± 3.8	0.793 ± 0.08
Lansoprazole 30 mg kg^{-1} b.w.	33.23^{bc} ±2.4	1.24^{b} ± 0.12

Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and GRAE/lansoprazole treated groups. Range was provided by Duncan multiple test at $P < 0.05$. a: Less significant; b: Moderately significant; c: Very significant and d: Most significant.

Table 3.2. Antioxidant / antioxidant enzymes and TBARS levels in Swim stress induced ulcer model

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	GPx (ηmoles/g)	GSH (U/mg)	TBARS ηmoles
Stomach						
Healthy	$2.23^{c} \pm 0.16$	092.9±08	46.5±4.6	28.6±2.4	376.6±37	0.82±0.07
Ulcerated	$1.39^{a} \pm 0.16$	201.3±21	22.8±2.1	68.6±5.6	216.2±23	2.16±0.19
GRAE 100 mg/kg	$1.68^{a} \pm 0.16$	161.6±18	38.6±3.4	32.4±3.5	306.5±32	1.12±0.08
GRAE 200 mg/kg	$2.46^{b} \pm 0.23$	136.4±14	43.1±4.5	26.9±2.8	351.5±34	0.91±0.06
Lansoprazole	$2.13^{b} \pm 0.13$	$124.3^{b} \pm 14$	$44^{ab} \pm 4.5$	$26.7^{c} \pm 2.3$	$325^{a} \pm 32$	$0.94^{b} \pm 0.08$
Serum						
Healthy	$6.621^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9$	$0.221^{a} \pm 0.04$	$23.6^{c} \pm 3.0$	$0.165^{a} \pm 0.01$
Ulcerated	$6.845^{a} \pm 0.53$	$264.6^{d} \pm 32$	$22.90^{a} \pm 3.1$	$0.286^{c} \pm 0.02$	$11.1^{a} \pm 1.8$	$0.326^{d} \pm 0.02$
GRAE 100 mg/kg	$6.663^{a} \pm 0.62$	$186.8^{c} \pm 21$	$34.23^{b} \pm 3.6$	$0.293^{d} \pm 0.03$	$16.5^{b} \pm 1.7$	$0.264^{c} \pm 0.02$
GRAE 200 mg/kg	$6.943^{a} \pm 0.61$	$148.6^{b} \pm 15$	$41.45^{c} \pm 4.3$	$0.254^{b} \pm 0.03$	$22.3^{bc} \pm 2.3$	$0.186^{a} \pm 0.02$
Lansoprazole	$6.632^{a} \pm 0.62$	$143.6^{bc} \pm 16$	$36.82^{b} \pm 3.4$	$0.246^{a} \pm 0.02$	$18.8^{a} \pm 2.3$	$0.188^{b} \pm 0.01$
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	$21.9^{a} \pm 0.23$	$142.4^{a} \pm 18$	$22.18^{bc} \pm 2.6$	$0.58^{c} \pm 0.05$	$221^{a} \pm 26$	$2.41^{d} \pm 0.23$
GRAE 100 mg/kg	$23.7^{b} \pm 0.27$	$196.6^{a} \pm 21$	$22.54^{bc} \pm 2.4$	$0.45^{ab} \pm 0.04$	$323^{b} \pm 33$	$1.98^{c} \pm 0.21$
GRAE 200 mg/kg	$24.2^{b} \pm 0.23$	$266.7^{d} \pm 36$	$26.67^{a} \pm 2.4$	$0.46^{a} \pm 0.04$	$382^{a} \pm 36$	$1.45^{b} \pm 0.27$
Lansoprazole	$23.7^{b} \pm 0.25$	$234.4^{cd} \pm 24$	$24.62^{a} \pm 2.3$	$0.41^{a} \pm 0.03$	$325^{a} \pm 31$	$1.64^{b} \pm 0.21$

SOD: Superoxide dismutase; GPx: glutathione peroxidase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and GRAE/lansoprazole treated groups.

Table 3.3. Antioxidant/antioxidant enzymes and TBARS levels in ethanol induced ulcer model (n = 6) mean ± SD:

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	GPx (ηmoles/g)	GSH (U/mg)	TBARS ηmoles
Stomach						
Healthy	$2.23^{a} \pm 0.21$	078.8±07	48.2±6.2	26.5±2.3	368.2±42	0.76±0.06
Ulcerated	$2.32^{a} \pm 0.09$	218.3±20	21.6±2.2	76.6±6.0	208.4±21	1.93±0.21
GRAE 100 mg/kg	$2.38^{a} \pm 0.24$	156.9±16	36.1±3.8	56.9±6.4	286.6±27	0.96±0.08
GRAE 200 mg/kg	$2.42^{a} \pm 0.26$	128.3±11	39.2±4.1	28.3±3.1	342.2±36	0.87±0.10
Lansoprazole	$2.42^{a} \pm 0.19$	168.6± 1.6	38.2 ±1.4	25.2 ± 2.03	252 ± 16	$0.96^{c} \pm 0.2$
Serum						
Healthy	$6.62^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9^{a}$	$0.221^{a} \pm 0.04$	$23.6^{d} \pm 3.0$	$0.165^{a} \pm 0.01$
Ulcerated	$6.52^{a} \pm 0.69$	$282.3^{d} \pm 26$	$28.36^{a} \pm 3.2^{b}$	$0.315^{c} \pm 0.03$	$09.6^{a} \pm 1.2$	$0.465^{d} \pm 0.03$
GRAE 100 mg/kg	$6.58^{a} \pm 0.62$	$198.6^{c} \pm 22$	$33.45^{ab} \pm 4.1^{b}$	$0.264^{b} \pm 0.02$	$15.4^{c}\pm 1.2$	$0.312^{c} \pm 0.03$
GRAE 200 mg/kg	$6.62^{a} \pm 0.67$	$136.4^{b} \pm 18$	$42.34^{b} \pm 3.3^{a}$	$0.251^{b} \pm 0.02$	$22.5^{c}\pm 2.1$	$0.172^{a} \pm 0.02$
Lansoprazole	$6.32^{a} \pm 0.69$	$210.7^{c}\pm28$	$34.12^{ab} \pm 4.6^{b}$	$0.252^{b} \pm 0.03$	$14.6^{b}\pm 1.6$	$0.214^{ab} \pm 0.02$
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	$24.3^{a} \pm 0.31$	$118.1^{a} \pm 16$	$19.64^{b}\pm 2.2$	$0.48^{bc} \pm 0.03$	$392^{bc} \pm 41$	$2.98^{d} \pm 0.31$
GRAE 100 mg/kg	$26.4^{a} \pm 0.23$	$127.4^{a} \pm 12$	$22.32^{b} \pm 2.3$	$0.43^{b} \pm 0.04$	$365^{b} \pm 34$	$2.63^{c} \pm 0.24$
GRAE 200 mg/kg	$26.8^{a} \pm 0.25$	$238.3^{c} \pm 24$	$25.23^{a} \pm 2.6$	$0.36^{a} \pm 0.03$	$396^{ab} \pm 36$	$1.36^{b}\pm 0.13$
Lansoprazole	$26.8^{a} \pm 0.29$	$254.5^{b} \pm 26$	$14.24^{a} \pm 1.8$	$0.31^{a} \pm 0.03$	$211^{a} \pm 28$	$1.61^{b} \pm 0.16$

SOD: Superoxide dismutase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and GRAE/lansoprazole treated groups.

3.3.4. Potential anti ulcer properties of pectic polysaccharide fractions from ginger (GRPP) and mango ginger (MGPP) *in vitro*

Various polysaccharide fractions were prepared from ginger and mango ginger and examined for potential H^+K^+-ATPase activity *in vitro.*

As indicated in Figure 3.3A & B, of all the fractions such as water soluble (GWSP/MWSP), Hemicellulose A (G Hem A/M Hem A), Hemicellulose B (Ghem B/ M Hem B) and extract of alkali insoluble residue (GAIR/MAIR) and pectic polysaccharides (GRPP/MGPP) from ginger and Mango ginger respectively, only GRPP and MGPP showed potent H^+, K^+-ATPase inhibitory properties similar to that of the known proton blocker - Lansoprazole. Hence elaborative studies were conducted in these fractions.

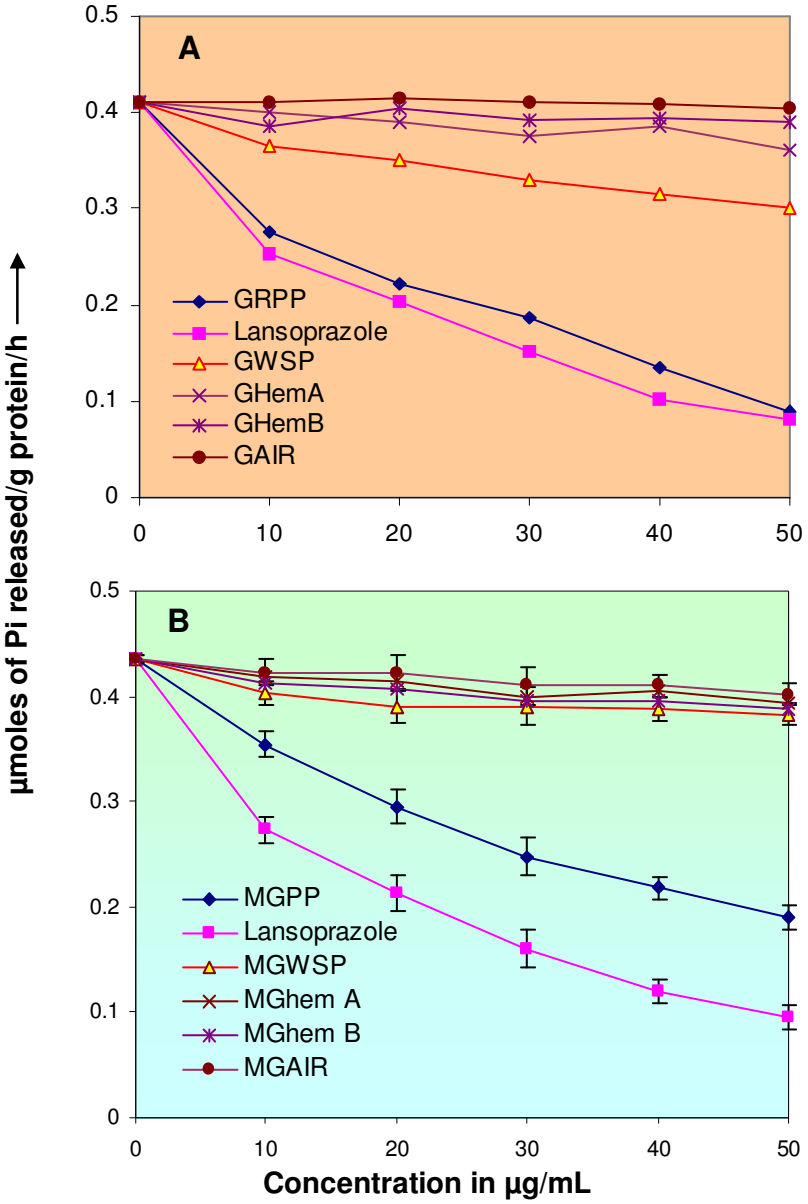
3.3.5. Yield of pectic polysaccharide fraction from ginger (GRPP) and mango ginger (MGPP)

Pectic polysaccharide from ginger (GRPP) and mango ginger (MGPP) resulted in ~ 6% and 4% of yield upon ammonium oxalate extraction respectively. Before considering these molecules for *in vivo* testing, we examined other ulcer preventive potentials for these fractions *in vitro* such as a) H^+, K^+-ATPase inhibitory activity; b) Mucin protection; c) *H. pylori* growth inhibition and d) Antioxidative effect.

a) H^+, K^+-ATPase inhibitory activity

Results showed potent H^+, K^+-ATPase inhibitory activity in both ginger and mango ginger pectic polysaccharide fractions. Pectic polysaccharide of ginger (GRPP) inhibited proton pump (proton potassium ATPase activity) at 27.2 ± 2.4 µg/mL concentration**,** potentially similar to that of lansoprazole (19.3 ± 2.2 µg w/w), where as MGPP has much lower activity (38 ± 3.5 µg/mL) compared to that of GRPP (Figure 3.3A & B).

Figure 3.3. Inhibition of proton potassium ATPase enzyme activity by ginger and mango ginger polysaccharide fractions

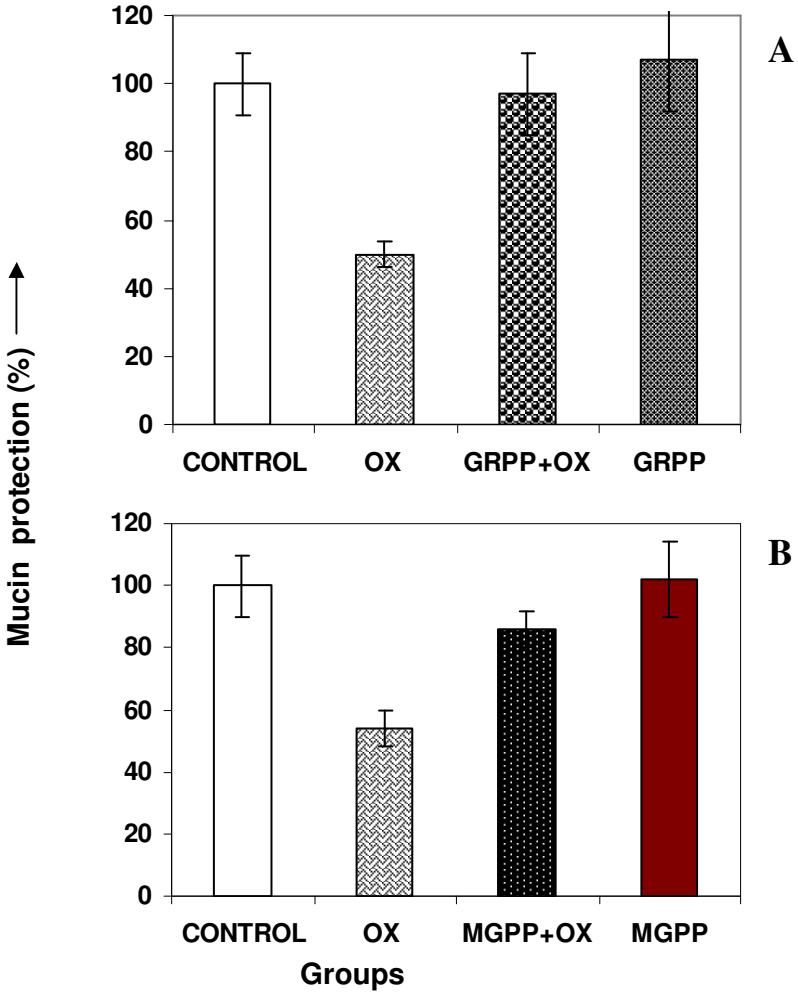


Sheep parietal cell extract was employed as gastric-H^+,K^+-ATPase source, and activity was determined employing the protocol described under materials and methods. 350 µg enzyme protein/mL of reaction volume was incubated with 10 - 50 µg/mL of samples. Enzyme activity is represented as µ moles of Pi released/mg enzyme protein/h. All data are the mean ± SD of triplicates

b) Gastric mucin protection

When we analysed the gastric mucin protection *in vitro,* GRPP and MGPP dose dependently protected oxidation induced damage of mucin. Figure 3.4 shows there was ~50 % reduction in the mucin level in oxidized cells and they were protected up to 97 % and 88 % by GRPP and MGPP at 200 µg/g of stomach membrane respectively. Again GRPP showed more potent activity than MGPP.

Figure 3.4. Protection of mucin layer by GRPP and MGPP

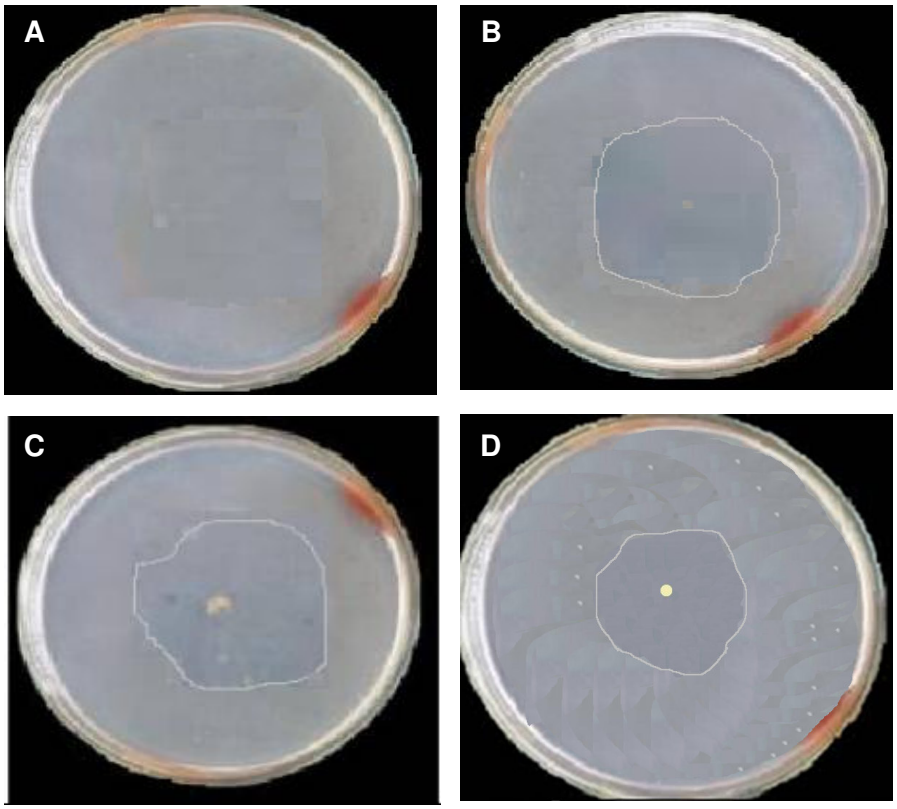


Fundic part of the sheep stomach membranes were pretreated with and without GRPP/MGPP extracts for 15 min, and subjected to oxidation with 4 mM $FeSO_4$ and 0.1mM Ascorbic acid for 1h and assay carried out as per the protocol mentioned in materials and methods. Mucin levels were measured in oxidized cells and in the polysaccharide pre-treated cells.

c) Inhibition of *H. pylori*

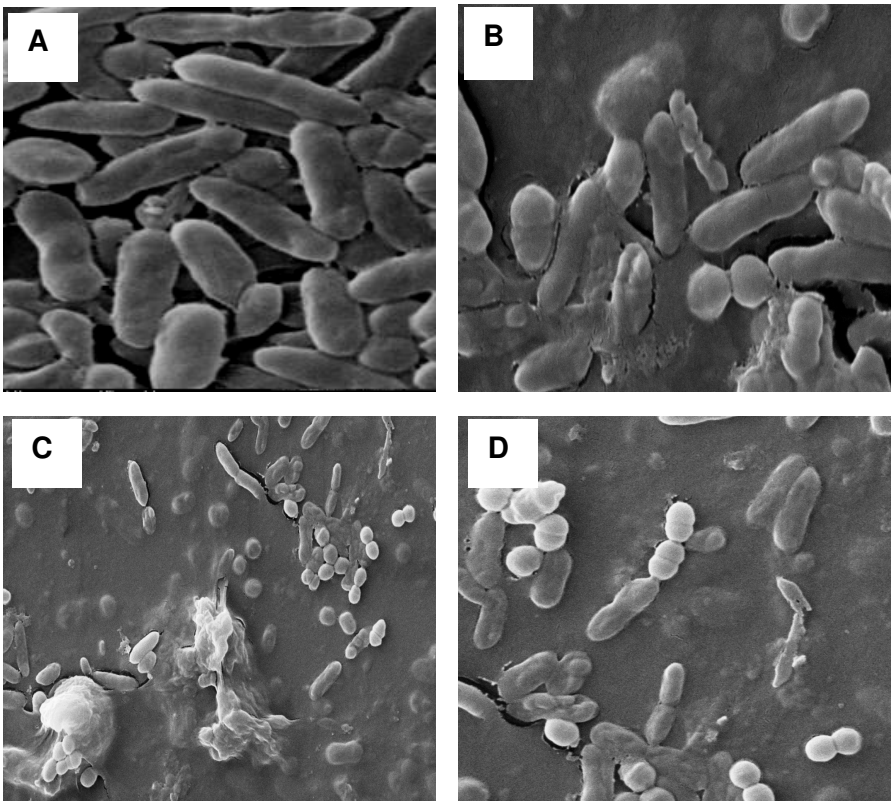
Initially anti-*H. pylori* activity was assayed by agar diffusion method. Firstly, complete growth was ensured (Figure 3.5A) in the plate that acted as control, where no inhibitors were added. Clear inhibition zones appeared around the disc containing susceptible antibiotic amoxicillin at 10 µg/mL (Figure 3.5B) and discs containing GRPP (Figure3.5C) and MGPP (Figure 3.5D) at 100 µg/mL concentration. Further Scanning electron microscopic data revealed that normal *H. pylori* possesses uniform rod shaped cells (Figure3.6A), whereas the cells treated with GRPP and MGPP changed from helical form to coccoid and became necrotic (showed in arrows in Figure3.6C&D). A similar coccoid form was observed with *H. pylori* treated with amoxicillin (Figure 3.6B) and this form is known to result in a loss of infectivity. Coccoid form with blebs in the bacterial surface, appearance of vacuoles, granules and an area of low electron density in the cytoplasm were observed in treated sample indicating the lysis of *H. pylori.* Substantiating to this, viability test indicates the loss of 97% and 82% viability upon treatment with GRPP and MGPP respectively, supporting antimicrobial nature of GRPP and MGPP. Minimum inhibitory concentration (MIC) values were also determined by broth dilution method and it indicated significant anti-*H. pylori* activity at 60 ± 5.2 µg/mL of GRPP and 85 ± 7.2 of MGPP with P ~ 0.003 (Table 3.4).

