Studies on Antiulcer Effects of *Hippophae rhamnoides*

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Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualification.

Signed Song Jingmei

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Abbreviations

ANOVA	analysis of variance
$CHCl_3$	chloroform
c-extract	chloroform extract of the seed of *Hippophae*
e-extract	ethanol extract of the seed of *Hippophae*
EtOH	ethanol
H^+	hydrogen ion
HCO_3^-	bicarbonate
H_2O	water
h-extract	hexane extract of the seed of *Hippophae*
HP	*Helicobacter pylori*
Hr	*Hippophae rhamnoides*
GC-MS	gas chromatography mass spectrometry
GMBF	gastric mucosal blood flow
HPLC	high pressure liquid chromatography
i.g.	intragastric
i.p.	intraperitoneal
NSAIDs	nonsteroidal anti-inflammatory drugs
PGs	prostaglandins
s.c.	subcutaneous
SEM	standard error of the mean

non-SH	non-protein sulfhydryls
TCM	Traditional Chinese Medicine
TLC	thin layer chromatography
UV	ultra-violet

Abstract

The anti-gastric ulcer activities of *Hippophae rhamnoides* (Hr) were evaluated in six ulcer models in the present study. Both gastric protective effect and ulcer healing activity of Hr were assessed, and the possible mechanisms involved were examined by various pharmacological tests.

Sprague-Dawley rats (210-230 g) were employed in the present study. The antiulcer effect of Hr seed extract was evaluated against acute gastric lesions induced by ethanol-(60%, 6ml/kg), aspirin-(200 mg/kg in 0.15N HCl), stress-(water immersion for 6 h at 24±1°C), pylorus ligation-(6h) and three necrotizing agents-(0.6N HCl; 25% NaCl; 0.2 N NaOH). The ulcer healing effect of Hr was also determined with an acetic acid-induced chronic ulcer model. To investigate mechanisms of the protective action of Hr, the content of mucus was measured by the Alcian Blue. The content of pepsin and protein in the gastric juice and the content of reduced glutathione (GSH) in gastric mucosa were determined by spectrophotometer methods. Gastric acidity was measured by an autotitration system. Gastric motility was assessed as the capacity to transfer resin pellets from the stomach to the intestine. Gastric mucosal blood flow (GMBF) was measured with a laser Doppler flow meter. Indomethacin (5 mg/kg, s.c.) was used to study the involvement of prostaglandins (PGs) in the antiulcer effect of Hr extract. The content of PGE_2 was measured by an immunoassay method. Chemical identification and analyses of constituents of Hr were performed by TLC, HPLC, GC and GC-MS.

Results indicated that pretreatment with Hr extract (300-600 mg/kg) significantly inhibited the formation of gastric lesions in various models except for the

damage induced by 0.2N NaOH. Hr markedly prevented the depletion of GSH in gastric mucosa induced by ethanol (1.514±0.038 vs 1.258±0.052 μmol/g tissue, $P<0.05$). The decreased gastric emptying rate induced by ethanol (32.08±4.24% vs 57.47±3.17%, $P<0.01$) was normalized by the treatment with Hr at a concentration of 600mg/kg (49.58±3.17%). In addition, Hr prevented the decrease in GMBF induced by ethanol. Pretreatment with indomethacin could partially inhibit the protective effect of Hr indicating that the activity of Hr may be partially mediated via endogenous PGs. The study on gastric secretion showed that Hr significantly inhibited gastric acid ($P<0.01$) and pepsin secretion ($P<0.05$). In the chronic ulcer model, gastric ulcers were healed by twelve days of Hr treatment (3.50±0.91 mm^2, $P<0.01$) compared with the control (11.76±1.99 mm^2), and the gastric mucus content was increased markedly with Hr treatment (259.8±22.3 vs 173.4±16.1 μg/g tissue, $P<0.05$). Analysis of chemical ingredients of Hr demonstrated the presence of vitamins A, E, and C, unsaturated fatty acids (e.g. linoleic acid 37.2%, linolenic acid 29.7%), and phytosterols.

The present findings suggest that Hr is a promising crude drug as a stomach-preventive agent. It possesses cytoprotective effect against various experimental gastric lesions, and the activities may be related to improvement of mucosal blood flow, modification of gastric motility, stimulation of mucus secretion and GSH generation, maintenance of mucosal integrity, increase of free radical scavenging, inhibition of gastric acid and pepsin secretion, and acceleration of tissue repair. Further investigations are needed to reveal other mechanism(s), if any, involved in the Hr cytoprotective and antisecretory activities.