Figure. 3.5. *H. pylori* inhibition by GRPP/MGPP.



H. pylori grown on nutrient agar plate in presence of GRPP and MGPP showed a clear inhibition zone around the applied sample at 100 µg/mL and the comparative amoxicillin was at 10 µg/mL.
A. without inhibitor, B. with amoxicillin, C. with GRPP and D. with MGPP.

Figure 3.6. Effect of GRPP/MGPP on *H. pylori* – Scanning electron microscopy

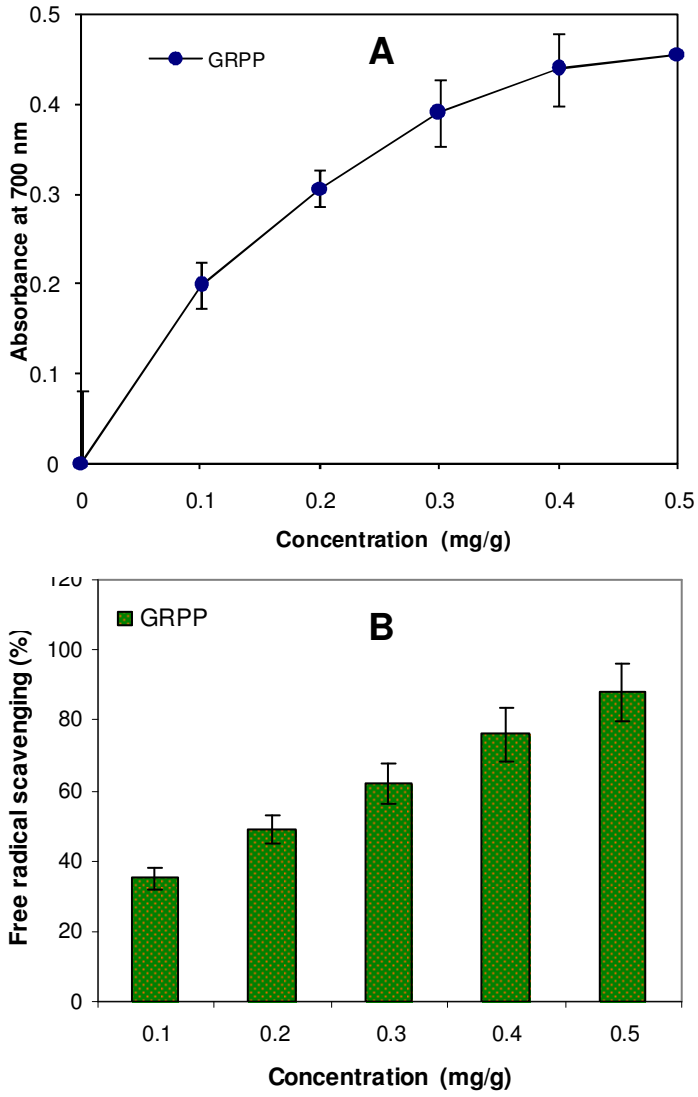


A-D indicates the scanning electron microscopic pictures of *H. pylori*. Untreated control cultures indicate uniform rod shaped *H. pylori* cells, (A). Amoxicillin treatment showed coccoid form with blebbing, fragmented and lysed cells (B). GRPP treatment (C) and MGPP treatment (D) showed disrupted structures.
A & B are at 15 k magnification, C & D are at 10 k magnification

d) Antioxidant potency of GRPP and MGPP

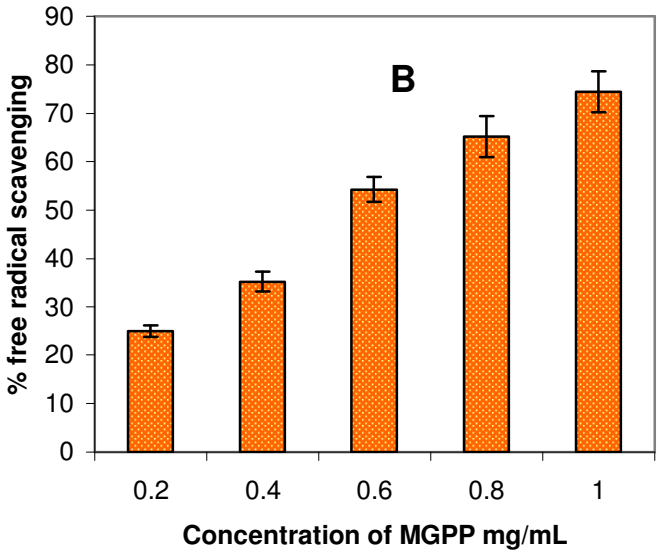
Both GRPP and MGPP showed dose dependent antioxidant activity where free radical scavenging activity with an IC_{50} of 205 µg/mL in GRPP (Figure 3.7B) and 378 µg/mL in MGPP (Figure 3.8B) was observed. Results were substantiated by expression of potent reducing power ability with 98.7 absorbance units/g of GRPP (Figure 3.7A) and 82.0 absorbance units/g (Figure 3.8A) of MGPP respectively.

Figure 3.7. Antioxidant potency of GRPP.



Dose dependent antioxidant activity evaluated as reducing power ability (A) and free radical scavenging ability (B) indicates potential antioxidant activity by phenolics of GRPP.

Figure. 3.8. Antioxidant potency of MGPP.



Dose dependent antioxidant activity evaluated as reducing power ability (A) and free radical scavenging ability (B) indicates potential antioxidant activity by phenolics of MGPP.

Table 3.4. Comparative evaluation of Antiulcer and Antioxidant potency of GRPP and MGPP.

	GRPP	MGPP
Antiulcer activity		
H^+, K^+-ATPase inhibition -IC_{50} (µg/mL)	27 ± 2.3 (1.4)	38 ± 3.5
H. pylori inhibition –MIC (µg/mL)	60 ± 5.4 (1.4)	85 ± 7.2
Mucin protection activity-IC_{50} (µg/mL)	102 ± 8.6 (1.14)	117 ± 10
Antioxidant activity		
Free radical scavenging -IC_{50} (µg/mL)	205 ± 18 (1.8)	378 ± 26
Reducing power ability – U/g	98 (1.2)	82

Antiulcer activity determined by in vitro assays with inhibition of H^+, K^+ -ATPase and *H. pylori* growth. Dose dependent antioxidant activity evaluated as free radical scavenging ability and reducing power ability. GRPP showed more potency than MGPP both in antiulcer and antioxidant activity. Numbers in the parenthesis indicate the fold of better activity in GRPP than MGPP.

Table 3.4 consolidates potential antiulcer properties includimg H^+, K^+ -ATPase inhibition, *H. pylori* growth inhibition, mucosal protection and antioxidant properties, *in vitro,* of both GRPP and MGPP. Data indicate that in all assays although not much difference in the activities was observed between GRPP and MGPP, under similar experimental conditions, GRPP showed better activity.

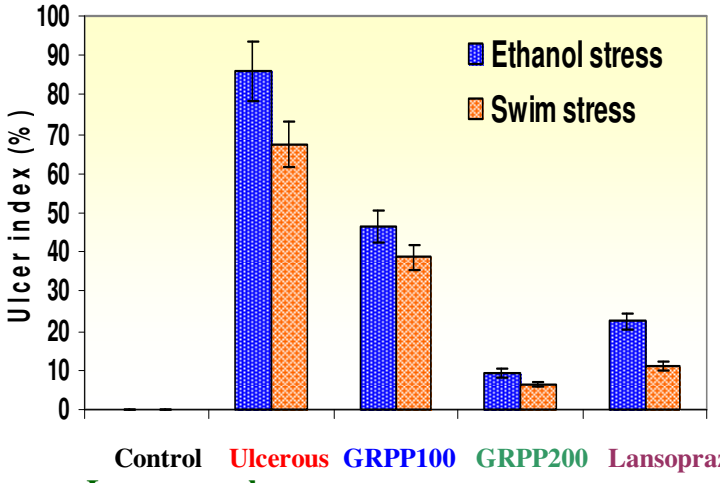
3.3.6. Effect of GRPP against ulcer *in vivo*: Ulcer preventive ability

In vivo experiments were conducted for only GRPP since it showed although slightly better activity than MGPP.

3.3.7. GRPP on Swim/Alcohol Stress Induced Gastric Lesions

Ulcer preventive effect of GRPP was evaluated using swim/ethanol stress induced ulcer models. Figure 3.10 A depicts the stomach of healthy or those treated with only lansoprazole (Figure 3.10 D) or GRPP (Figure 3.10G), which showed no damage or lesions. In swim/ethanol stress induced ulcers the lesions were characterized by multiple hemorrhagic red bands of different size along the long axis of the glandular stomach. Figure 3.10 B and C depict the damage in the gastric wall. Oral treatment of GRPP at 200 mg/kg b.w. (Figure 3.10 H & I) as well as lansoprazole at 30 mg/kg b.w. (Figure 3.10 E & F) showed protection in a dose dependent manner with no intraluminal bleeding and insignificant number of gastric lesions.

Figure 3.9. Effect of GRPP on Ulcer index

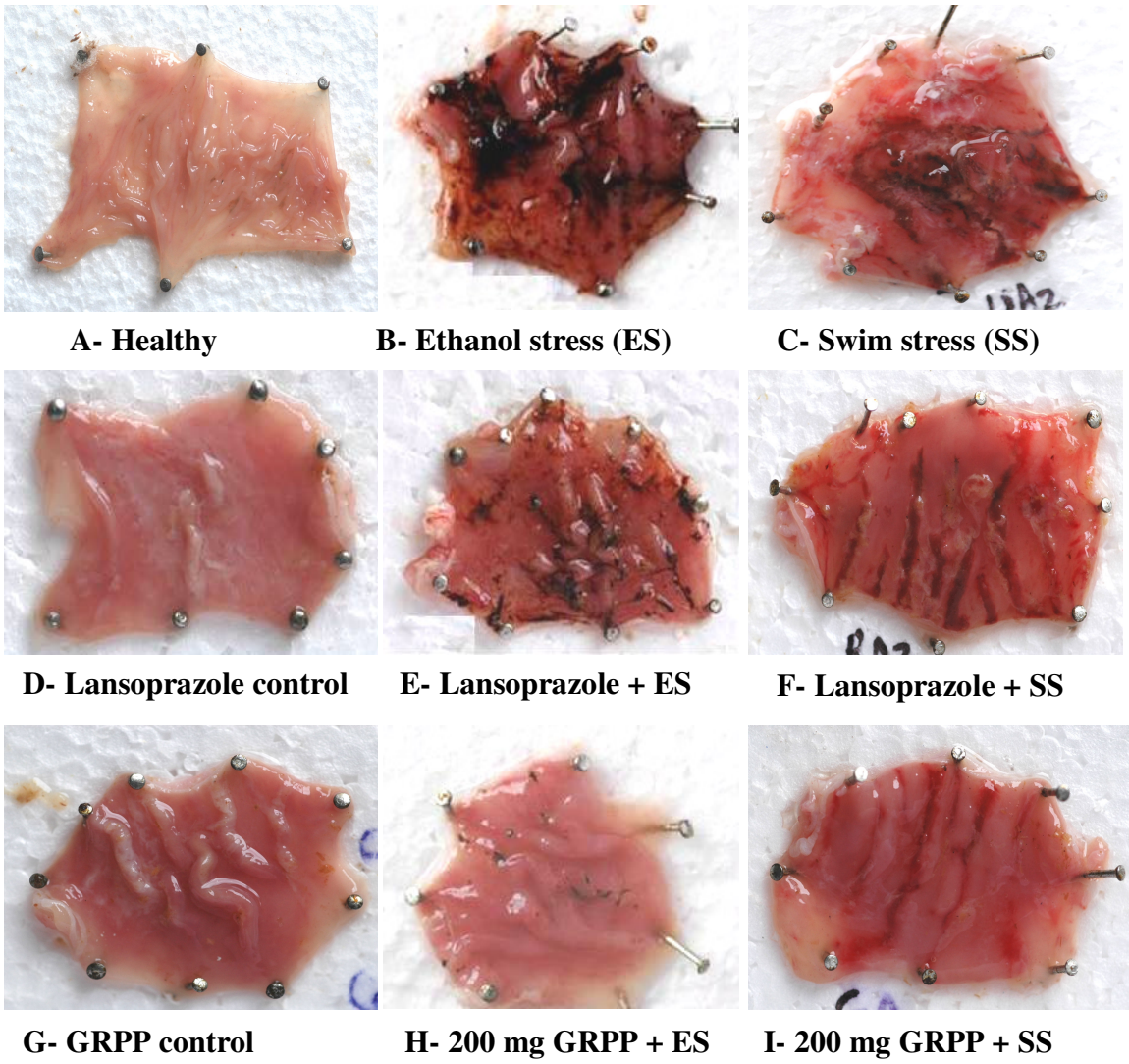


	Control	ulcerous	GRPP100	GRPP 200	Lansoprazole
Ethanol stress	**0**	**86**	**46.6** [a]	**9.1** [b]	**22.4** [b]
Swim stress	**0**	**67.4**	**38.8** [a]	**6.6** [b]	**11.2** [b]

Maximum ulcer index observed during stress induction was controlled in a concentration dependent manner. Reduction in ulcer index and percent protection is depicted. [a]$P < 0.05$ and [b]$P < 0.01$ between ulcerated and treated groups.

Quantitative reduction in ulcer index in treated rats compared to either ulcer induced or healthy is calculated and depicted in Figure 3.9. Data indicated that GRPP protected dose dependently and it was 91 - 95% protection at 200 mg/kg b.w.

Figure 3.10. Macroscopic observation of Ulcers in ulcer induced/protected stomachs in swim stress/ethanol stress induced ulcer models.



A- Healthy **B- Ethanol stress (ES)** **C- Swim stress (SS)**

D- Lansoprazole control **E- Lansoprazole + ES** **F- Lansoprazole + SS**

G- GRPP control **H- 200 mg GRPP + ES** **I- 200 mg GRPP + SS**

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**), GRPP control (G), lansoprazole control (D)-no ulcer lesions or damage in the stomach tissue was observed. In ethanol stress (B) and swim stress (C) induced animals ulcers score were very high. Whereas in Lansoprazole (E&F) and GRPP (H&I) treated animals showed reduced stomach lesions.

3.3.8. Effect of GRPP on H^+, K^+ -ATPase Activity *in vivo*

The results indicated that swim stress and ethanol stress increases the H^+, K^+-ATPase activity, and oral pre-treatment of GRPP -dose dependently inhibited the activity and showed 84% and 78% inhibition (~2.4 fold reduction) at 200 mg/kg b.w. Slightly decreased level of H^+, K^+-ATPase activity was observed in case of lansoprazole treated animals (Table 3.5).

Table 3.5. Gastric mucin and H^+, K^+-ATPase levels in healthy, ulcerated and protected rats.

Group, n=6	Mucin content (mg/g)	H^+,K^+-ATPase activity (μ moles Pi released/mg/h)
Healthy	$45.04^{d} \pm 4.1$	$0.826^{a} \pm 0.09$
Swim stress induced ulcer model		
Swim stress induced	$17.78^{a} \pm 2.5$	$2.606c \pm 0.19$
GRPP 100 mg kg^{-1} b.w.	$31.13^{b} \pm 3.8$	$1.763^{b} \pm 0.22$
GRPP 200 mg kg^{-1} b.w.	$41.41^{c} \pm 3.6$	1.106 ± 0.08
Lansoprazole 30 mg kg^{-1} b.w.	$29.54^{bc} \pm 3.1$	$1.320^{b} \pm 0.14$
Ethanol stress induced ulcer model		
Ethanol stress induced	$16.32^{a} \pm 3.2$	$2.621^{d} \pm 0.211$
GRPP 100 mg kg^{-1} b.w.	$36.26^{b} \pm 3.8$	$1.802^{c} \pm 0.192$
GRPP 200 mg kg^{-1} b.w.	$43.43^{bc} \pm 3.8$	$1.216^{b} \pm 0.113$
Lansoprazole 30 mg kg^{-1} b.w.	$32.15^{b} \pm 2.5$	$1.132^{b} \pm 0.124$

(n = 6) mean ± SD: Different letters a to d in the column represents that values are significantly different when compared ulcer induced with healthy control and GRPP/lansoprazole treated groups. Range was provided by Duncan multiple test at $P < 0.05$. a: Less significant; b: Moderately significant; c: Very significant and d: Most significant.

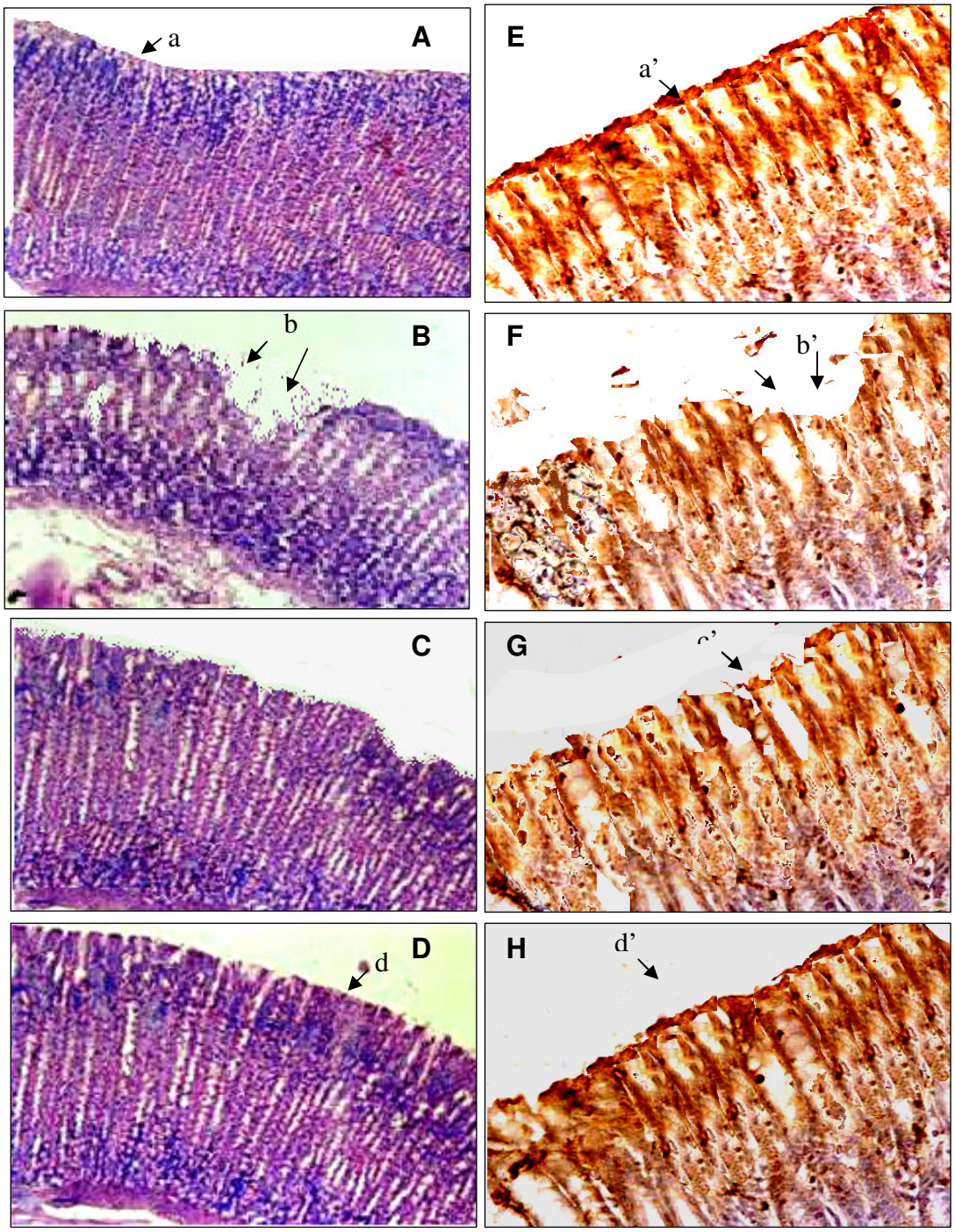
3.3.9. GRPP Prevents Gastric Mucosal Damage

Gastric mucin is a large glycoprotein which plays a major role in the protection of the gastrointestinal tract from acid, proteases, pathogenic microorganisms, and mechanical trauma. During ulcer, 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 GRPP on protection to gastric wall mucus during ulceration induced by swim/ethanol stress. Since Alcian blue binds to carboxylated mucopolysaccharides, sulfated and carboxylated glycoproteins, any disruption in mucin, results in reduction in the dye binding, which can be quantitated. The swim stress/ethanol stress induced ulcerous rats showed decreased levels of gastric mucin (16.32 ± 3.2 mg/g and 17.78 ± 2.5 mg/g), where as healthy rats showed 45.04 ± 4.1 mg/g and it was recovered up to 91 to 95 % in GRPP treated animals with 41.41 ± 3.6 and 43.43 ± 3.8 mg/g respectively (Table 3.5).

Results were substantiated by histopathological and immunohistological studies. Hematoxylin and eosin staining showed intact tissue without any damage to the epithelium in healthy control (Figure 3.11A). In ulcerated rats the damage in mucosal epithelium (Figure 3.11B) was clearly observed with very high inflammatory exudates, proliferated fibroblasts, infiltration of leucocytes and cellular debris. Figure 3.11C & D clearly reveals the recovery of mucosal layer dose dependently and complete, upon treatment with lansoprazole and GRPP respectively.

Further, increased gastric mucin content was also evidenced by monoclonal antibody-based immuno histological studies**.** Immunostaining of tissue sections showed an intact regular mucosal epithelium in healthy stomachs (Figure 3.11E) and in the ulcer induced, showed damage in the mucosal epithelium, destruction of regular glandular organization (Figure 3.11F). GRPP treated rats showed recovery in the mucosal epithelium, regained glandular structure and mucosal regeneration in lansoprazole at 30 mg/kg b.w. (Figure 3.11G) and 200 mg/kg b.w. (Figure 3.11H) respectively.

Figure 3.11. Histopathologic/Immunohistopathologic observation of stomach from ulcer induced/GRPP and Lansoprazole treated animals.

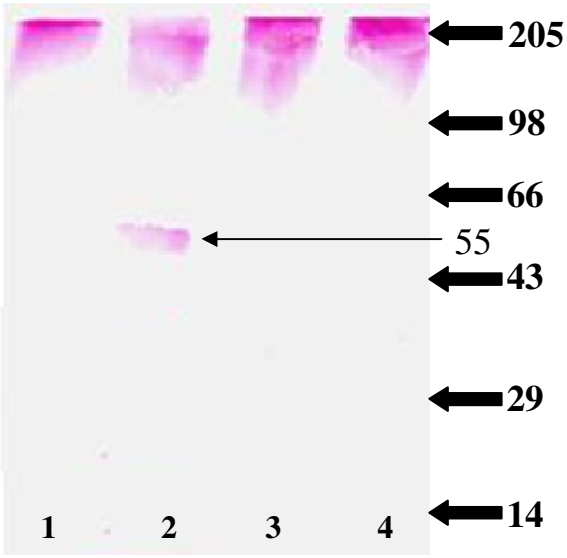


A-D indicates HE staining sections (x40), while **E**-**H** reveal anti-gastric mucin stained sections (x40, and magnified the selected portion in computer photoshop). Control (**A, E**) shows intact mucosal epithelium with organized glandular structure (a) and intense brown staining for gastric mucin by antibody (a'). Ulcer induction (**B, F**) showed damaged mucosal epithelium (b, b') and loss of gastric mucin. Partial recovery by lansoprazole (c and c' of **C, G**) and complete recovery of mucosal damage (d and d' of **D, H**) by GRPP was observed.

3.3.10. Electrophoretic Pattern of Ulcerated and Normal Gastric Mucin

A large molecular entity was observed at the start in the running gel. On treatment with 0.2 mol/L 2- mercaptoethanol, the ulcerated mucin yielded less larger polymeric mucin of molecular size ~55 kDa (Lane 2 in Figure 3.12) and reduction in the quantity of high molecular weight – 200 kDa bands. Mucin from stomach of the GRPP (Lane 4) and lansoprazole (Lane 3) treated samples yielded no small molecular weight band while displayed increase in 200 kDa band suggesting that the ulceration induced degradation of mucin is inhibited by both Lansoprazole and GRPP. The increased content of mucin also suggests that there may be enhanced synthesis of mucin substantiating the histological and immunohistological recovery of mucin layer. The demonstration of a band of 55 associated with mucin from ulcerated sample was similar to that found in gastric carcinoma and peptic ulcer conditions[30].

Figure 3.12. Electrophoretic pattern of gastric mucin



SDS-PAGE analysis showed inhibition of mucin degradation, it shows presence of ~55 kDa fragment in ulcerated stomach (lane 2) and it is absent in healthy, lasoprazole and GRPP pretreated (lane 1, 3 and 4 respectively) groups.

3.3.11. GRPP Interferes with *H. pylori* Agglutination with RBCs; Interaction Between GRPP with *H. pylori* and Gastric Mucin

H. pylori is known to bind to blood group polysaccharide preferably the 'O' antigens which carry fucosyl oligosaccharide unit. We also could observe similar results, where 70 % cells were agglutinated. After establishing that the *H .pylori* strain collected from endoscopic samples from ulcer patients of local hospitals mimic similar affinities for blood group antigens, particularly to 'O' group antigens, the effect of GRPP-polysaccharides on this interaction was examined. Incubation of mucin with *H. pylori,* reduced RBC agglutination significantly; this was recovered when GRPP + mucin was added to *H. pylori* suggesting that probably GRPP and gastric mucin interaction may block the binding of *H. pylori* to gastric mucin and hence may allow RBC agglutination (Table 3.6).

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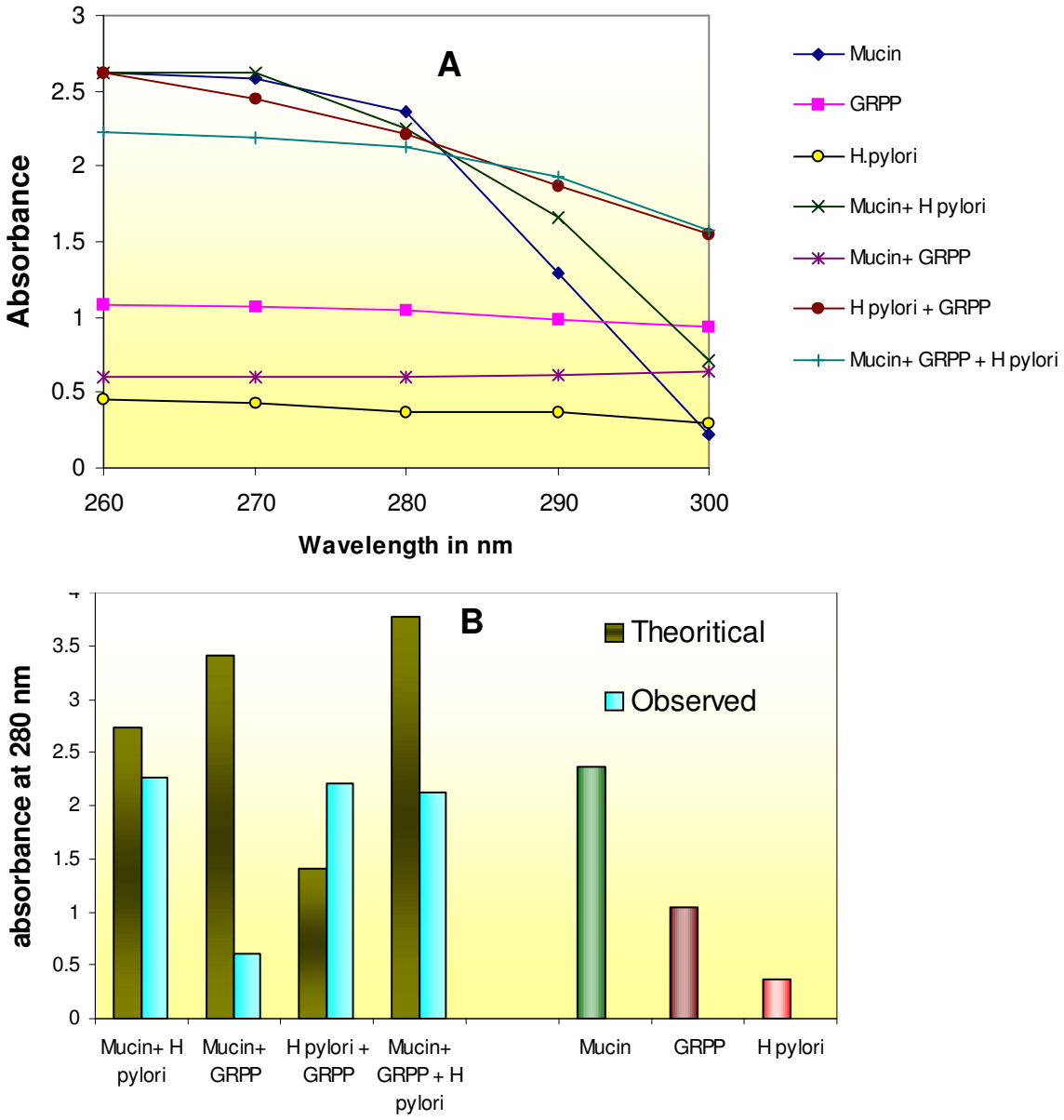
Table 3.6. Interaction of GRPP with *H. pylori* and gastric mucin

S. No	Assay system	Agglutination score (%)
1	*H. pylori* + RBC	**70**
2	(*H. pylori* + Gastric mucin) + RBC	**<10**
3	(*H. pylori* + GRPP) + RBC	**<15**
4	(Gastric mucin + GRPP)+ *H. pylori* + RBC	**60**

Interaction of GRPP with *H. pylori* and gastric mucin was studied using RBC hemagglutination method as described in materials and methods.

The possibility of binding was examined by spectral studies. As indicated in Figure 3.13, *H. pylori* and GRPP with selected concentrations showed poorer absorbance while mucin showed ~2.5 fold higher absorbance.

Figure 3.13. Interaction of GRPP with gastric mucin and *H. pylori* (A and B)



Gastric mucin isolated from healthy control group of experimental animals and *H. pylori* from fresh culture were examined for spectral profile in presence and absence of GRPP (A); B. extent of interaction was measured as the fold variation between theoretical and observed values- indicative interaction has been depicted.

However ~ 2 fold decreased absorbance was observed when mucin was complexed with GRPP. Reduction in absorbance than the expected value suggested that mucin molecule must have been enveloped with GRPP. On the other hand addition of GRPP to *H. pylori* increased the absorbance to ~4 fold. Further addition of *H. pylori* to gastric mucin + GRPP slightly alters the binding (Figure 7B), suggesting the possibility of GRPP binding with *H. Pylori* and gastric mucin.

3.3.12. Evaluation of GRPP Potential on Oxidant and Antioxidant Status in Ulcerous and Treated Animals

Antioxidant (GSH) and antioxidant enzymes (SOD, catalase, glutathione peroxidase) were estimated in stomach/liver homogenate and the serum of swim/ethanol stress models. Table 3.7 & 3.8 indicates antioxidant, antioxidant enzymes and TBARS levels in swim and ethanol stress induced ulcer stomachs. SOD and GPX levels increased in stomach (2 fold) and CAT & GSH decreased (1.8 fold) during stress induced ulcerous conditions were normalized upon treatment with GRPP in a dose dependent manner. Approximately 4 fold increase in TBARS levels depicts lipid peroxidation or damage of stomach tissue in ulcerous animals and was recovered up to 85% upon treatment with GRPP. Similar kind of changes in antioxidant enzymes except catalase was also observed in serum and liver homogenate.

Table 3.7. Effect of GRPP on antioxidant/antioxidant enzymes and TBARS levels in swim stress induced ulcer model (n = 6) mean ± SD:

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	Glutathione Peroxidase (ηmoles/g)	GSH (U/mg)	TBARS ηmoles
Stomach						
Healthy	$2.23^{c} \pm 0.16$	$9.86^{a} \pm 1.1$	$829^{c} \pm 41$	$0.061^{a} \pm 0.009$	$224^{c} \pm 10.0$	$0.38^{a} \pm 0.01$
Ulcerated	$1.39^{a} \pm 0.16$	$19.01^{c} \pm 1.8$	$462^{a} \pm 30$	$0.540^{d} \pm 0.01$	$121^{a} \pm 18.9$	$1.08^{c} \pm 0.20$
GRPP 100 mg/kg	$1.89^{a} \pm 0.19$	$14.23^{bc} \pm 1.6$	$507^{ab} \pm 20$	$0.376^{b} \pm 0.02$	$186^{b} \pm 20.3$	0.87 ± 0.10
GRPP 200 mg/kg	$2.14^{b} \pm 0.21$	$12.68^{b} \pm 1.8$	$647^{b} \pm 31$	$0.125^{a} \pm 0.01$	$216^{c} \pm 20.2$	$0.45^{ab} \pm 0.03$
Lansoprazole	$2.28^{b} \pm 0.12$	$14.33^{b} \pm 1.4$	$516^{ab} \pm 42$	$0.145^{c} \pm 0.01$	$151^{a} \pm 11.1$	$0.58^{b} \pm 0.10$
Serum						
Healthy	$6.621^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9$	$0.221^{a} \pm 0.004$	$23.6^{c} \pm 3.0$	$0.165^{a} \pm 0.01$
Ulcerated	$6.845^{a} \pm 0.53$	$264.6^{d} \pm 32$	$22.90^{a} \pm 3.1$	$0.286^{c} \pm 0.02$	$11.1^{a} \pm 1.8$	$0.326^{d} \pm 0.02$
GRPP 100 mg/kg	$6.512^{a} \pm 0.62$	$188.5^{c} \pm 22$	$32.32^{b} \pm 4.1$	$0.279^{d} \pm 0.04$	$15.8^{b} \pm 1.8$	$0.248^{c} \pm 0.03$
GRPP 200 mg/kg	$6.821^{a} \pm 0.51$	$146.4^{b} \pm 18$	$42.31^{c} \pm 4.6$	$0.241^{b} \pm 0.04$	$21.2^{bc} \pm 2.5$	$0.161^{a} \pm 0.01$
Lansoprazole	$6.43^{a} \pm 0.62$	$193.5^{bc} \pm 19$	$29.61^{b} \pm 2.8$	$0.232^{a} \pm 0.02$	$13.8^{a} \pm 2.3$	$0.166^{b} \pm 0.01$
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	$21.9^{a} \pm 0.23$	$142.4^{a} \pm 18$	$22.18^{bc} \pm 2.6$	$0.58^{c} \pm 0.05$	$221^{a} \pm 26$	$2.41^{d} \pm 0.23$
GRPP 100 mg/kg	$24.6^{b} \pm 0.31$	$188.4^{a} \pm 20$	$20.96^{bc} \pm 2.1$	$0.41^{ab} \pm 0.04$	$320^{b} \pm 32$	$2.14^{c} \pm 0.11$
GRPP 200 mg/kg	$21.3^{b} \pm 0.24$	$234.4^{b} \pm 23$	$18.22^{a} \pm 1.9$	$0.38^{a} \pm 0.03$	$261^{a} \pm 26$	$1.38^{b} \pm 0.14$
Lansoprazole	$23.6^{b} \pm 0.26$	$314.4^{cd} \pm 36$	$17.34^{a} \pm 1.9$	$0.32^{a} \pm 0.02$	$254^{a} \pm 28$	$1.41^{b} \pm 0.12$

SOD: Superoxide dismutase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and GRPP/lansoprazole treated groups.

Table 3.8. Effect of GRPP on antioxidant/antioxidant enzymes and TBARS levels in ethanol stress induced ulcer model (n = 6) mean ± SD

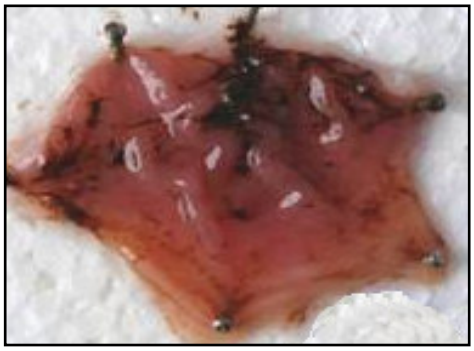
Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	Glutathione Peroxidase (ηmoles/g)	GSH (U/mg)	TBARS ηmoles
Stomach						
Healthy	$2.23^{a} \pm 0.21$	$09.86^{a} \pm 1.1$	$829.2^{c} \pm 41.6$	$0.21^{a} \pm 0.009$	$224^{d} \pm 23.2$	$0.31^{a} \pm 0.1$
Ulcerated	$2.32^{a} \pm 0.09$	$17.86^{c} \pm 2.4$	$201.5^{a} \pm 18.9$	$0.30^{c} \pm 0.01$	$102^{a} \pm 12.6$	$1.26^{d} \pm 0.3$
GRPP 100 mg/kg	$2.25^{a} \pm 0.21$	$14.36^{c} \pm 1.5$	$321.4^{a} \pm 35.2$	$0.28^{b} \pm 0.01$	$166^{b} \pm 15.8$	$0.86^{c} \pm 0.1$
GRPP 200 mg/kg	$2.48^{a} \pm 0.22$	$10.12^{b} \pm 1.2$	$621.2^{b} \pm 58.5$	$0.22^{c} \pm 0.02$	$208^{c} \pm 21.2$	$0.48^{b} \pm 0.1$
Lansoprazole	$2.42^{a} \pm 0.19$	$13.42^{b} \pm 1.4$	$476.6^{c} \pm 41.6$	$0.24^{a} \pm 0.02$	$172^{b} \pm 14.3$	$0.91^{c} \pm 0.1$
Serum						
Healthy	$6.62^{a} \pm 0.51$	$112.3^{a} \pm 28$	$44.20^{c} \pm 4.9^{a}$	$0.221^{a} \pm 0.04$	$23.6^{d} \pm 3.0$	$0.165^{a} \pm 0.01$
Ulcerated	$6.52^{a} \pm 0.69$	$282.3^{d} \pm 26$	$28.36^{a} \pm 3.2^{b}$	$0.315^{c} \pm 0.03$	$09.6^{a} \pm 1.2$	$0.465^{d} \pm 0.03$
GRPP 100 mg/kg	$6.42^{a} \pm 0.65$	$202.2^{c} \pm 28$	$32.32^{ab} \pm 3.2^{b}$	$0.272^{b} \pm 0.02$	$14.3^{c} \pm 1.2$	$0.302^{c} \pm 0.03$
GRPP 200 mg/kg	$6.48^{a} \pm 0.62$	$142.3^{b} \pm 21$	$41.21^{b} \pm 4.11^{a}$	$0.242^{b} \pm 0.02$	$20.3^{c} \pm 2.5$	$0.188^{a} \pm 0.02$
Lansoprazole	$6.63^{a} \pm 0.64$	$218.7^{c} \pm 25$	$32.15^{ab} \pm 3.5^{b}$	$0.226^{b} \pm 0.03$	$13.4^{b} \pm 1.5$	$0.221^{ab} \pm 0.02$
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	$24.3^{a} \pm 0.31$	$118.1^{a} \pm 16$	$19.64^{b} \pm 2.2$	$0.48^{bc} \pm 0.03$	$392^{bc} \pm 41$	$2.98^{d} \pm 0.31$
GRPP 100 mg/kg	$24.8^{a} \pm 0.22$	$124.5^{a} \pm 13$	$21.21^{b} \pm 2.1$	$0.41^{b} \pm 0.04$	$361^{b} \pm 31$	$2.32^{c} \pm 0.32$
GRPP 200 mg/kg	$25.2^{a} \pm 0.28$	$243.4^{c} \pm 28$	$24.25^{a} \pm 2.3$	$0.38^{a} \pm 0.03$	$392^{ab} \pm 34$	$1.16^{b} \pm 0.12$
Lansoprazole	$27.6^{a} \pm 0.27$	$248.4^{c} \pm 25$	$15.41^{a} \pm 1.5$	$0.33^{a} \pm 0.02$	$221^{a} \pm 24$	$1.52^{b} \pm 0.14$

SOD: Superoxide dismutase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and GRPP/lansoprazole treated groups.

3.3.13. Effect of GRPP against ulcer *in vivo*: Ulcer healing ability

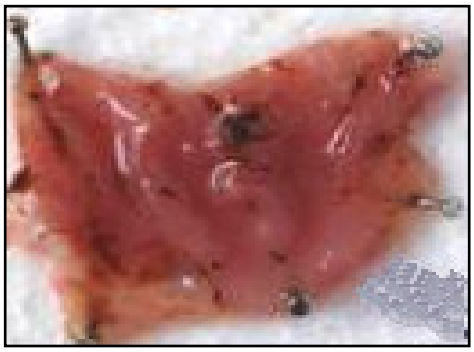
Results of the experiments indicated that GRPP could effectively prevent gastric ulcers induced by swim and alcohol stress. Data is also supported by observing normalization of activated H^+, K^+ -ATPase, antioxidant and antioxidant enzymes when compared to those of healthy controls in a statistically significant manner. In order to understand the ulcer curative ability of GRPP, experiments were conducted where GRPP was ingested after the induction of ulcers and evaluated the curative of ulcer healing ability.

Figure 3.14. Macroscopic observation of Ulcers in, ulcerated day 1, day 3, day 5 and day 10 protected rats.

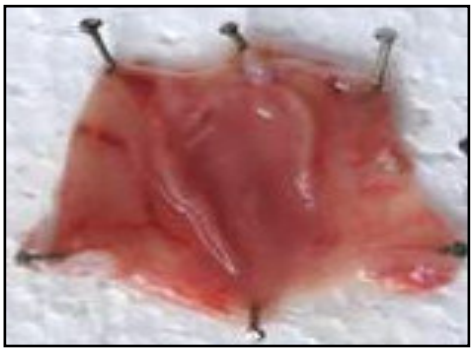


Swim stress + GRPP-1 day

Swim stress + GRPP-3 day



Swim stress + GRPP-5 day

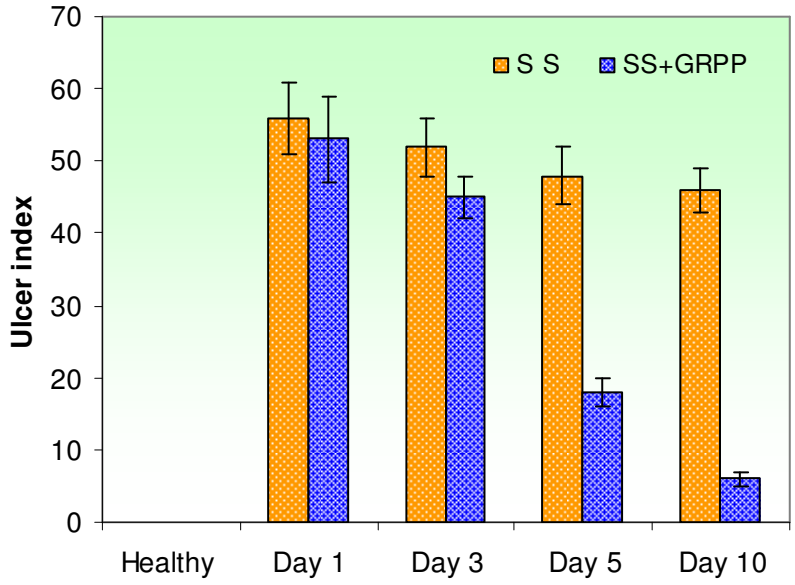


Swim stress + GRPP-10 day

Ulcers were scored as described under the methods and expressed as ulcer index.