摘要

本文应用不同的动物模型研究了沙棘对胃溃疡的预防和治疗作用，以及可能的作用机理。

SD 大鼠(体重 210-230 g)用于本研究。沙棘提取物的胃保护作用用五种胃损伤模型进行评价，即乙醇(60%, 6mg/kg)，阿斯匹林(200mg/kg, 悬浮于 0.15N 盐酸溶液)，应激(浸水 6 小时, 24±1° C)，幽门结扎(6 小时)，及胃粘膜损伤物(0.6N 盐酸, 0.2N 氢氧化钠, 25%氯化钠) 诱导的急性胃损伤模型。沙棘对胃溃疡的治愈作用则用乙酸(30%, 0.05ml) 诱导的慢性胃损伤模型研究。同时，用下列的生理和生化指标研究了沙棘的胃保护作用机理。胃粘膜粘液含量用阿力新蓝染料结合法测定。胃粘膜中的谷光甘肽含量及蛋白含量用分光光度法测定。胃运动以树脂球的胃排空量为指标。胃粘膜血流用雷射激光多普勒血流计测定。胃液酸度用自动滴定系统测定。前列腺素与沙棘胃保护作用的关系用引哚美辛(5mg/kg, s.c.)研究。胃粘膜前列腺素 E_2 的含量用酶联免疫法测定。沙棘的抗溃疡活性成分用薄层层析，柱层析，高效液相色谱及气相色谱-质谱等方法分析和鉴定。

实验结果表明，沙棘提取物(300-600mg/kg, 口服)显著抑制各种实验性胃溃疡的形成，但对 0.2N 氢氧化钠诱导的胃损伤无效。沙棘显著抑制 (1.514±0.038 μmol/g 湿粘膜重， $P<0.05$) 乙醇诱导的胃粘膜还原型谷光甘肽的降低 (1.258±0.052 μmol/g 湿粘膜重)，并可使乙醇诱导胃排空率的降低 (32.08±4.24%, $P<0.01$) 恢复正常(49.58±3.17%, $P<0.05$)。对胃分泌研究的结果表明，沙棘显著抑制胃酸($P<0.01$)及蛋白酶分泌($P<0.05$)。此外，给予沙棘提取物可有效地防止乙醇引起的胃粘膜血流下降。皮下注射引哚美辛显著地减弱但未完全抑制沙棘的胃保护作用，揭示沙棘的胃保护作用可能与内源性前列腺素的合成有关。给予沙棘提取物十二天，可显著治愈乙酸诱导的慢性胃损伤，使溃疡指数由对照组的 11.76±1.99mm^2 降至 3.50±0.92 mm^2 ($P<0.01$)，同时粘液含量显著升高，从对照组的 173.4±16.1 (μg/g 湿粘膜重) 提高到 259.8± 16.1 (μg/g 湿粘膜重) ($P<0.05$).

化学组分的分析和鉴定结果证明，沙棘提取物中含有维生素 A，维生素 C，

维生素 E，植物脂肪酸（亚油酸 37.2%，亚麻酸 29.7%）及植物甾醇（β-谷甾醇）等。

目前的研究结果表明，沙棘是一个具有发展潜力的预防胃溃疡的植物药。沙棘显示细胞保护作用并对多种实验性胃损伤具有抑制作用。其主要作用机理为改善胃粘膜血流及胃运动，促进粘液分泌及还原型谷光甘肽的生成，清除自由基，抑制胃酸及蛋白酶分泌，以及促进溃疡组织的修复。然而，沙棘对内源性前列腺素的作用及其机制目前尚不明确，有待进一步研究。

Chapter 1

Introduction

Gastric ulcer is a common disease with complex pathogenesis. Although extensive studies have been conducted, the etiology of gastric ulcer is still not fully understood. It has been widely accepted that imbalance between aggressive factors and defensive factors (Table 1.1) in the stomach is the major cause for the induction of gastric ulcer; and conditions related to mucosal resistance are determinants in the pathogenesis of gastric ulceration (Chacin, 1990). Nonetheless, the current treatments for peptic ulcer are mainly focused on reduction of aggressive factors, e.g. inhibition of gastric acid secretion or neutralization of excessive acid. Only limited therapeutic agents are clinically used for strengthening the defensive factors of the stomach (Bettarello, 1985; Andersen, 1988).