Gastric lesions in, ulcer induced (swim stress), untreated animals and GRPP ingested animals are depicted in Figure 3.14. As graphically represented in Figure 3.15, only 18 % healing was observed in animals induced with ulcers but not treated with any compound. In other words that 18 % healing observed has been attributed to the spontaneously healing mechanism. On the contrary in the animals treated with GRPP, 5-fold increase in healing was observed (Figure 3.15) suggesting that GRPP can also cure gastric ulcers to a significant extent.

Figure 3. 15. Effect of GRPP on Ulcer index



% protection	**Swim stress**	**100**	**0**	**8**	**15**	**18**
	SS + GRPP	**100**	**6**	**27**	**68**	**90**

5-fold increase in healing was observed in GRPP treated animals compared to spontaneous healing after 10 days.

Table 3.9. Gastric mucin and H^+, K^+-ATPase levels in healthy, ulcerated day 1, day 3, day 5 and day 10 protected rats.

Group, n=6	Mucin content (mg/g)	% recovery	H^+,K^+-ATPase activity (μ moles Pi released/mg/h)	% normalization
Healthy	66 ± 5.2	100	0.721±0.02	100
Swim stress induced ulcer model				
Day 1	21 ± 2.2	34	2.213±0.19	0
Day 3	22 ± 2.4	35	1.982±0.17	11
Day 5	26 ± 2.1	42	1.961±0.17	12
Day 10	34 ± 3.3	55	1.821±0.19	18
Swim stress + GRPP 200				
Day 1	22 ± 2.2	35	1.960±0.17	0
Day 3	36 ± 3.3	58	1.682±0.16	14
Day 5	43 ± 4.2	69	1.356±0.15	31
Day 10	52 ± 5.1	84	1.081±0.09	55

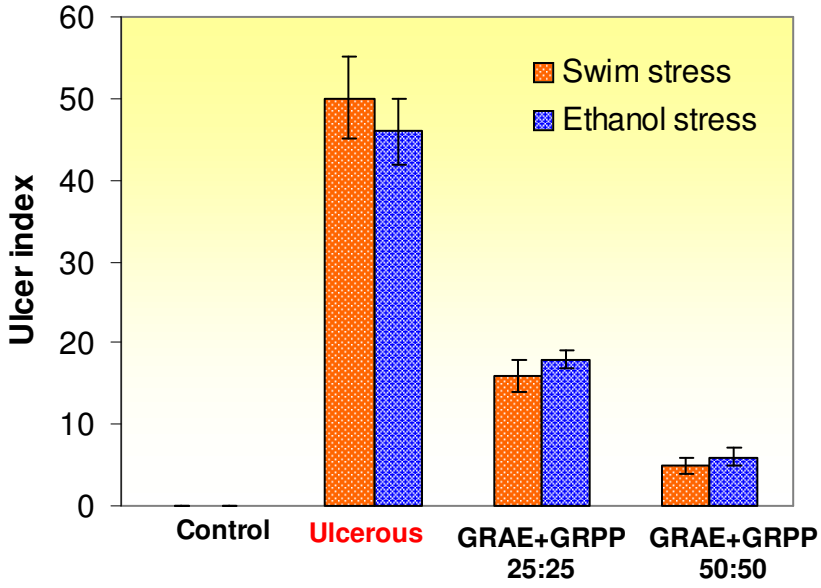
84% recovery of gastric mucin as apposed to only 55 % in the spontaneous healing after day 10 and of a 3 fold better inhibition H^+ K^+-ATPase activity in GRPP treated groups was observed compared to untreated or spontaneous healing groups.

Observed results were supported by the data provided in Table 3.6 and depicts the recovery of gastric mucin and *in vivo* H^+, K^+- ATPse inhibition status in different experimental groups. 84% recovery of gastric mucin as apposed to only 55 % in the spontaneous healing after day 10 and of at least 3 fold better inhibition activity in GRPP treated groups of animals than those allowed to heal spontaneously suggest the role of GRPP also in healing the gastric ulcers. Despite 3 fold better inhibition of ulcer index, H^+, K^+-ATPase inhibitory levels were only partial. Higher than 200 mg/kg b.w. of GRPP may be essential for complete ulcer healing ability. Also suggesting that ulcer healing effect may by some other mechanisms than inhibition of H^+, K^+-ATPase. Further, it makes sense also in that since H^+, K^+-ATPase is already induced leading to ulcers, curing requires a regeneration process. Further in depth study in this direction may unravel the mechanism of ulcer healing by GRPP. As reported by others (Matsumoto et al, 2002) and recently established in our laboratory (unpublished observation) for another source, ulcer healing effect was due to the ability of polysaccharide to enhance mucin synthesis. GRPP also has potentials to enhance mucin synthesis.

3.3.14. Combinational effect of GRAE and GRPP against ulcer *in vivo*: Ulcer preventive ability

Since GRAE and GRPP are potent in inhibiting ulcers *in vivo*, and they carry different constituents such as mainly phenolic acids in GRAE and polysaccharide with little 5 % of phenolic acids in GRPP, attempt was made in this experiment to understand the effect of both the components in combination. 90 % protection in ulcer index was observed at 50:50 (GRAE+GRPP) mg/kg b.w. combination (Figure 3.17) and the result was substantiated by the gastric mucin protection and down regulation of H^+, K^+-ATPase enzyme activity (Table 3.10). Data presented in Figure 3.17 and Table 3.10 & 3.11 suggests that ~ 1.3 -1.7 fold or ~ 25 – 42% increase in protection was observed both in terms of inhibiting ulcer index (Figure 3.17), inhibition of H^+, K^+-ATPase enzyme activity, mucosal protection (Table 3.10) and normalization of antioxidant and antioxidant enzymes. (Table 3.11), in GRAE+GRPP system rather than in groups treated with either GRAE or GRPP alone. Data thus suggest that there may be some synergistic effect of both GRAE and GRPP constituents present in them. Precise mechanism of synergism and interaction needs to be elucidated

Figure 3. 16. Effect of GRPP on Ulcer index



SS- ulcer index	**0**	**50±6**	**14±1.8**	**5±0.6**
% protection	**-**	**0**	**59**	**90**
ES- ulcer index	**0**	**46±4**	**17±1.6**	**6±0.7**
% protection	**-**	**0**	**57**	**87**

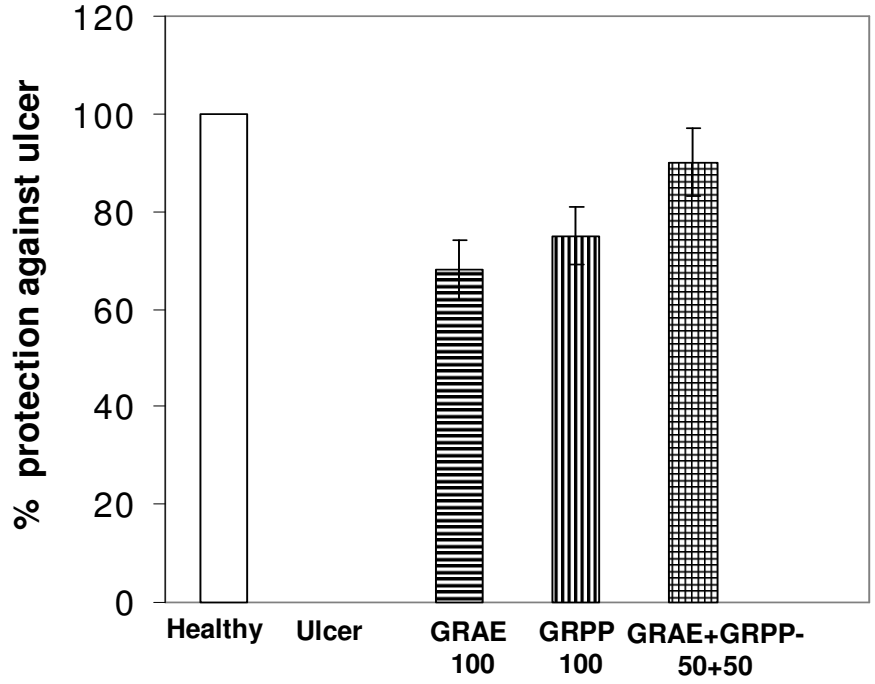
Dose dependent protection observed in Combinational treatment

Table 3.10. Gastric mucin and H^+, K^+-ATPase levels in healthy, ulcerated and protected rats. (n = 6) mean ± SD.

Group, n=6	Mucin content (mg/g)	H^+,K^+-ATPase μ moles Pi released/mg/h
Healthy	62.05^{d} ±5.1	0.721^{a}± 0.02
Swim stress induced ulcer model		
Swim stress induced	18.42^{a} ± 3.4	2.610^{d} ± 0.21
GRAE+GRPP (25:25)	40.41^{b} ±4.2	1.418^{b} ±0.13
GRAE+GRPP (50:50)	49.61^{bc} ±4.4	1.012^{a} ±0.09
Ethanol stress induced ulcer model		
Ethanol stress induced	22.37^{a} ±2.3	2.318^{C}± 0.24
GRAE+GRPP (25:25)	38.34^{b} ±3.8	1.368^{b} ±0.11
GRAE+GRPP (50:50)	51.65^{c} ± 4.5	0.961^{a} ±0.08

Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and GRAE+GRPP (with different ratio) treated groups. Range was provided by Duncan multiple test at $P < 0.05$. a: Less significant; b: Moderately significant; c: Very significant and d: Most significant.

Figure 3.17: Combinational effect of GRAE and GRPP on antiulcer activity – ulcer index



At a dose of 100 mg/kg b.w. GRAE protects up to 68 % and GRPP 75% but at the same dose in combination it protected up to 90% showing the improved synergestic effect of phenolics from GRAE and polysaccharide from GRPP.

Table 3.11. Individual and combinational effect of GRAE and GRPP on ulcer preventive properties *in vivo*

A	Ulcer Index	% Protection	Gastric mucin (mg/g)	% Protection	$H^+ K^+$ ATPase
Healthy	0	100	62	100	0.72
Ulcer induced*	50	0	18	0	2.6
GRAE-100 mg/kg b.w.	16	68	43	69	0.83
GRPP-100 mg/kg b.w.	13	75	31	50	1.76
GRAox + GRPP (50+50) mg/kg b.w.	5	90	54	87	1.01

* Ulcers were induced by swim stress in various

Table 3.12. Individual and combinational effect of GRAE and GRPP on ulcer preventive properties *in vivo*

B	CAT	SOD	GPx	GSH	TBARS
Healthy	22.8±2.1	201±21	68.6±5	216±23	2.16±0.19
Ulcer induced	48.2±6.2	78±7	26.5±2	368±42	0.76±0.06
GRAox-100	38.6±3.4	161±18	32.4±3	306±32	1.12±0.08
GRPP-100	24±2.2	142±12	37.6±2	297±20	0.87±0.03
GRAox + GRPP (50+50)	36.1±2.9	160±13	44.6±4	248±19	1.42±0.11

* Ulcers were induced by swim stress in various

3.3.15. Toxicity study

Toxicity studies with aqueous solution of GRPP carried out in rats for safety evaluation indicated no lethal effect upto 1 g/kg b.w. when orally fed for 14 days. There were no significant differences in total protein, TBARS levels, ALP, SGPT and SGOT between normal and GRPP treated rats (Table 3.9) indicating no adverse effect on the major organs. Animals after above treatment schedule remained healthy as that of control animals with normal food and water intake, body weight gain and behavior.

Table 3.13. Toxicity studies with ginger pectic polysaccharide (n = 6) mean ± SD:

Parameters	**Control**	**GRPP treated**
Total protein	$348^{a} \pm 32.21$	$366.51^{a} \pm 32.1$
SGOT (U/mg protein)	$18.34^{a} \pm 1.55$	$17.16^{a} \pm 1.94$
SGPT (U/mg protein)	$21.31^{a} \pm 2.70$	$19.36^{a} \pm 2.14$
ALP (U/mg protein)	$35.52^{a} \pm 3.879$	$36.25^{a} \pm 3.29$
TBARS (n moles/mg protein)	$0.166^{a} \pm 0.08$	$0.155^{a} \pm 0.09$

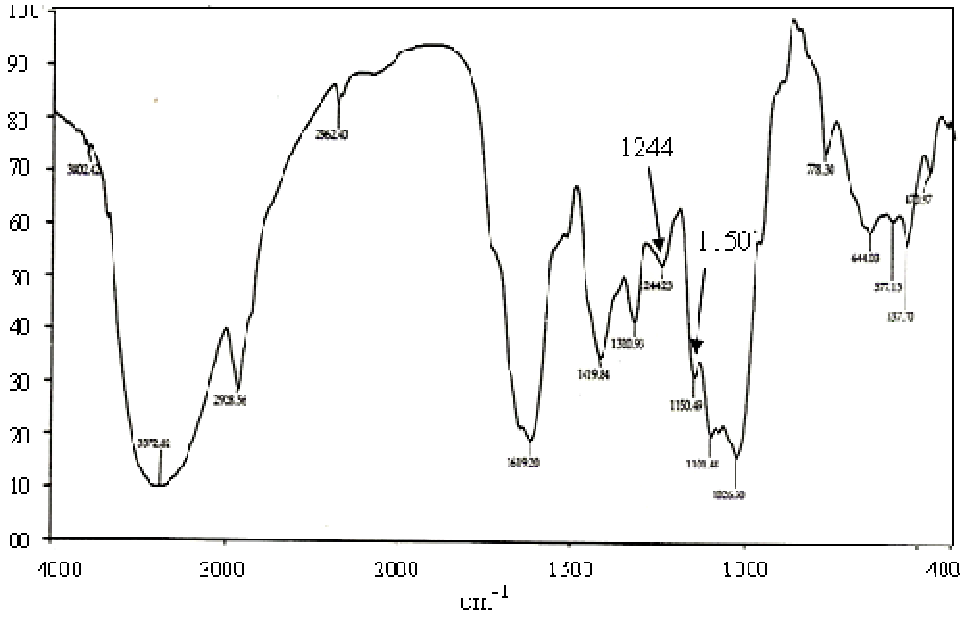
SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase; ALP: Alkaline phosphatase; TBARS: Thiobarbituric acid reactive substances. $^{a}P <$ 0.05 between control and GRPP treated groups.

3.3.16. Characterization of GRPP and its structure - function relation to anti-ulcer activity

3.3.16.1 FTIR spectra

FTIR spectra obtained using a FTIR spectrometer (Perkin-Elmer 2000 spectrophotometer) equipped with TGS detector with solid samples at a concentration of 1-10 mg provides a signal at ~ 1244 and 1150 cm^{-1} indicating the presence of sulfonamides where sulfate may be found attached to aminosuagars of pectic polysaccharide (Figure 3.18).

Figure 3.18. Fourier Transform Infra-Red Spectroscopy of GRPP and MGPP



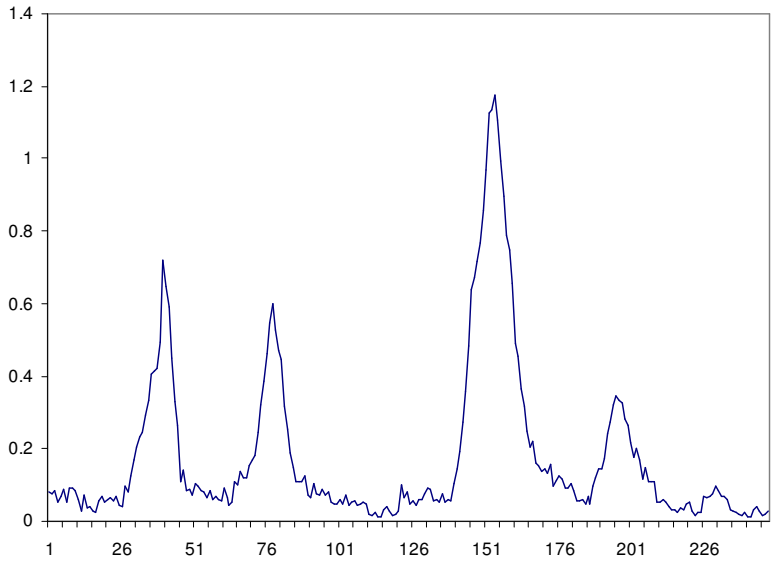
Arrow at 1329 and 1145 cm-1 indicate the presence of sulfonamide group in FTIR spectrum.

3.3.16.2. Phenolic content and sugar composition

GRPP being a pectic polysaccharide contained higher levels of carbohydrate of specific composition. However GRPP also contained phenolics that were bound to sugar chain. Thus the composition of both carbohydrate and phenolics of GRPP and GRPP fractions ($GRPP_{0.05M}$, $GRPP_{0.10M}$, $GRPP_{0.15M}$, and $GRPP_{0.20M}$) were determined and established correlation coefficient with bioactivity such as antioxidant, inhibition of H^+, K^+-ATPase and inhibition of *H. pylori* growth, to understand the structure – function analysis.

The individual neutral sugar composition of GRPP and its DEAE cellulose fractions were determined by hydrolysis of pectic fractions followed by derivatization and analysed as their alditol acetates by GLC (Figure 3.19 & Table 3.15).

Figure 3.19. Fractionation of GRPP on DEAE cellulose column chromatography.



GRPP was fractionated on DEAE – cellulose column chromatography and fractions resolved using 0.05 M-2 M Ammonium carbonate.

Results indicated the presence of Rhamnose (4%), Arabinose (24%), Xylose (8%), Mannose (3%), Galactose (<1%) and Glucose (60%) in GRPP. Arabinose and glucose were present in higher concentration in all the fractions whereas galactose and xylose were negligible. $GRPP_{0.05M}$ and $GRPP_{0.20M}$ fractions however contained higher (33%) levels of mannose and rhamnose (21%) as a predominant sugar respectively (Table 3.15).

Table 3.14. Yield of GRPP fractions (%)

Fractions (Amm carb)	Yield (%)
0.05M	16 ± 2.0
0.10M	15 ± 1.6
0.15M	40 ± 3.2
0.20M	10 ± 0.8
0.25M	05 ± 0.4

GRPP was fractionated on DEAE – cellulose column chromatography and fractions resolved using 0.05 M-2 M Ammonium carbonate.

Table 3.12 provides total phenolic content and bioactivity – particularly antiulcer potentials, since we have shown in our previous papers that antioxidant activity followed by inhibition of H^+, K^+-ATPase and *H. pylori* represents the potential anti-ulcer property (Siddaraju & Shylaja, 2007a,b & Srikanta et al, 2007, Yougender et al, 2007). GRPP contained 5.76 mg/g phenolics and this was distributed at different levels ($GRPP_{0.05M}$ ~45%, $GRPP_{0.10M}$ ~14%, $GRPP_{0.15M}$ ~31%, $GRPP_{0.20M}$ ~16%) in GRPP fractions. Relatively better (1.5 to 4 fold) antioxidant activity and H^+, K^+-ATPase inhibitory activity was observed in GRPP than in GRPP fractions; while *H. pylori* inhibition activity was better in $GRPP_{0.15M}$ fraction (~ 2 fold better than GRPP) suggesting that differential composition may be responsible for differential activity. Among the fractions, $GRPP_{0.05M}$ showed ~ 2 to 3 fold better antioxidant and H^+, K^+-ATPase inhibitory property than the other fractions. All the activities were poor in $GRPP_{0.10M}$ fraction.

Table 3.15. Sugar composition of GRPP and its fractions

	Rham	Arab	Xyl	Man	Gal	Glc	Uronic acid
GRPP	4	24	8	3	7	54	24
0.05M	10	25	3	33	2	27	2
0.10M	15	49	7	12	1	16	5
0.15M	8	10	1	2	1	78	9
0.20M	21	45	12	3	2	16	3

Relative percent of each sugar of GRPP fractions were determined employing Gas Liquid chromatography.

A Significant correlation observed between total phenol content & antioxidant activity (R^2 ~0.829); between antioxidant and H^+, K^+-ATPase inhibition (R^2 ~0.997). There was no significant correlation found with different sugars in them suggesting that phenolics of polysaccharide exhibit antioxidant property and antioxidants present in polysaccharide may be responsible for the H^+, K^+-ATPase inhibitory property. Contradictory to this, correlation with R^2 of ~ 0.888 was observed with glucose content in the fraction of GRPP with *H. pylori*. Results were substantiated by better anti-*H. pylori* activity (1.5 fold) in $GRPP_{0.15M}$ fraction than in GRPP and other *H. pylori* inhibitory fractions suggesting that polysaccharide with higher glucose may be responsible for the activity.

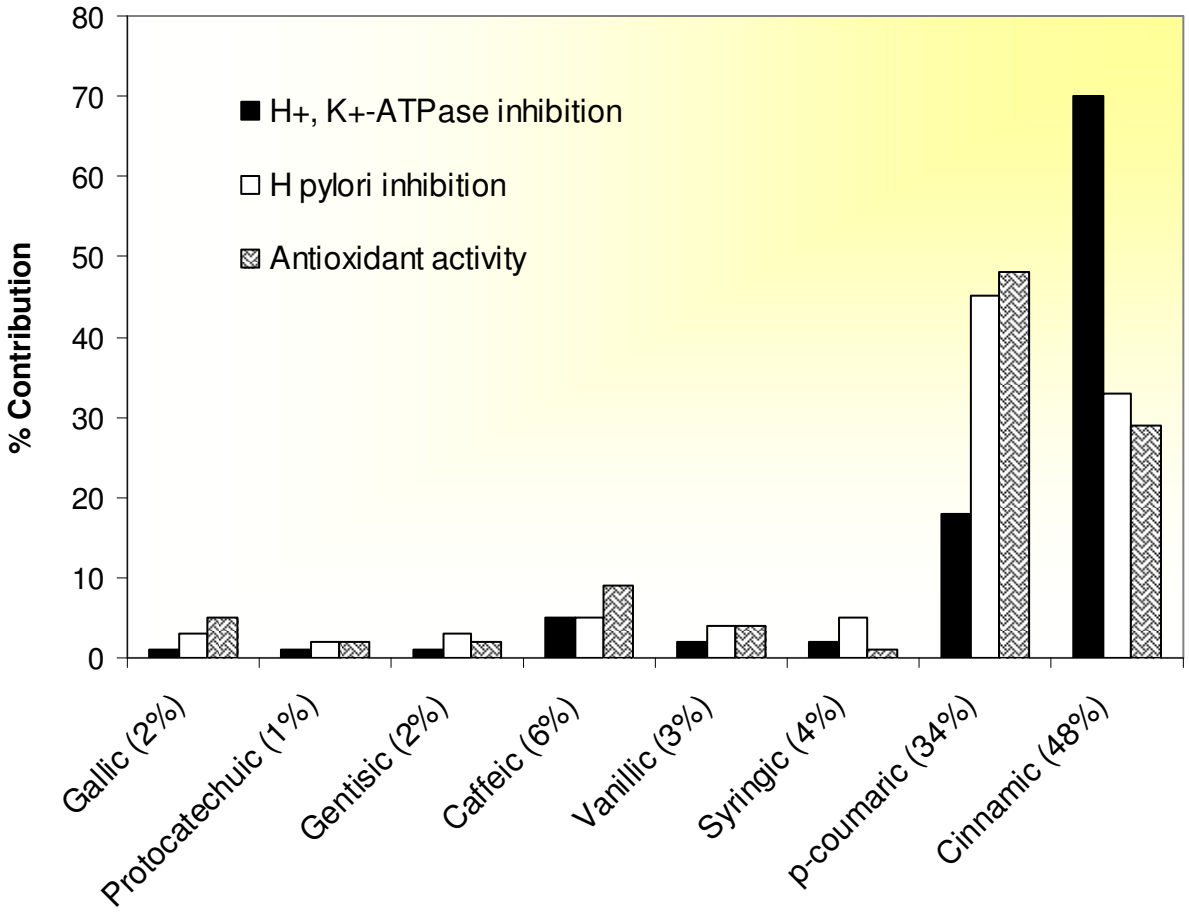
Table 3.16. Consolidated table depicting bioactivity of GRPP fractions.

DEAE – Fractions	Phenolics (mg/g)	AOX activity * - IC_{50} (in µg)	H+K+ATPase inhibition - IC_{50} (in µg)	Inhibition of *H pylori* growth – MIC (in µg)
GRPP	5.76	205	27	60
0.05M	02.6	380	54	86
0.10M	0.8	812	155	140
0.15M	01.8	556	102	44
0.20M	00.9	784	150	100

Role of phenolics of GRPP towards its contribution to potential ulcer preventive properties - antioxidant, inhibition of H^+, K^+-ATPase and *H. pylori* growth has been depicted. Cinnamic acid followed by p-coumaric acid contributed to these activities significantly.

Further total contribution by phenolic acids to *H. pylori* and H^+, K^+-ATPase inhibition was calculated reveals that cinnamic acid followed by p-coumaric acid offers better activity against *H. pylori* and H^+, K^+-ATPase inhibition in addition to antioxidant activity (Figure 3.20). Although gallic acid has better antioxidant activity than p-coumaric acid and cinnamic acid, their total contribution to antioxidant activity was higher due to their higher abundance.

Figure 3.20. Percent contribution of GRPP bound phenolics to bioactivity of GRPP



Role of phenolics of GRPP towards its contribution to potential ulcer preventive properties - antioxidant, inhibition of H^+, K^+-ATPase and *H. pylori* growth has been depicted. Cinnamic acid followed by p-coumaric acid contributed to these activities significantly.

3.4. Discussion

Ulcer results from an imbalance between aggressive factors and the maintenance of mucosal integrity through the endogenous defense mechanisms. To regain the balance, different therapeutics including spice and plant extracts have been used. In chapter II we have shown that free and bound phenolics of ginger possessed potential ulcer preventive activity *in vitro,* including inhibition of H^+, K^+-ATPase and *H. pylori* growth. However in view of addressing a question whether the traditional practice of using crude ginger extract in either boiled water or cold water extract can yield compounds which are gastroprotective in nature; we evaluated *in vitro* and *in vivo* ulcer preventive properties of ginger aqueous extract -GRAE and determined whether it also contained phenolic acids that favors gastroprotection as reported in our previous papers (Siddaraju & Shylaja, 2007a, b).

Traditional systems of medicine have been used throughout the world for centuries. Certain ancient systems, such as traditional Chinese medicine, Ayurveda (the holistic system of medicine from India), and Tibetan medicine, are still used extensively, particularly in their country of origin. In developing countries, interest in the therapies of such systems, particularly for the treatment of chronic illness, is growing. These therapies, usually referred to as complementary or alternative medicine, range from medicinal herbs to acupuncture to massage. Most of them have not been studied scientifically, and nearly all are unregulated.

GRAE at 200 mg/Kg b.w. protected swim stress/ethanol induced ulcer lesions up to 86% similar to that of lansoprazole (80%), a known antiulcer drug at 30 mg/Kg b.w. Bloody streaks, inflammations, oozing of blood into the lumen of the stomach etc., observed in ulcerous animals were not found in GRAE ingested animals, similar to those of healthy rats indicating the gastroprotective effect of GRAE. Further, we followed the protective effect investigating the biochemical parameters such as alterations in the gastric mucin, oxidants, GSH, H^+, K^+-ATPase and antioxidant enzymes level including catalase, superoxide dismutase, peroxidase etc., in the ulcerated organ-stomach as well in the metabolizing organ-liver in all groups of rats-healthy, ulcerated and GRAE/lansoprazole treated. Preventive antioxidant enzymes such as superoxide dismutase and catalase are the first line of defense against reactive oxygen species. Administration of GRAE resulted in a significant increase in the SOD, catalase and

reduced GSH levels (Table 3.1 and 3.2) similar to those of control animals, suggesting the efficacy of GRAE in preventing free radical-induced damage during ulceration.

In our experimental model ~ 3.4 fold reduction in gastric mucin and 2.4 fold reduced glutathione as well as 2.6 fold increased oxidative product- TBARS in the stomach were normalized by GRAE (Table 3.1, 3.2 & 3.3) treatment. Gastric ulcers are often a chronic disease and it may persist for 10-20 years as characterized by repeated episodes of healing and re-exacerbations. Stress induced ulcer better resembles clinical ulcers in chronicity, severity and practicality of experiencing stress due to varietal patterns of lifestyle in day to day life and serves the most reliable model to study ulcer healing process (Brady et al, 1979, Mohri et al, 1970). The incidence of swim stress induced ulcer is predominant in the glandular part of the stomach leading to gastric mucosal/mucin damage. GRAE significantly prevented ulcers both by reducing the oxidative stress as well as boosting the mucosal defense. Further, during our study, we evaluated the possible mechanism of protection to gastric ulcer apart from upregulation of antioxidant and antioxidant enzyme levels. Gastric H^+, K^+-ATPase located in the apical membrane of parietal cells, pumps protons into the gastric lumen, using energy derived from the hydrolysis of ATP, and is thus involved in gastric acid secretion. Accordingly, the activity of gastric H^+, K^+-ATPase was measured in the stomach homogenate, which showed 3-fold upregulation of the enzyme in ulcer condition and was normalized by treatment with GRAE (Table 3.1). Results were further substantiated by sheep H^+, K^+-ATPase inhibition by GRAE with an IC_{50} of 16.5 ± 1.2 µg/mL on par or better than lansoprazole (19.3 ± 2.2 µg w/w), indicating the potential multi-targeted effect of GRAE in preventing swim stress induced ulcers in experimental rats. GRAE may find itself more useful as a H^+, K^+-ATPase (proton pump) inhibitor than the existing pump inhibitors, since they have adverse effects as reported particularly under conditions of pregnancy/lactation and alcohol or any other drug consumption. Least toxicity of GRAE may also find GRAE as useful alternative source for ulcer healing therapeutics.

Also it is intriguing to observe that cinnamic acid is acting as a potent inhibitor of H^+, K^+-ATPase, and also *H. pylori* probably by both binding effect of cinnamic acid than gallic acid (Figure 3.20). Lack of correlation between fold inhibitory activity v/s binding between gallic/cinnamic acid still do not rule out the proposed mechanism. Because being hydrophobic

cinnamic acid binding ability may be higher with H^+, K^+-ATPase and *H. pylori* which carry membrane domains than HSA alone which is devoid of this hydrophobic domain.

GRAE also exhibited reducing power and prevented free radical induced lipid and DNA Peroxidation. This antioxidative property also contributes significantly to reduce ulcer condition and justifies the ethno medical claims.

The most commonly used alternative therapy is dietary supplements, which include medicinal herbs and nutraceuticals. Most dietary supplements used in alternative medicine are derived from plants; some are derived from animals. Pectic polysaccharides have been identified as the functional molecule since they contribute to various biological properties, in addition to offering protection to plants. They are generally constituted by various sugars such as rhamnose, arabinose, mannose, xylose and glucose in addition to uronic acids and phenolics which can amplify the crosslinking. Crosslinking gives a characteristic structural conformation to a polysaccharide, which in turn is dependent on the nature and arrangement of sugar and phenolic residues present in them (Mary et al, 2005). So far feruloyl-polysaccharide crosslinks have been reported where ferulic acid is crosslinked with pectic polysaccharides. Occasionally p-coumaric acid has also been found to be associated with pectic polysaccharides (Jim et al, 1992). In the current chapter we report a non-feruloyl pectic polysaccharide with the composition of rhamnose, arabinose, mannose, xylose, galactose and glucose with uronic acid and contained 5.76 mg/g of phenolics. Cinnamic acid (48%) and p-Coumaric acid (34%) were found to be the major phenolic constitutents of the polysacchride and contributed significantly to the inhibition of H^+, K^+-ATPase and *H. pylori* growth. Similar observations were made earlier from our laboratory (Siddaraju & Shylaja, 2007) substantiating the observations of Vattem *et al*. (2005)

In another study we had reported phenolic bound pectic polysaccharide from Swallow root (SRPP) that could offer potential ulcer preventive properties with multi-step action. During our continuous survey, a pectic polysaccharide from ginger –GRPP also showed a potent H^+, K^+-ATPase inhibitory activity with an IC_{50} of 27 µg/mL; similar to that reported from Omeprazole-known proton pump blockers which had IC_{50} of 27 µg/mL. Current study was undertaken to precisely understand the role of GRPP in ulcer preventive ability *in vivo* and *in vitro* to elucidate the probable mechanism of action.

Crude ginger polysaccharide showed promising antiulcer activity when examined for H^+, K^+-ATPase inhibition. This was further fractionated into water soluble polysaccharide, pectic polysaccharide (GRPP), hemicellulose A, hemicellulose B and nonsoluble alkali residue. They were screened for antiulcer potency. Only pectic polysaccharide showed the inhibition of H^+, K^+-ATPase, an enzyme responsible for influx of protons into the stomach lumen causing ulcers. Further antiulcer effect of ginger pectic polysaccharide was confirmed by multiple *in vitro* assay systems such as protection against mucosal damage, *H. pylori* growth and anti-oxidative action.

GRPP possessed H^+, K^+-ATPase inhibitory activity (IC_{50} ~ 27 µg/mL). Phenolics present in GRPP together with those reported in the literature reveal that phenolic antioxidants are potent H^+, K^+-ATPase blockers (Siddarajuu & Shylaja, 2007a,b & Srikanta et al, 2007, Ricardo et al, 2006). Significant levels (~6%) of phenolics present in GRPP were found to contribute to inhibition of H^+, K^+-ATPase activity, a key player in gastric protection. Of the reported phenolic acids in GRPP, cinnamic acid followed by p-coumaric acid contributes significantly - ~70 and 20% to H^+, K^+-ATPase inhibition respectively (Figure 3.20). Inhibition of H^+, K^+-ATPase envisages the neutralizing action against acidic condition in the gastric lumen. Results were further supported by *in vivo* studies where the GRPP treated group showed 2.5 folds reduction in up-regulated H^+, K^+-ATPase activity that occurred during ulcerous condition.

Further, during *in vivo* studies, oral administration of 100 and 200 mg/kg b.w. reduced gastric lesions. It is evident from our data that swim and ethanol stress - induced gastrointestinal disturbances, such as gastric erosions, gastric or duodenal ulcerations, gastrointestinal hemorrhages and perforations (Figure 3.9 and 3.10); were modulated including inhibition of up-regulated H^+, K^+-ATPase, and enhancement of down regulated gastric mucin / antioxidant & antioxidant enzyme levels (Table 3.5, 3.7 and 3.8). Histological studies indicated that characteristic ulcerogenic pathogenicity with distinct ulcer margin formed by the adjacent non-necrotic mucosa – the epithelial component, and granulation tissue at the ulcer base was normalized upon treatment with GRPP. In addition, GRPP is a safer source since toxicity studies indicated no lethal effect at least up to an oral dose of 1 g/kg b.w. for 14 days.

Besides, GRPP protected gastric mucin, an essential event to keep up the mucosal integrity and gastro protective properties. Understanding the potential role of GRPP in gastric mucosal protection is important since mucin is an insoluble adherent mucus gel, which is quite stable and has significant buffering capacity for neutralization of luminal acid in presence of bicarbonate and hence a key regulator of gastric ulcer. Also gastric mucin has been used by *H. pylori* as a media to invade into the host. GRPP showed 96 % recovery of damaged gastric mucin as revealed by immunohistological / biochemical and ELISA methods indicating the stabilization of the mucosal layer by GRPP during induction of ulcers by swim and ethanol stress. The enhancement of gastric mucin contents in GRPP treated group as measured by ELISA and Alcian blue binding may suggest that enhancement is most probably due to both prevention / protection of mucosal injury during ulceration and enhanced synthesis. This is supported by up-regulation of gastric mucin in GRPP controls where animals were fed with GRPP alone without inducing ulcers. Similar results were observed with previously reported antiulcer polysaccharide – SRPP, where we could show that increased synthesis was due to increased proliferation of gastric mucosal cells (unpublished observation). However, regulated synthesis might also occur which may be evaluated by studying the integration of sugar residues of GRPP to gastric mucin employing tracer techniques. Gastric mucin being the target for *H. pylori* attack, interaction between *H. pylori* and mucin was studied in presence and absence of GRPP. Binding studies indicated that isolated rat gastric mucin binds to *H. pylori* as evidenced by alterations in UV spectral profile (Figure 3.13A). In an independent experiment hemagglutination assay, GRPP has also shown to bind to gastric mucin very effectively (Table 3.6). It is possible that GRPP by virtue of its anionic nature may bind effectively to positively charged amino acid residues of gastric mucin as that of sucralfate and other polysaccharides (Rees, 1991). Further when GRPP + mucin were preincubated with *H. pylori* the result of hemagglutination suggests that more or less *H. pylori* and GRPP may bind to the similar region on the mucin. However reduction in binding due to steric hinderance cannot be ruled out. Detailed studies would envisage the precise binding site of GRPP and *H. pylori* on gastric mucin. This binding of GRPP may avoid gastric mucin damage and hence may prevent ulcers as has been clearly demonstrated by *in vivo* data. In other words GRPP may possess the capability to dissociate *H. pylori* binding to gastric mucin suggesting the potential ulcer healing or curative ability of GRPP. *H. pylori* attack *in vivo* may eventually be avoided. Revival of agglutination of RBCs by *H. pylori* supports this observation (Table 3.6).

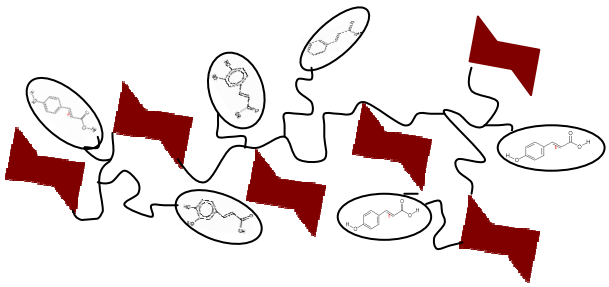
GRPP showing potential anti-*H. pylori* activity adds to the ulcer preventive potency of GRPP. This is the second polysaccharide reporting from our laboratory regarding anti-*H. pylori* effect. Results are in accordance with the observation made by Lee et al., (2006) and from our laboratory (Siddaraju & Shylaja, 2007a,b), where inhibition of *H. pylori* growth by pectic polysaccharides is reported. GRPP phenolics may inhibit microbial activity by following mechanisms a) hyper acidification at the plasma membrane interface of the *H. pylori* b) intracellular acidification, resulting in disruption of H^+, K^+-ATPase required for ATP synthesis of microbes or; c) inactivation of cellular enzymes causing membrane permeability changes (Shahidi et al, 2004, Sung-Sook et al, 2005, Vattem et al, 2005). Phenolics may inactivate the urease enzyme, which is essential for neutralizing hyper acidification for *H. pylori* to survive in the gastric environment of the stomach. In the experimental evidences provided in Figure 2 it is thus clear that GRPP is creating a cavity in the organism with the loss of cellular contact resulting in loss of viability of *H. pylori*. Indeed such a killing of *H. pylori* by GRPP may necessiate the binding of GRPP to *H. pylori.*

The observation that GRPP has the ability to bind to gastric mucin and *H. pylori* has lots of implications in potential *in vivo* antiulcerative effect, even with the major ulcerogen – *H. Pylori.* It is pertinent to mention here that gastric mucin play a protective role against ulcer inducing factors such as free radical induced oxidative injury and *H. pylori* infection. The damaged mucin is more susceptible for *H. pylori* adhesion and successful infection. In both swim and alcohol stress induced oxidative injury results in degradation of mucin. Results are supported by a). reduction in gastric mucin content as indicated by histological and immunohistological studies (Figure 3.11) and b). degradation of mucin resulting in low molecular weight form upon SDS gel electrophoresis (Figure 3.12). However, upon treatment with GRPP, normalization of all these events reveals that GRPP not only prevented gastric mucin damage but also enhanced the biosynthesis which compensated for damaged mucin. Of all the fractions better inhibition of *H. pylori* was by $GRPP_{0.15M}$ fraction containing high glucose is intriguing (R^2 ~ 0.888), and suggest that *H. pylori* may bind effectively to glucose enriched-polysaccharide than those of GRPP fractions with lesser glucose content. Implications of this result further strengthen the displacement effect of *H. pylori* to mucin. Extrapolation of *in vitro* data may reveal that *H. pylori* attack may potentially blocked by GRPP. There is a possibility of binding of GRPP also in *in vivo* condition that may envisage gastric protection against ulcers.

$GRPP_{0.05M}$ fraction has better AOX and PPI activity; while *H. pylori* inhibition in $GRPP_{0.15M}$ fraction as revealed by correlation coefficient studies may suggest the differential role of constituent sugar and phenolics present in them.

Results of the experiments thus enable us to propose probable steps acted upon by GRPP that can inhibit ulcer formation. As proposed in scheme -2, GRPP inhibited oxidative stress induced H^+, K^+-ATPase activation which controls acidity and hence gastric mucin damage and attack by *H. pylori*. Direct action of GRPP on *H. pylori* inhibition and gastric mucosal protection adds to the significance. Also antioxidative properties of phenolics (cinnamic and p-coumaric) of GRPP has an additional advantage of acting at the site of mucosal damage to offer protection in *in vivo* situation also.

Since phenolic antioxidants are attached to polysaccharide it can target directly the affected cell and protect gastric mucin against mucosal damage. It is possible that as per the concept of *Craig Fleming et al.* (2005) polysaccharide may bind to cells by virtue of carbohydrate – carbohydrate interaction and once internalized may release antioxidants that can now freely work. In GRPP therefore, it can bind to cells (possibly also to parietal cells) may internalize polysaccharide and release cinnamic or p-ccoumaric acid that are now free to act on eliminating oxidative stress and hence; normalization of H^+, K^+-ATPase leading to reduction in activity.



Phenol bound polysaccharides

Multi-potency of GRPP together with non-toxic nature enables its use as a safer and promising multi-step ulcer blocking alternative to allopathic drugs that pose side effects. This is the first report on ginger polysaccharide with effective anti-ulcer potency. Combinational use of both GRPP and GRAE may reduce the load of these components to be used for effective healing or prevention of ulcers. Phenolics attached to pectic polysaccharide in GRPP may offer additional advantages in targeting and preventing pathogenesis related steps.

3.5. Summary and conclusions

- Antioxidant (GRAE) and pectic polysaccharide (GRPP) fractions of ginger showed potential ulcer preventive abilities *in vivo* against swim and alcohol stress induced ulcer models. ~ 80 – 90% protection was offered at 200 mg/kg b.w.

- Since GRAE is constituted mainly by cinnamic acid (50%) and gallic acid (46%) they appear to play antiulcerative role *in vivo*; Indeed they contributed significantly in inhibiting H^+, K^+ -ATPase, *H. pylori* inhibition and offering antioxidant activity *in vitro* that are required to exhibit antiulcer properties.

- GRPP on the other hand contained 5.76 mg/g of phenolics and rhamnose (4%), arabinose (24%), xylose (8%), mannose (3%), galactose (<1%) and glucose (54%) in its sugar moieties. Based on the studies both phenolics and sugar residues are implicated in *in vivo* antiulcerative properties.