In the early 1910's, Schwartz suggested that " Peptic ulcer is a product of self-digestion, it results from an excess of autopeptic power in gastric juice over the defensive power of gastric and intestinal mucosa" (Schwartz, 1910). The Schwartz' dictum of "no acid-no ulcer" dominated the therapeutic strategy for the treatment of gastric ulcer for decades. Consequently, antacids have been used as main therapeutic agents for many years in the clinical practice. In the last two decades, the discovery of H_2-receptor antagonists (e.g. cimetidine) and proton-pump inhibitors (e.g. omeprazole) provides a selection of potent acid-reducing agents that further improve

the management of peptic ulcers (McGuigan, 1983; Meryn et al., 1983; Walan, 1984; Andersen, 1988; Giercksky; 1989; Schunack, 1989; Guerreiro et al., 1990).

However, previous clinical investigations have suggested that there was no definite correlation between acid output and ulcer induction, or between ulcer recurrence rate after vagotomy and preoperative acid secretion (Hojgaard et al., 1996). In addition, excess secretion of gastric acid was found to be insufficient to cause damages in the gastric mucosa; but with assistance of pepsin, development of gastric lesions may occur and duration for ulcer healing may be prolonged (Alphin, 1977; Wallace et al., 1986). Despite gastric acid and pepsin are the major aggressive factors in the formation of ulcers, other pathogenic factors, such as *Helicobacter pylori* and nonsteroidal anti-inflammatory drugs can also contribute to the ulceration (Trevethick et al., 1995; Peek et al., 1997).

Table 1.1 The aggressive, defensive and repairing factors related to gastric ulceration

Aggressive factors	Defensive factors	Repairing factors
Gastric acid	Mucus	Mucoid cap
Pepsin	Bicarbonates	Proliferation
Gastrin	Mucosal blood flow	Growth factors
Proteases	Prostaglandins	Epithelial renewal
Free radicals	Sulfhydryls	
Ischemia	EDRF (NO)	
Leukotrienes	Gangliosides	
Dysmotility	Dopamine	
Ethanol	SDO/catalase	
Smoking	Interleukins	
NSAIDs	Polyamines	
Stress	Hydrophobicity	
Helicobacter pylori	Luminal cell membrane	
Bile salts	Cell junctions	
Platelet-activating factor	Apical resistance	
	Balance between base-acid in tissue	
	Mucosal immune system	

Refs: Robert, 1979; Gannon et al., 1984; Brain et al., 1988; Konturek, 1990 (a; b); Wallace, 1990

Helicobacter pylori

Infection of *Helicobacter pylori* (HP) has been identified as one of the major causative factors in the pathogenesis of gastroduodenal ulceration (Nakajima et al., 1997; Parsonnet, 1998). The bacteria can release a variety of bacterial enzymes, toxins, and inflammatory mediators formed in response to bacterial colonization. These can stimulate an inflammatory response and gastric mucosal metaplasia (Peek et al., 1997). The treatment of HP infection can help in the ulcer healing process and significantly decreases the recurrence rate of ulcer and degeneration of epithelium (Peura, 1996). The effectiveness of anti-HP agents is even better than antisecretory drugs in reducing the relapse of peptic ulcers (Hunt et al., 1995). Nonetheless, it was suggested that only 1-2% of people infected by HP might develop peptic ulcers, thus infection of HP may not be the sole cause inducing peptic diseases (Glavin et al., 1992; Wallace et al., 1996).

Nonsteroidal anti-inflammatory drugs (NSAIDs)

Despite their therapeutic benefits, the use of NSAIDs is accompanied by a number of side effects in the gastrointestinal mucosa, such as bleeding and perforation (Giercksky, 1989). The NSAIDs-induced gastrointestinal impairment results from diffusion of non-ionized NSAID agents (e.g. aspirin) from gastric lumen to the mucosal cells. This can induce cell damage and inhibit cyclooxygenase (COX_1 and COX_2), subsequently prostaglandin synthesis (Trevethick et al., 1995). Although inhibition of COX_2 is an important feature of NSAIDs to exert analgesic and anti-inflammatory effect, inhibition of COX_1, however, is involved in the pathogenesis of gastric ulceration. The incidence and severity of gastric lesions induced by NSAIDs

are dose-dependent and associated with the existence of acid in the gastric lumen (Konturek et al., 1981). In addition, NSAIDs can decrease gastric blood flow (Kauffman, 1980b), inhibit mucus and bicarbonate secretion, impair mucosal cells (Vane, 1971), delay ulcer healing and reduce epithelial proliferation. Moreover, administration of NSAIDs causes higher risk in the gastroduodenal ulceration in patients infected with HP (Russell, 1997).