- GRAE and GRPP fractions in combination, showed better antiulcer activity than when they were given individually, in *in vivo* testing models, suggesting that there may be synergistic interaction between the constituents present in GRAE such as phenolic acids and constituents typically found in polysaccharide fractions – sugar residues.

- Fractionation of GRPP followed by structure function analysis revealed that pectic polysaccharide contain several fractions which differ in their structure and function. Based on the results phenolic acid particularly cinnamic and p-coumaric acid and glucose rich fractions are implicated in inhibition of H^+, K^+ -ATPase and *H. pylori* respectively.

- Besides GRPP protected gastric mucin, gastro protective factors. The interaction studies revealed that GRPP may possess the capability to dissociate

H. pylori binding to gastric mucin, which is a putative step to prevent severity in ulcer pathogenicity.

- GRPP was not only effective in preventing ulcers; but could also heal ulcers. This observation is significant to explore the curative potential of GRPP.

- GRPP thus reported with multi potent activity as H^+, K^+ -ATPase inhibition to inhibit acid flux, to protect regenerate gastric mucin to potential gastroproventive ability and the potential to inhibit *H. pylori,* a major ulcerogen adds to the significance of GRPP to potential ulcer protection.

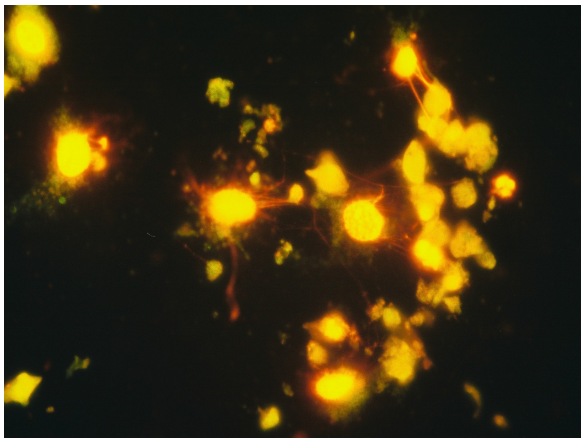
- This is second report about the multistep active but non toxic polysaccharide from our laboratory and first report on cinnamyl polysaccharide. Cinnamic acid being a good inhibitor of H^+, K^+ -ATPase and *H. pylori* growth, the binding of cinnamic acid adds further to the scope of the polysaccharide as antiulcer agent.

- Since oxidative damage is initiator of ulcer pathogenesis, via several mechanisms including mucin oxidation/degradation, binding of GRPP although by virtue of sugar residues to gastric mucin; cinnamyl and p-coumaric derivatives present in GRPP may neutralize oxidation.

- Multi-step active GRPP together with non-toxic nature enables its use as a safer and promising ulcer blocking alternative.

Chapter 4

Anticancer Attributes of Antioxidant and Polysaccharide Fractions



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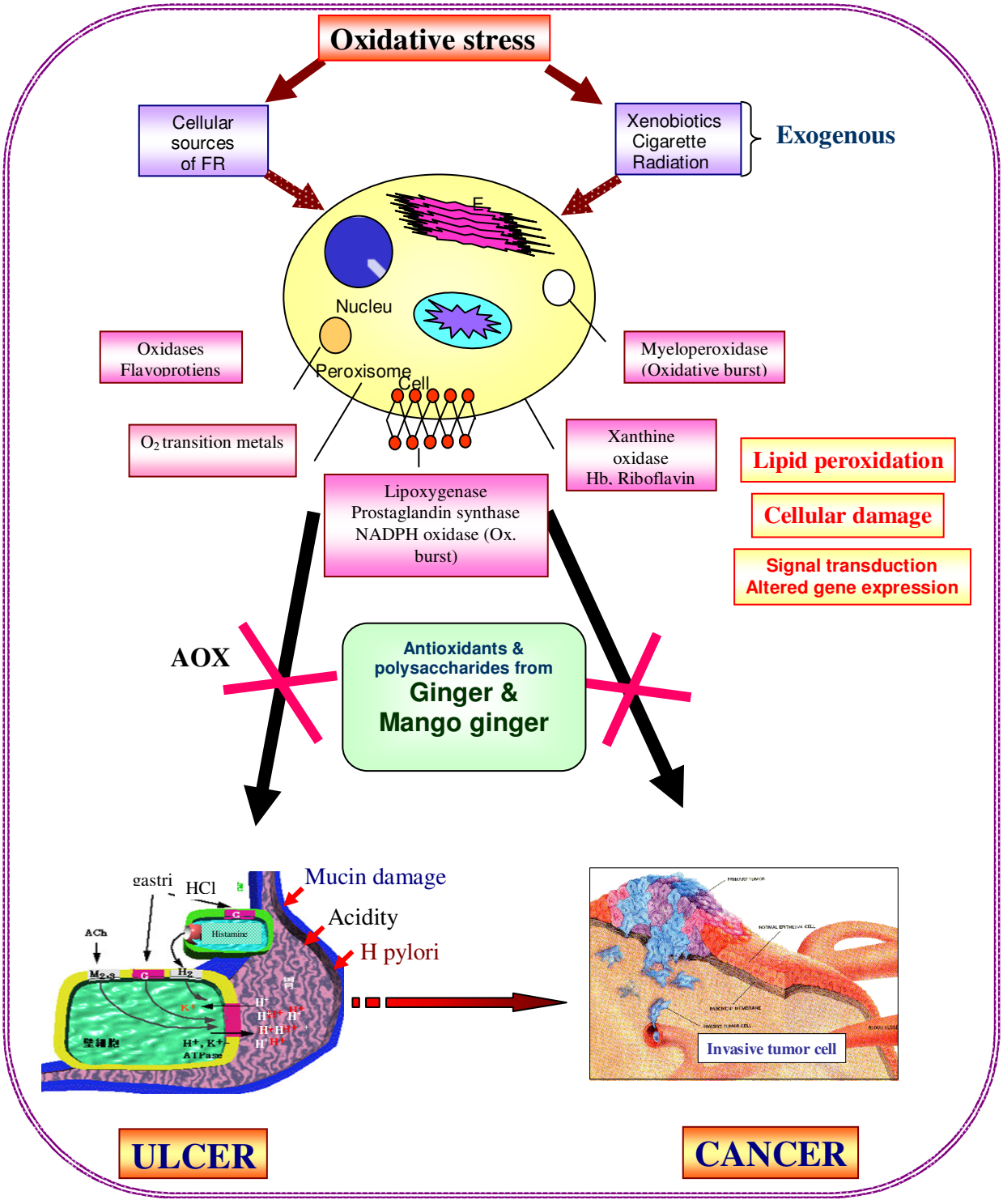
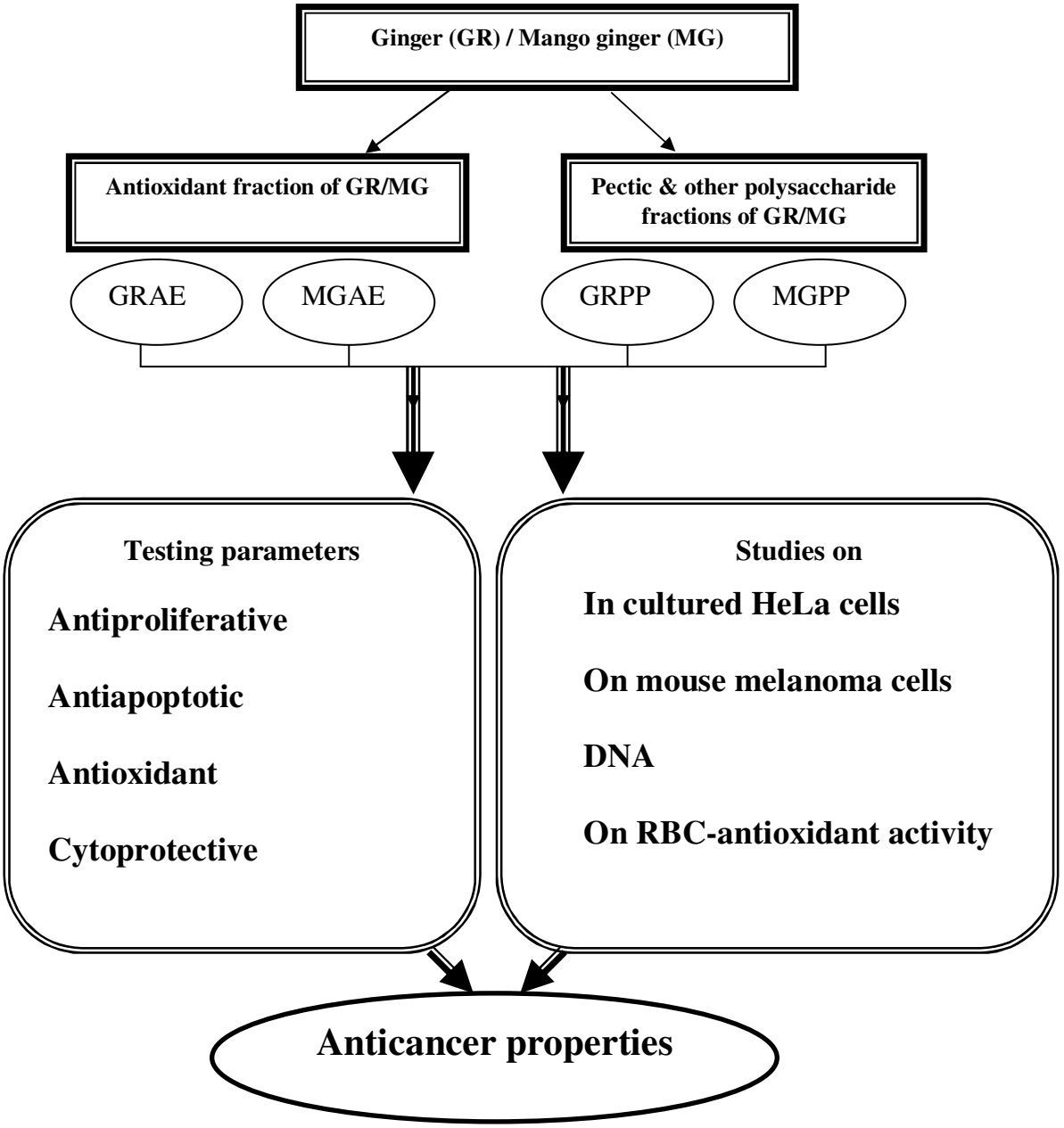


Can antiulcer compounds from Ginger and Mango ginger be anticancer also ?

WORK PLAN



Anticancer properties in Ginger and Mango ginger fractions

4.1. Introduction

Ginger (GR) and Mango ginger (MG) belongs to the family Zingiberaceae and several members of this have been shown to exhibit anticancer properties (Rhode et al, 2007 & Kirana et al, 2003*).* Cancer incidence is constantly raising in both developing and developed countries attributing to several factors such as free radical generation, repeated infection, long term exposure to environmental pollutants, drugs etc. Pungent components of ginger such as vallinoids, [6]-gingerol and [6]-paradol, shogaol and zingerone have been demonstrated to exhibit anticancer properties in ginger (Surh et al, 1999) while in mango ginger very few reports are available. Anticancer property was found to be due to antioxidant components in them (Yogeshwer & Madhulika, 2006).

Several polysaccharides extracted from traditional Chinese herbs also found to exhibit array of bioactivities including anti-inflammatory, anticancer, antitumor, antioxidant properties etc,. We could also show previously from our laboratory that, unlike reported compounds-gingerone, zinzeberone in ginger and curcumin in mango ginger, phenolic compounds playing a major role in exhibiting bioactivity (Siddaraju and Shylaja, 2007a&b).

Oxidative Stress (OS) induced ulcer pathogenicity was found to be effectively inhibited by phenolics of ginger (Siddaraju and Shylaja, 2007a) and mango ginger (Siddaraju and Shylaja, 2007b). Cancer disease is known to be mediated by Reactive Oxygen Species (ROS) induced cellular/nuclear damage (Wiseman & Halliwell, 1996). Cancer cell manipulation leading to advanced and serious stages in cancer i.e metastasis makes the disease pathogenicity a complex one and difficult to treat. In the current study we attempted to determine the anticancer attribute of antiulcer polysaccharide and antioxidants from ginger and mango ginger. Aqueous extracts of GR-GRAE and MG-MGAE containing significant levels of antioxidants and, polysaccharides were examined for anticancer properties. Anticancer attributes were addressed in fractions of GR and MG by examining antiproliferative, DNA protective, cytoprotective and apoptotic effects. Understanding of the role of antioxidants in GRAE/MGAE and pectic polysaccharides of ginger (GRPP) and mango ginger (MGPP) in offering to anticancer properties have been emphasized in this chapter. Polysaccharides are also included in the study because they found to contain significant levels of phenolic

antioxidants and antioxidant properties besides exhibiting various health beneficial properties (Srikanta et al, 2007, Wei et al, 2006, 2003, Gao et al, 2004)

Objective of the present study was to examine whether antiulcer phenolics and pectic polysaccharides from Ginger/Mango ginger exhibit potential anticancer activity against cancer cells *in vitro*. Results of the study indicated that both antioxidant and polysaccharide fractions exhibited anticancer property with different degree with respect to antioxidant and pectic polysaccharide fractions of ginger and mango ginger.

Objective :

Determination of potential Anti-cancer properties of Anti-ulcer antioxidants and polysaccharides from Ginger and Mango ginger.

4.2. Materials and Methods

4.2.1. Preparation of GR and MG fractions

Aqueous (antioxidant rich) fractions of ginger and mango ginger were prepared as described in **Chapter 2** and designated as GRAE and MGAE respectively. Similarly pectic polysaccharide fractions of ginger and mango ginger were prepared as described in **Chapter 3** and designated as GRPP and MGPP respectively.

The total phenolic contents in GRAE/MGAE and GRPP/MGPP were determined using a modified Folin-Ciocalteu method (Singleton and Rossi, 1965) as described in **Chapter 2.**

Total carbohydrate content was quantified in GRPP and MGPP by phenol-sulfuric acid method (Dubois et al., 1956) as described in **Chapter 3.**

4.2.2. Determination of inhibition of HeLa cell proliferation

HeLa –Human cervix carcinama cells were grown in 75 mm Flask as monlayer culture in DMEM high glucose containing 4.5g/L with 2 mM L-glutamine, 3.7 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% FBS and Antibiotics – streptomycin (100 units/mL) and fungizone (10 units/mL) were included in the medium to avoid microbial contaminations. Cells were grown to confluence at 37 ºC in humidified atmosphere containing 5% CO_2 in air and were passed weekly using 0.25% trypsin.

Antioxidant fractions -GRAE/MGAE at 5 - 25 µg GAE/mL and polysaccharide fractions - GRPP/MGPP at 100 – 500 µg/mL concentrations were used to measure their ability to inhibit cell proliferation (Nakano et al, 1998). Cells were exposed to the indicated concentrations of the extracts during a 48 h growth period. Cell proliferation was measured by the ability of viable cells to reduce 3-(-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, whose absorbance could be analyzed spectrophotometrically at 570 nm. All measurements were performed in triplicates. The absorbance was measured using the Hewlett Pakaard UV-Vis spectrophotometer (Palo Alto, CA). The results were expressed as the percentage of viable cell proliferation with respect to the control.

Cell proliferation (%) = [Mean absorbance of the control - Mean absorbance in presence of sample(s)) / Mean absorbance of the control] x 100; In other words Mean absorbance of the control is taken as 100%.

The effective median dose (EC_{50}), which is the amount of sample required to inhibit cell proliferation by 50%, was calculated graphically for each sample on cell proliferation curve and expressed as milligrams of ginger or mango ginger samples ± SD.

4.2.3. Determination of Viability of cells by Trypan blue assay

Number of viable cells was counted using the Trypan blue dye exclusion test. Briefly, cell suspensions both control and sample treated were mixed with an equal volume of Trypan blue solution (400 mg Trypan blue/100 mL PBS), and cell number in the mixtures was counted under an inverted microscope to discriminate the viable cells and dead cells. Viable cells look transparent without blue dye and dead cells appeared blue by uptake of dye. Three separate experiments for each extracts were conducted in duplicates. Growth inhibition (%) of cells was calculated using the following equation (Hendrik et al, 2003), Growth Inhibition (%) = [1-(cell number of test group/cell number of control group)] x 100%.

4.2.4. Morphological examination

B16F10 mouse melanoma cells were obtained from black tumor colonies from mouse which were prior injected with cultured B16F10 cells. Cells from tumor colonies were dispersed in sterile PBS pH 7.4 soon after its removal from the animal and subjected to morphological observation. Cells were observed under the inverted microscope before and after treatment with GRAE/MGAE (5 – 25 µg GAE/mL) and GRPP/MGPP (100 – 500 µg/mL). Cells were stained with acridine orange (AO) and ethidium bromide (EB) and examined under fluorescent microscope. Treated and untreated cells were photographed and compared the colour, nuclear localization, shape and size of cells treated with samples with those of control cells.

4.2.5. Determination of cytoprotective effects of GR and MG fractions

Cytoprotective role of ginger and mango ginger aqueous and pectic polysaccharide fractions were studied and quantitated by determining the percent protection offered to Red Blood Cells which are very fragile, by both colorimetric and microscopic methods.

4.2.5.1. RBC protective ability of GR and MG fractions against oxidant induced cell lysis

Red Blood Cells (RBC), a fragile blood cell known to be affected during various oxidative stress conditions were employed as a model to evaluate the cytoprotective ability of ginger and Mango ginger fractions as described earlier (Ana et al, 2003). For treatment with RBCs, both oxidants and test samples were prepared in PBS, pH 7.4 to maintain the appropriate osmolarity, so that nonspecific damage by such effects can be eliminated. Appropriate controls were set up during the experiment. A 10% suspension of erythrocytes in PBS, pH 7.4 was added to the same volume of oxidants (50 mM ascorbic acid and 80 mM $FeSO_4$) in presence and absence of 5 - 25 µg GAE of GRAE/MGAE and 20 – 100 µg/mL of GRPP/MGPP. After incubating the sample at 37 ºC for 2 h, the absorbance was read at 540 nm. The percent hemolysis was determined using the equation (1-A/B) X 100; where A = Sample treated and B = control.

4.2.5.2. Microscopic analysis of RBCs

In order to confirm that inhibition of lysis is due to intactness of RBCs after treatment with samples, microscopic examination was performed. Cell morphological pictures were captured at 0, 5, 10 and 20 min duration in both oxidant and sample treated systems. Cells were preincubated with indicated concentrations of samples. Both oxidants and samples were prepared in PBS, pH 7.4. Morphological changes observed in controls and tested samples were photographed at different time intervals (0 - 20 min). Differences between controls (untreated), oxidant treated and cells pretreated with test samples followed by oxidants treatment were recorded.

4.2.6. Effect of GR and MG fractions against H_2O_2 induced HeLa cell damage:

H_2O_2 is well-known to cause cellular damage via $OH^{\cdot}$ & $O^{\cdot}$ radicals (Mello et al, 1984). HeLa cells $1x10^8$ cells/mL were treated with 20 - 100 µM H_2O_2 concentration to understand the cellular damage at least up to 80% which is an essential step prior determining the effect of GR and MG fractions on H_2O_2 induced cellular damage. The concentration of H_2O_2 required to induce at least ~ up to 80% damage was selected for further studies.

4.2.6.1. Cytoprotective effect of GR and MG fractions against H_2O_2 induced cellular damage

$1x10^6$ cells washed with Hank's balanced salt solution (HBSS) first followed by PBS, pH 7.4 were treated with 5 – 25 µg GAE/mL and 20 – 100 µg/mL concentration of GRAE/MGAE and GRPP/MGPP, incubated at 37 ºC for 30 min. In the control and oxidant induced samples, cells were either treated with PBS alone or with 100 µL of H_2O_2 to obtain a final concentration as 80 µM. After 2 h of treatment cell viability was measured by MTT assay as described earlier. Results are expressed as % viability in presence and absence of the potential cytoprotective components.

4.2.6.2. Establishment of oxidant and antioxidant levels in cells treated with and without GR and MG fractions in healthy and oxidative stressed cell system.

Malondialdehyde (MDA) levels: Malondialdehyde one of the well – known secondary products of lipid peroxidation after exposure to Reactive Oxygen Species (ROS) and Free Radicals (FR) was used as indicator for cell membrane injury; since this is commonly encountered in cancer. The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm following a methodology previously described (Alejandro et al, 2005). The results are expressed as nmol MDA/mg of protein using a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1.}$

Glutathione levels: The glutathione levels from the cell cultures were determined by the DTNB GSSG reductase recycling assay as previously described (Anderson, 1985). The results are expressed as nmol GSH/mg of protein.

4.2.7. Determination of antimetastatic acitivity of GR and MG fractions.

Microplate agglutination assay was performed for the evaluation of potential dietary antimetastatic compounds employing the protocol of Nowak et al. (1976). Briefly, Human erythrocytes were prepared from 10 mL of fresh blood (collected in Alsever's medium) that has been washed 4 times with 5 volumes of 0.15 M NaCl. A 4 % erythrocyte suspension in 0.02 M PBS pH 7.4 containing 1 mg/mL trypsin was incubated at 37 ºC for 1h. The trypsin treated cells were then washed with 5 volumes of 0.15 M NaCl and fixed in 5 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 7.2 at 4 ^{o}C and the fixed erythrocytes were employed for hemagglutination assay. Hemagglutination assays were done in microtitre agglutination assay plate using serial dilutions of pectic polysaccharides of GR and MG in 0.15 M NaCl. The reaction mixture volume of 150 µL contained 20 µL of media (galectin containing), 20 µL of 0.15 M NaCl and 20 µL of 4 % erythrocyte suspension. The pectic polysaccharide fractions tested for hemagglutination inhibition were diluted in 0.15 M NaCl and replaced the 20 µL of 0.15 M NaCl in the wells. Effective inhibitory concentration (ICe) of the substances was determined to compare the bioefficacy of the dietary sources. Inhibition of galectin-3 of metastatic cell (MDA-MB-231) mediated agglutination was observed under the microscope.

4.3. Results

4.3.1. Antiproliferative activity of GR and MG fractions; inhibition of HeLa cell proliferation

The aqueous extracts (GRAE/MGAE) and polysaccharide fractions (GRPP/MGPP) of Ginger and Mango ginger were measured for their ability to inhibit HeLa cells proliferation. Cells were exposed to various concentrations at 10 and 20 µg/mL GAE of GRAE/MGAE and 250 & 500 µg/mL (dry wt) of GRPP/MGPP during a 48 h growth period. Results indicated dose dependent inhibition of cell proliferation as determined by MTT assay. At 20 µg/mL concentration ~ 2 fold increased antiproliferative activity was observed for GRAE when compared to that of MGAE (Figure 4.1A). GRPP however, on the dry weight basis, at 250 µg/mL showed 1.3 fold better activities than GRAE suggesting that both phenolics and polysaccharide components may contribute to the antiproliferative activity (Figure 4.1B). The mechanism of action needs to be elucidated.

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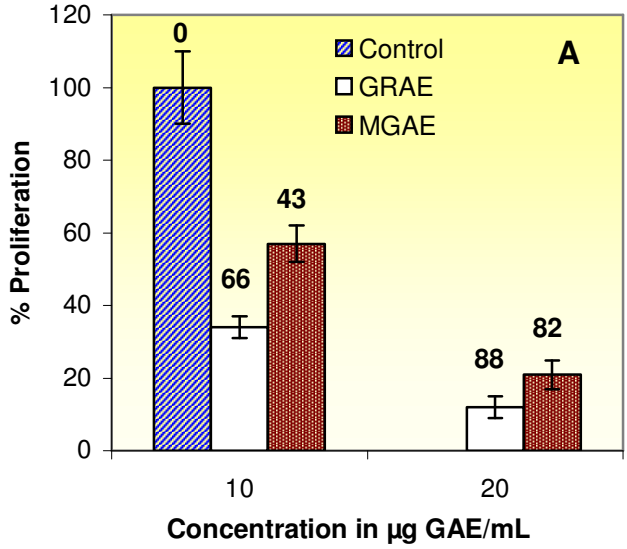
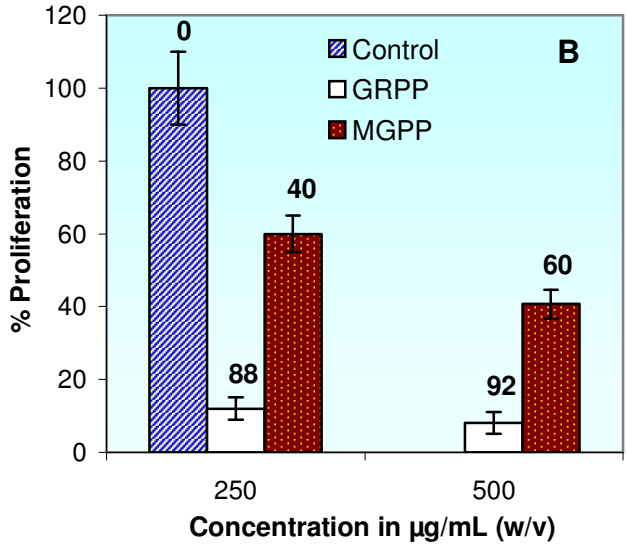
Since GRAE/MGAE enriched in antioxidant activity, the effective median dose (EC_{50}) was expressed as micrograms GAE/mL ± SD for polysaccharide fractions EC_{50} was expressed in µg/mL (dry wt) ± SD. EC_{50} of antiproliferation of HeLa cells was found to be 9.4 ± 0.8 and 11.8 ± 1.0 µg/mL for GRAE and MGAE respectively. No significant difference was found between these two sources. Standard antioxidant – tannic acid however exhibited an EC_{50} of 2.0 ± 0.1 µg/mL. Similarly polysaccharide fraction yielded 50% at an EC_{50} of 200 ± 22 and 360 ± 34 µg/mL (w/v) of GRPP and MGPP respectively (Table- 4.1).

Figure 4.1. Antiproliferative activity of Ginger and Mango ginger





(A)- Antiproliferative activity of aqueous extracts of GR- GRAE and MG- MGAE, (B)- Antiproliferative activity of polysaccharide fractions of GR- GRPP and MG- MGPP. % inhibition of proliferation is indicated on top of the bars.

Table 4.1. Effect of GR and MG fractions on HeLa cells

	EC_{50} value (µg/mL)
Antioxidant rich fraction	
Std. Tannic acid	2.0 ± 0.1
GRAE	9.4 ± 0.7
MGAE	11.8 ± 1.2
Polysaccharide fraction	
GRPP	200.6 ± 2.1
MGPP	360.0 ± 2.7

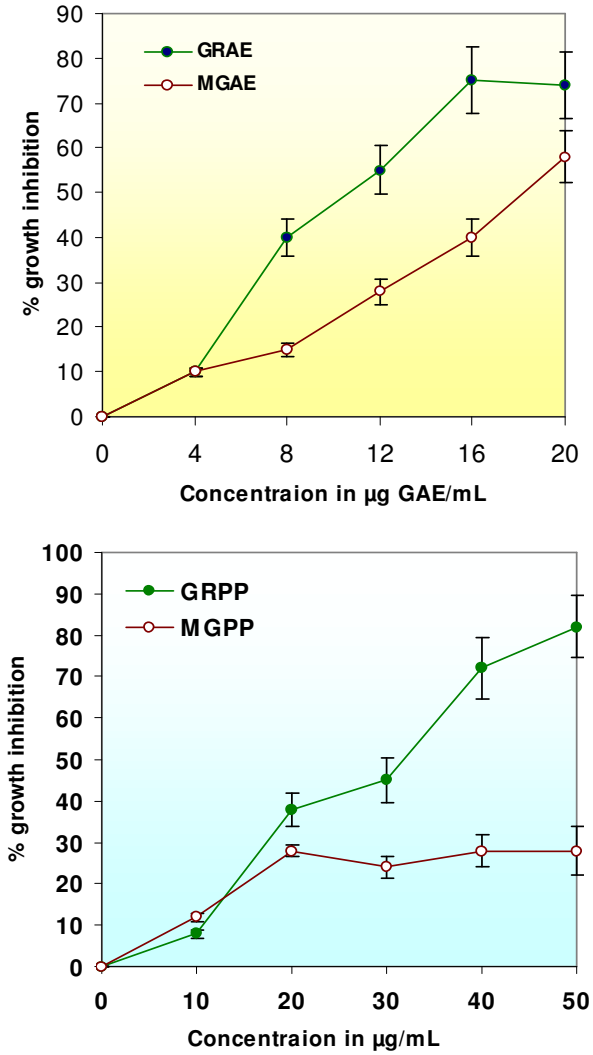
5 - 25 µg/mL of GRAE/MGAE and 100 – 500 µg/mL GRPP/MGPP concentrations were tested for antiproliferative activity. Effective dose to inhibit 50% of HeLa cell proliferation was studied.

4.3.2. Effect of GR and MG fractions on cell viability by Trypan blue exclusion assay

Figure 4.2 represent the effect of HeLa cell viability and number of viable cells by trypan blue exclusion method. Loss of viability was observed due to cell death. Results are expressed as % growth inhibition.

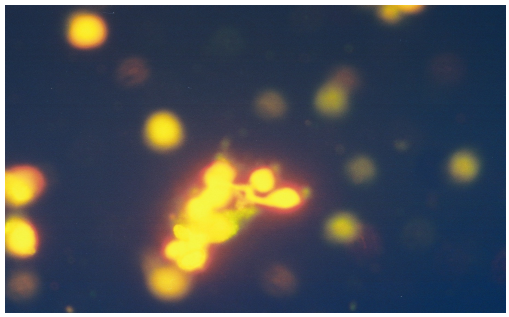
At linear range of activity, % growth inhibition was compared between GR and MG fractions. GRAE inhibited cell growth up to ~ 70% while that of MGAE showed at least ~ 2 fold lesser activity. This difference in activity could be attributed to probable differences in their ingredients that are reported in chapter II.

Figure 4.2. Effect of GR and MG fractions on growth inhibition.

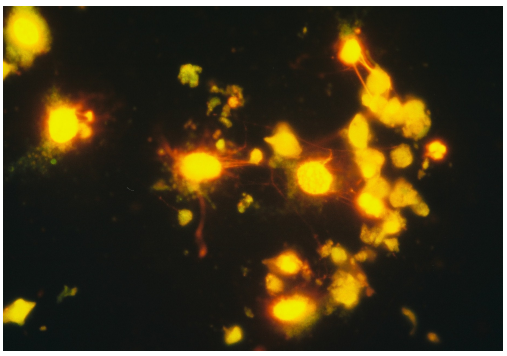


HeLa cell viability as examined by Trypan blue assay as per the protocol described under material methods Growth inhibition of Antioxidant fraction (A) of Ginger and Mango ginger and polysaccharides (B)

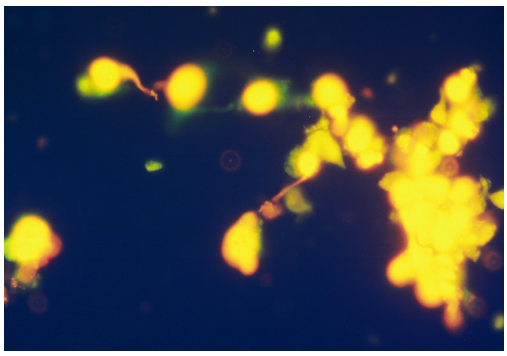
Figure 4.3. Morphological observation of B16F10 cells in presence and absence of GR and MG fractions.



A- Control



A- GRPP



B- GRAE

B16F10 cells in – untreated (A); GRPP treated (B); GRAE treated (C) after treat of cells with respective compounds. They were stained with Ethidium bromide and Acridine orange. Control cells looked greenish with very little ethidium bromide staining; In GRPP treated cells (B) Vigorous apoptosis with apoptotic bodies and programmed nucleus was observed; GRAE treated also induced cells to apoptosis

4.3.3. Effects of GR and MG fractions on B16F10 melanoma cells

Overall data suggest the phenomenon of apoptotic bodies, Shrunken and Necrotic cells were also observed. Stained by EB and AO, the color of control cell is green while the color of apoptotic cell is yellowish red under fluorescence microscope. After 4 h of treatment of GRAE/MGAE and GRPP/MGPP at the dose of 10 – 30 µg GAE/mL and 80 – 240 µg/mL concentrations respectively, the cells were much brighter (Figure. 4.3B) than the control cells (Figure. 4.3A). There was a shift in the nuclear stain towards the periphery of the cell or outside the cell in treated samples, in addition to fragmented pattern of the nucleus leading to multi nuclear state of cells, suggest the phenomenon of apoptosis. The data coincides with the

loss of cell viability and decrease in cell growth indicating the effect of extracts on cancer cells by apoptotic route.

4.3.4. Cytoprotective ability of Ginger antioxidant and polysaccharide fraction

Free radical generated by $FeSO_4$ and Ascorbic acid induced cell lysis was measured by the released haemoglobin pigment at 540 nm. Water lyses RBCs readily and instantly; while oxidants induced lysis is time dependent. Figure 4.4A Protection to RBC lysis was studied with different fractions and data presented in Figure 4.4B suggest dose dependent inhibition of cell lysis. Microscopic observations of human erythrocytes incubated with oxidants showed loss of cellular shape and eventually cell lysis as observed in Figure 4.5A. Reaction was time dependent. In oxidant treated lane (Figure 4.5 B, C & D) majority of cells were shrunken and disorted. In 20 minutes, ~ 90 lysis was observed and this was effectively protected by ginger antioxidant (GRAE) at 12 µg GAE/mL (Figure 4.5 E.F.G) and polysaccharide (GRPP) Figure 4.5 H.I.J) fractions at 80 µg/mL. Recovery of morphological structure of RBCs after treatment corroborates with the inhibition of cell lysis. Data thus suggest that GRAE with enriched antioxidant activity may quench free radical hence may prevent cellular damage; while polysaccharide may stabilize the cell membrane so that it becomes resistant to oxidant and hence may be protection.

Figure 4.4 (A) Oxidant induced RBC lysis

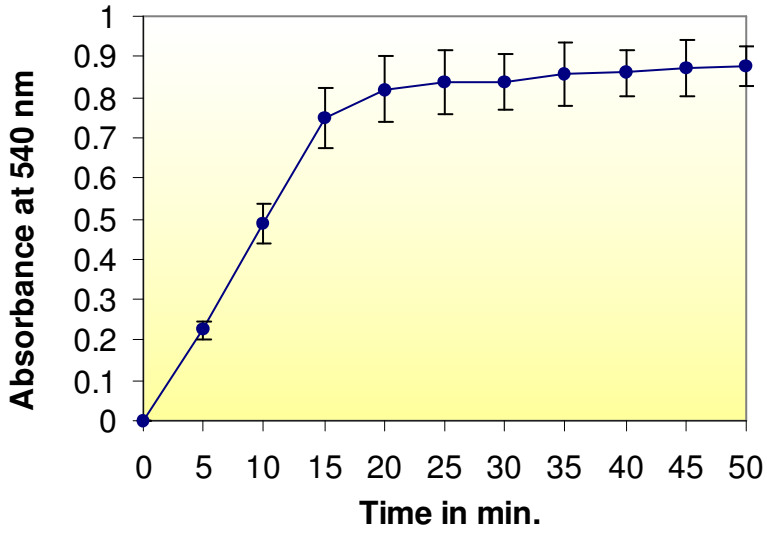


Figure 4.4. A. RBCs were isolated from fresh human blood, subjected to oxidants treatment. Oxidants induce cellular damage resulting in the release of Hb pigment. This is quantitated colorimetrically. Maximum damage (~90% lysis) with respect to water treated cells was observed with in 20 min.

Figure 4.4. (B)

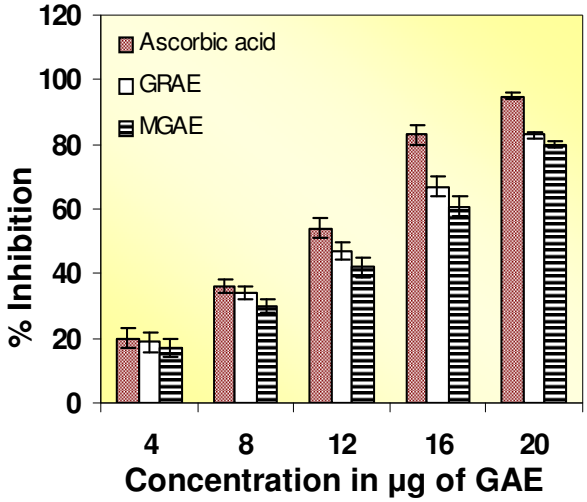
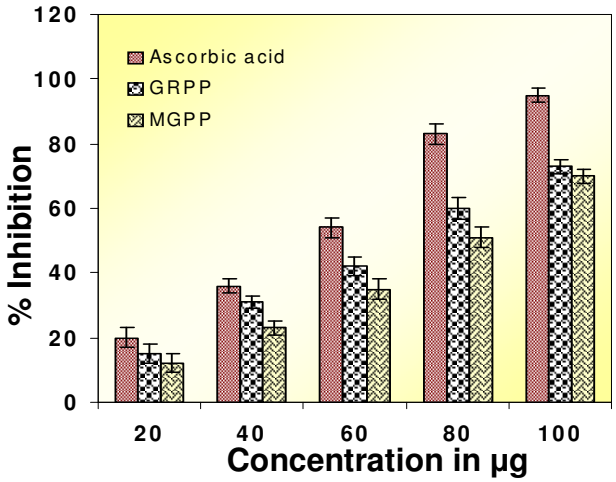
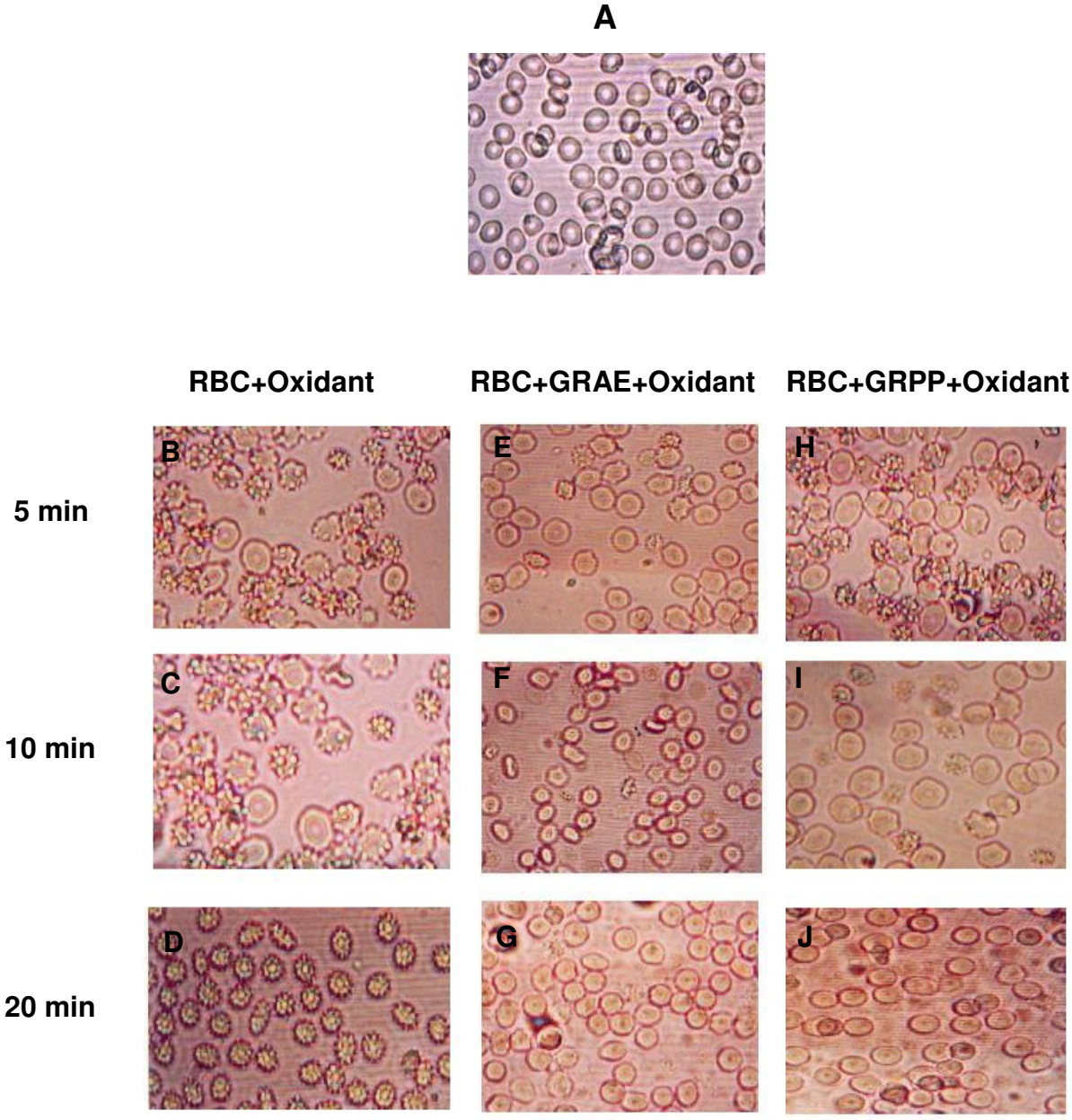


Figure 4.4. (C)



RBCs were preincubated with various concentrations of GRAE (B) and polysaccharide fractions (C). Dose dependent inhibition of lysis was observed 1-5 µg of Ascorbic acid was incubated as positive control.

Figure 4.5 Morphological observation of RBC lysis in presence and absence of GRAE and GRPP fractions.

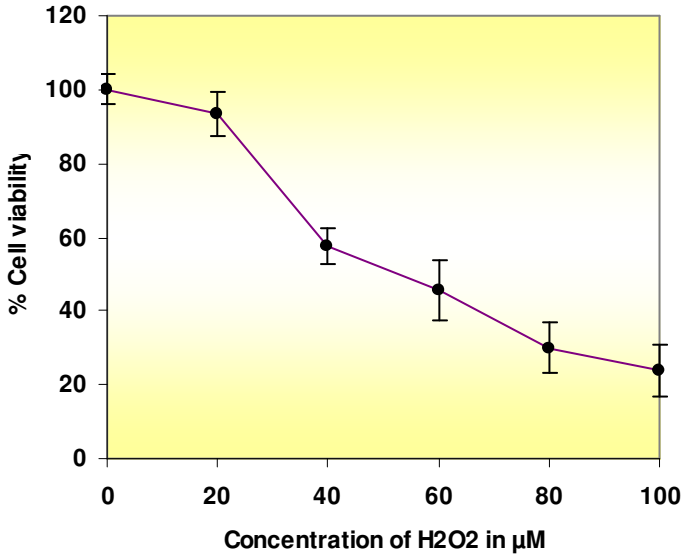


A- Control cells are uniform size, intact with no release of haemoglobin; B-D oxidant treated, maximum damage was observed in 20 min; E-G and H-J showing protection in presence of 12 µg GAE/mL and 80 µg/mL concentrations of GRAE and GRPP respectively.

4.3.5. Effect of GR and MG fractions against H_2O_2 induced HeLa cell damage

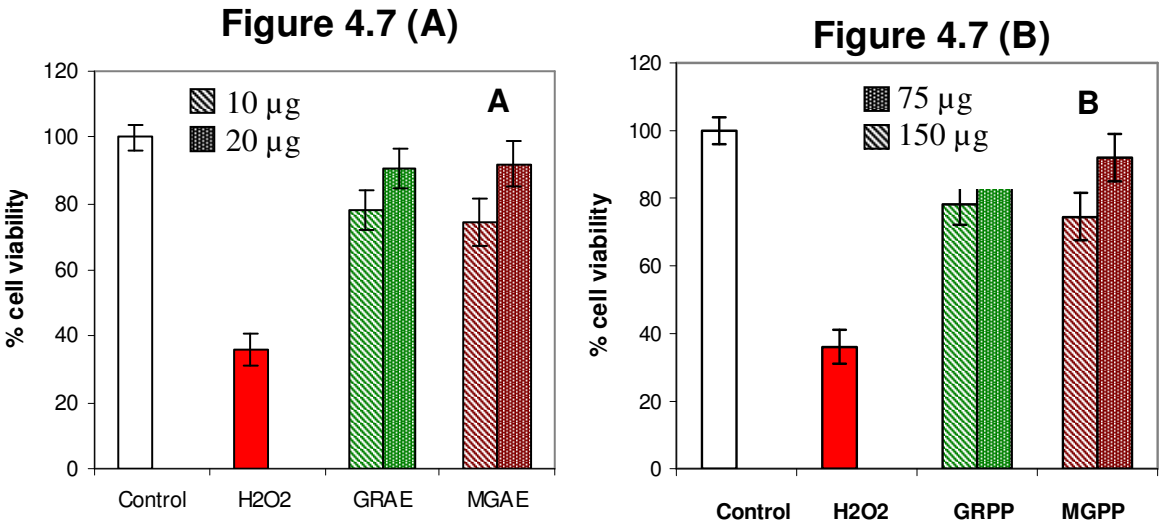
Figure 4.6 provides the dose dependent decrease in cell viability with the increase in concentration of H_2O_2 from 20 - 100 µM. At 80 µM concentration of H_2O_2 decreased the HeLa cell viability by atleast >70%. This concentration was selected to evaluate the cytoprotective ability of GR and MG fractions against oxidative damage. Loss of cell viability due to oxidative damage caused by H_2O_2 was recovered up to 85 and 71 % by GRAE and MGAE at 20 µg GAE/mL concentration respectively (Figure 4.7A). Similarly 76 and 68% recovery in viability by GRPP and MGPP at 150 µg/mL concentration respectively (Figure 4.7B).

Figure 4.6 H_2O_2 induced HeLa cell damage



HeLa cells were exposed to H_2O_2 at range 20 - 100 µM concentrations to induce oxidative damage. At 80 µM concentration of H_2O_2 decreased the HeLa cell viability by at least >70%.

Figure 4.7 Cytoprotective effects of GR and MG fractions



HeLa cells were exposed to 80 µM H_2O_2 to induce cellular damage and cytoprotectivity was assessed (**A**) in presence of 10 & 20 µg GAE/mL concentration of GRAE/MGAE and (**B**) in presence of 75 & 150 µg/mL concentration of GRPP/MGPP.

Figure 4.8 Effect of GR and MG fractions on lipid peroxidation in HeLa cells

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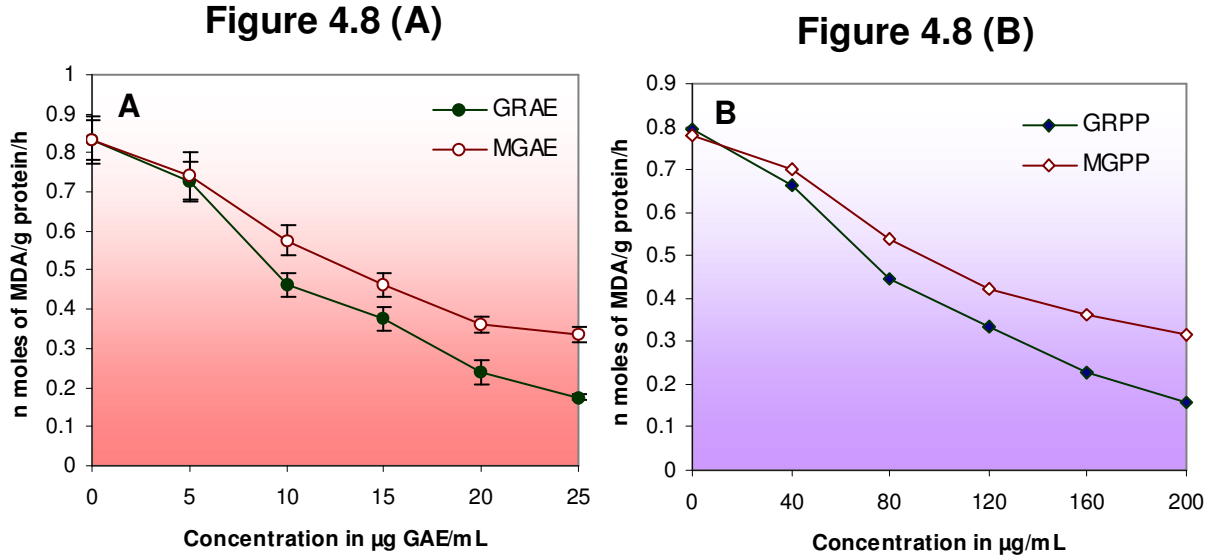


HeLa cells were exposed to 80 µM H_2O_2 to induce cellular damage and lipid peroxidation was measured as nmoles of MDA molecules formed by TBARS method (**A**) in presence of 5 - 25 µg GAE/mL concentration of GRAE/MGAE and (**B**) in presence of 40 - 200 µg/mL concentration of GRPP/MGPP.

4.3.5.1 Lipid Peroxidation

The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay. Both antioxidant and polysaccharide fractions showed dose dependent protection against lipid peroxidation with varying degrees (Figure 4.8 A & B). GRAE showed better inhibition with IC_{50} value of 13 ± 0.8 µg GAE/mL compared to MGAE (IC_{50} value 18 ± 1.2 µg GAE/mL) and even in polysaccharide fractions GRPP showed better activity compared to MGPP. IC_{50} values for lipid peroxidation are given in the Table 4.2.

Table 4.2. IC_{50} value of GR and MG fractions on lipid Peroxidation in HeLa cells

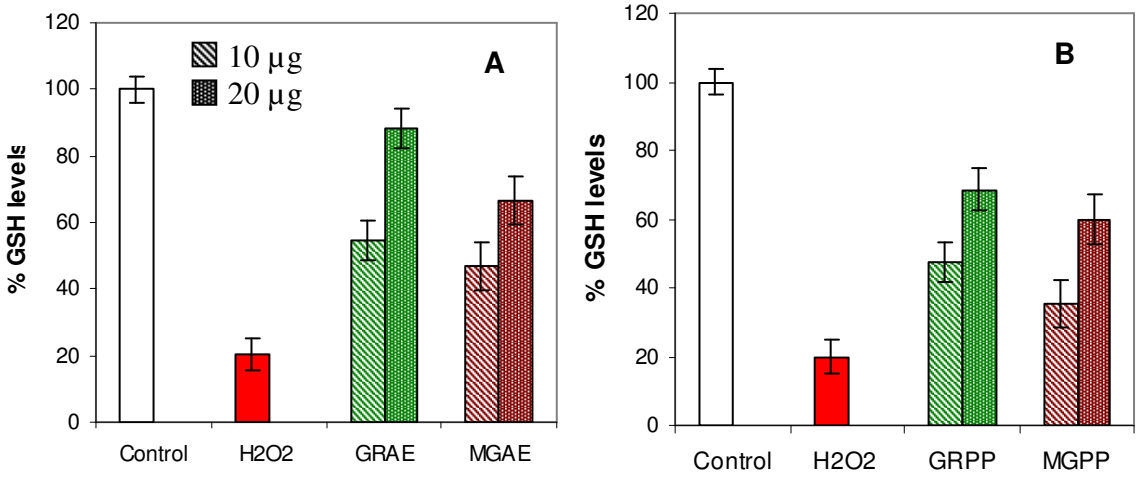
	Lipid Peroxidation IC_{50} value
Antioxidant fraction	**(µg GAE/mL)**
GRAE	13 ± 0.8
MGAE	18 ± 1.2
Polysaccharide fraction	**(µg/mL)**
GRPP	116 ± 10
MGPP	134 ± 12

HeLa cells were exposed to 80 µM H_2O_2 to induce cellular damage and lipid Peroxidation was measured as nmoles of MDA molecules formed by TBARS method. IC_{50} values were calculated as the amount of test samples required to inhibit 50% of the lipid peroxidation.

4.3.5.2. Glutathione levels

Glutathione is a tripeptide with antioxidant properties in cells. Upon exposure to H_2O_2, there is an oxidative burst and this utilizes the readily available cellular GSH to protect against oxidation. Therefore, there is depletion of GSH level and there was ~ 5 fold reduction in GSH levels were observed. The depleted GSH levels were recovered up to 88 and 66 % by GRAE and MGAE at 20 µg GAE/mL concentration respectively (Figure 4.9 A). Similarly 68 and 60% recovery by GRPP and MGPP at 150 µg/mL concentration respectively. (Figure 4.9 B). Data was substantiated by cytoprotectivity that was observed before.

Figure 4.9. Effect of GR and MG fractions on glutathione levels



HeLa cells were exposed to 80 µM H_2O_2 to induce cellular damage and glutathione molecules were depletion up to 20% was observed. Recovery in GSH in presence 10 - 20 µg GAE/mL concentration of GRAE/MGAE (**A**)and in presence of 75 - 150 µg/mL concentration of GRPP/MGPP(**B**) was observed .

4.3.6. Effect of pectic polysaccharide of GR and MG on antimetastatic activity

Recently from our laboratory as well as from other laboratories, pectic polysaccharides have been shown to exhibit antimetastatic potential by blocking the ability of anticancer cell to interact with either the basement membrane or normal extra cellular matrix molecules. In the current study therefore we examined whether pectic polysaccharides of ginger and mango

ginger exhibit any antimetastatic potential. Simple hemagglutination method was employed for this assay. Minimum inhibitory concentration of the polysaccharide (MIC) in inhibiting the galectin mediating agglutination of RBC was determined and results were compared with standad galectin specific sugars- galactose and lactose. Results indicated that GRPP and MGPP did not show any antimetastatic activity even at concentration higher than 600 µg to 1 mg, as they do not contain or they have very less amount of galactose. GRPP even at >6 mg/mL did not show any inhibition.

4.3.7. Galectin inhibitory property of GRPP and MGRPP

Samples	Agglutination	MIC µg/mL
RBC alone	-	-
RBC + galectin	+	-
RBC + Gactose + galectin	-	27.10
RBC + Lactose + galectin		4.16
RBC + GRPP > 600 µg/mL + galectin		> No inhibition at 600 µg/mL
RBC + MGPP + galectin		> No inhibition
RBC + SRPP* + galectin		1.85

*Swallow root pectic polysaccharide was found to be a very potent antimetastatic compound. This was there fore incubated as a positive control.

4.4. Discussion

Ginger (GR) and mango ginger (MG) have been known world wide as spice, food and traditional herb. Multi-health beneficial properties have been depicted by various workers including our own laboratory (Bordia et al, 1997, Surh et al, 1999, Ernst & Pittler, 2000, Siddaraju & Shylaja, 2007a,b). Although the ability of ginger constituents in exerting anticancer property is known, the role of phenolics which are present in abundant levels with multi-potent antioxidant properties are not clearly established.

In the present study we investigated whether antiulcer fractions also possesses anticancer properties since both ulcer and cancer are known to be initiated by oxidative stress as revealed in the hypothesis. Further plant sources and compounds showing multiple health beneficial properties are not uncommon.

We had shown antiulcer properties both *in vitro* and *in vivo* in aqueous extract (GRAE) and pectic polysaccharides (GRPP) fractions of Ginger – *Zingiber officinale.* Anti-ulcer activity was found to be mediated via inhibition of parietal cell – plasma membrane H^+, K^+-ATPase (*in vitro* and *in vivo*), inhibition of *H. pylori* growth (*in vitro*) and gastric mucosal protection (*in vitro* and *in vivo*) in addition to activation of antioxidant enzymes and antioxidant status (**Chapter 2 and 3**). In mango ginger however, antiulcer potentials have been proved by *in vitro* models (**Chapter 2 and 3**). In both the cases, phenolics appear to play a significant role.

Further in order to understand the ingredients that are responsible for the activity, phenolic compositions and polysaccharide compositions were determined. In the current study we explored the potentials of antioxidant and polysaccharide fractions of ginger and mango ginger (GRAE/MGAE and GRPP/MGPP) in exhibiting anticancer properties. Studies addressed the ability of the selected fractions to exhibit anti-proliferative, loss of cell viability, apoptotic, antimetastatic and cytoprotective properties. Many a times the anticancer properties were found to be due to antioxidant potencies. Hence antioxidant effect of the test extracts against cellular damage was also studied. Both antioxidant and polysaccharide fractions inhibited proliferation of HeLa cells. The anti-proliferative effect occurred after only 2 h of treatment and exerted its maximal effect after 10 h to the cell (Data not shown). The effective median dose is the concentration causing 50% reduction in cell proliferation (EC_{50}) was between 9 to 12 µg

GAE/mL of GRAE and MGAE. No significant differences were found in the antiproliferative activity in ginger and mango ginger which can be attributed to the presence of almost all similar phenolic acid profiles in GRAE and MGAE (Chapter 2). This is in accordance with the previous investigation where in various human tumor cells; the EC_{50} was described to be between in the same range. In other words, ginger showed better activity than mango ginger.

Data also corroborates with the literature that the extracts containing enriched amounts of phenolic acid are anticancerous in nature (Li Chen et al, 2006). Cinnamic acid has been implicated in offering potential anticancer activity also (Lei Liu et al, 2006). In that perspective GR and MG both containing > 40 % of cinnamic acids when compared to those of other phenolic acids may contribute significantly to antiproliferative effect. Antiproliferative activity of pectic polysaccharides can also be attributed to such phenolic compound found to be present in the covalently bound form pectic polysaccharide of ginger- GRPP and Mango ginger- MGPP (**Chapter 3**). In both the cases again, cinnamic acid has been found as a major ingredient among phenolics. However, the role of polysaccharide per se in stabilizing the cellular membrane and hence cytoprotection can not be ruled out.

The antiproliferative effect was most probably not the result of the possible cytotoxic action on plasma membrane integrity or cellular metabolic activity as revealed by cytoprotective effect of the extracts on Red Blood Cells. Antiproliferative effect of actively proliferating cancer cells and cytoprotective ability of ginger fractions even on fragile cells like RBCs, are cncouraging to employ as a safer anticancer compounds, where it affects only the cancer cells and not normal cells. Further, this differential property could be due to interaction of cancer-specific molecules and cancer mediating molecules such as activated NFkB, increased oxidative stress, depleted antioxidant enzyme and antioxidants including reduced glutathione. Recent report by Ishiguro indicated that 6-gingerol, a phenolic alkanone in ginger induced apoptosis by activating caspases-3/7, suppresses NFkB etc (Ishiguro et al, 2007). Another component of ginger-6-shogaol alone has been known to reduce the viability of cancer cells by affecting microtubules thereby inducing mitotic arrest (Badreldin et al, 2008).

Further, although the mechanism resulting in tumor growth inhibition are not definitely clear at the moment it seems that cinnamic acid partly exert its antiproliferative effects probably via an inhibition of protein isopenylation as indicated by Lei Liu et al, (2006) which in turn inhibits

mitogenic signal transduction. It is thus possible that ginger extracts being rich not only cinnamic acids, but cinnamyl derivatives, may also exert their anticancer potential in a similar manner.

Evidence indicated that hydroxylated flavonoids without the C2-C3 double bond could not inhibit the growth of melanoma cell line (*B16F10*). Whereas, the presence of atleast three adjacent methoxyl groups would confer, a more potent antiproliferative effect (Rodriuez et al, 2002). Moreover, flavonoids such as myricetin, baicalein and gallic acid were found to significantly inhibit the growth of B16F10 after 72 h of exposure, which led to the suggestion that the presence of C2-C3 double bond and three adjacent hydroxyl groups in the flavonoids a-or b- rings confer greater antiproliferative activity to the flavonoid. In this aspect, the GRAE potency may be attributed to the presence of gallic acid (46%) and cinnamic acid (50%) in predominant levels. Remaining 4% of other phenolic acids may also contribute also to some extent towards anticancer properties.

Induction of apoptosis of cancer cells is again intriguing. Apoptosis has been characterized as a fundamental cellular activity in maintaining the physiological balance of the organism. It is also involved in immune defense machinery and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells which are proliferated. Emerging evidence (Tadashi et al, 2004 & Koyama et al, 2006) has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer.

Antiproliferative activity observed in GR and MG fractions could be due to apoptotic properties of the test fractions; of course with different extents, resulting in cell death. Pushing of nucleus towards the periphery, nuclear fragmentation, disortion of cells etc., indicate the death of melanoma cells by tipical induction of apoptosis. Formation of apoptotic body, shrinking of cells upon treatment with test extracts supports the fact that there is an induction of apoptosis. Vijaya et al, (2007) have indicated that induction of apoptosis by ginger in Hep-2 cell line is mediated by reactive oxygen species.

Organic solvent soluble components such as 6-gingerol (Ishiguro et al, 2007), Zingerone, Zingeberone etc., although were implicated in bioactivity, the contribution of phenolics has

been believed to be higher due to multi-potency as well as abundance. Also in traditional medicine, water decoctions are used to find the remedy; therefore particular attention in this study has been paid to explore the role of water soluble components against chronic diseases such as ulcer and cancer. Recently we could show that phenolic constituents present aqueous extracts (reported in **Chapter 2**) playing a significant role in preventing ulcers *in vitro* and *in vivo*.

Cancer is characterized by active proliferation of cells followed by changes in cellular morphology including the changes in biochemicals in those cells *in vivo*, ultimately enforces on metastasis. Metastasis is a serious stage of the disease and is responsible for the death of cancer patients. Several synthetic and natural molecules have been identified to employ as anticancer compounds. Chemotherapeutic drugs were the drug of choice in killing cancer cells; however, they do not discriminate between normal cells and cancer cells hence results in lots of side effects. Alternative medicines are therefore gaining importance. More recently however, sugars both in single form and polymers acting against cancer either as antiproliferative or antimetastatic, catches attention of traditional therapist for remedies.

Polysaccharides particularly pectic polysaccharides have been depicted with multiple bioactives including anticancer, antiulcer, antidiabetic, anti-inflammatory diseases (Srikanta et al, 2007 & Sathisha et al, 20070. Polysaccharides with immunomodulatory effects have recently been isolated from different mushrooms. With investigating side effects from allopathic drugs, polysaccharides from edible mushrooms have become alternative to tumor therapy. The critical structures for these polysaccharides were proven to be branched glucans with 1-3, 1-4 and 1-6 linkages.

Polysaccharides from ginger/mango ginger were found to have bioactivities such as antioxidant and antiulcer properties as revealed from our laboratory (unpublished data). Ginger has been shown to posse's anticancer properties. The compositional studies reported the presence of precise levels of various sugars in GRPP it is composed of rhamnose (4%), arabinosse (24%), Xylose (8%), mannose (3%), galactose (> 1%) and glucose (54%) and in addition it contained 5.7% phenolic, particularly cinnamic acid and p-coumaric acid exhibiting antioxidant property. Cinnamic acid of polysaccharide has also been reported to possess anti proliferative activity.

However, when we examined the antimetastatic activity, it did not show any activity. This was attributed to suggesting that lower content of galactose, which appear to be crucial for antimetastatic activity and may be responsible for the observed result. In our previous study we could show that the polysaccharides rich in arabinose and galactose, only are antimetastatic. Galectin-3 appears to be a crucial molecule for metastasis (Sathisha et al, 2007). Galactin-3 found only in cancer cell and has been known to interact with β-galactoside of normal cell during invasion and metastasis. As indicated in the table the potent antimetastatic activity of polysaccharide from another source –*Decalepis hamiltonii*, equivalent are better than standard galactin-3 specific sugars; lack of activity in GRPP and MGPP may be explained by lack of galactose levels or only trace amounts of galactose and arabinose in GRPP and MGPP (reported in **Chapter 3**). Current study thus supports the previous observation of the laboratory (Sathisha et al, 2007) that the arabinose and galactose may be important for potential galactin-3 inhibitory activity or inhibition of galactin-3 mediated aglglutination.

4.5. Summary & Conclusions

- GRAE/MGAE and GRPP/MGPP exhibited antiproliferative activity in HeLa cells. Effective medium inhibition concentration to inhibit 50% proliferative activity (EC_{50}) under similar experimental conditions reveal the EC_{50} value of 9.4 ± 0.8 and 11.8 ± 1.0 µg/mL for GRA/MGAE and 200 ± 22 / 360 ± 34 µg/mL (w/v) of GRPP/MGPP respectively. Standard tannic acid had an EC_{50} value of 2.0 ± 0.1 µg/mL. Phenolic rich GRAE and MGAE showed better activity, than their respective polyaccharide fractions, suggesting that phenolics may play potential role in antiproliferative activity.

- GRPP and GRAE induced apoptosis in B16F10 – mouse melanoma cells. Hence antiproliferative / anticancer activity could be due to apoptotic property of the extract.

- Major phenolic constituents - cinnamic acid, gallic acid, syringic, caffeic, ferulic, gentesic and p-coumaric acid present in GRAE and MGAE may be responsible for anticancer (antiproliferative + apoptotic) activity.

- GRAE and MGAE showed cytoprotective ability in RBCs; suggesting that selected fractions are not toxic to normal cells; but toxic to cancer cells - HeLa and B19F10 ells.

- GRPP and MGPP did not exhibit antimetastatic property as that of SRPP. Low levels of galactose in GRPP and MGPP may be attributed to reduced inhibition of galectin-3 mediated agglutination.

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