In spite of the fact that current treatment of peptic ulcer mainly focuses on inhibition of the aggressive factors in the stomach, mucosal integrity actually plays an important role against attacks of various pathogenic factors. Thus it is important to maintain integrity of mucosa, strengthen the mucosal barrier, improve gastric blood flow, enhance secretion of mucus and bicarbonate in the stomach for the management of peptic ulcer.

Mucosal barrier

The gastric mucosal barrier is important in preventing the stomach from damages induced by harmful substances. The injury of gastric mucosal layer leads to diffusion of free hydrogen ions to the deep mucosa layer, and efflux of sodium, potassium, water and protein into the gastric lumen, which subsequently develops mucosal lesions in the stomach (Hung et al., 1997). In addition, the aggressive foreign substances in the stomach can readily get into epithelium in the impaired mucosal layer, which may induce acute inflammatory responses (Wallace et al., 1997). There are a number of substances, such as bile salts, aspirin and ethanol, that have been demonstrated to be able to impair the mucosal integrity and increase the permeability

of the barrier (Ritchie, 1981; Cheung, 1982). Therefore to strengthen gastric mucosal barrier is necessary to defense attacks induced by various pathogenic factors (Andersen, 1988).

Gastric Mucus

It is believed that mucus may play a major role in protection of the gastric epithelium by providing an unstirred layer on the surface of the mucosa. It strengthens mucosal resistance and prevents the digestion of acid and pepsin in the deeper layer of mucosa. Mucus is secreted from mucous cells of gastric glands, foveolar cells and cells of the glandular neck border the cylindrical cell on the superficial epithelium. Mucus contains 95% water with less than 5% glycoproteins (Allen et al., 1980). It forms a flexible gel adhering to the surface of gastric mucosa that builds the first line of defense against acid and pepsin (Rees et al., 1982). Bicarbonate ions are trapped in mucus and these consist of a mucous-bicarbonate barrier (Heatley, 1959). Studies have suggested that both superficial and intracellular mucus is responsible for reduction of hydrogen-ion back diffusion and pepsin activity (Levey et al., 1954; Slomiany et al., 1985). Clinical investigations indicated that patients with peptic ulcer usually possessed lowered level of glycoproteins (Pearson et al., 1986). Thus, mucus contributes to the defense of gastric mucosa in various aspects, such as lubrication of mucosal layer, protection of the gastric mucosa from the mechanical attack of ingested foods and maintenance of a pH gradient across the mucus layer. It retains an almost neutral layer on the superficial epithelium although the intraluminal pH is about 2 or 3 (Hemstrom et al., 1984). Continuous mucus-bicarbonate secretion by surface cells provides an uninterrupted layer to prevent H^+ diffusion from the lumen and obstructs

the epithelium from erosion of acid and pepsin (Heatley, 1959; Takeuchi et al., 1983; Tasman, 1985).

Gastric Mucosal Blood Flow (GMBF)

GMBF is important to sustain mucosal barrier by retaining gastric pH at physiological levels and providing sufficient oxygen and nutrition. When gastric mucosa is impaired, the GMBF will be responsible for maintaining normal cellular function and healing of the ulcer (Jacobson, 1985). Under physiological conditions, the level of basal gastric secretion and food stimulated gastric secretion is associated with an increase in GMBF (Kauffman, 1982). It has been suggested that abnormal alterations in GMBF can be found in mucosal damage (Varhaug et al., 1979) and increase of GMBF possesses beneficial effect on mucosa against harmful substances (Jacobson, 1985; Shorrock et al., 1988).

Bicarbonate

It is believed that bicarbonate is secreted from the epithelial cells on the surface of gastroduodenal mucosa and plays an important role in mucosal defense, cooperating with the mucus gel. The protective mechanisms of bicarbonate on gastric mucosa involve neutralization of H^+ diffused from the gastric lumen, and maintenance of a pH gradient across the mucus gel that strengthens the physic-chemical property of the mucus (Takeuchi, 1996). The regulation of bicarbonate secretion is associated with gastric motility and neural activity. Previous studies have suggested that the secretion of bicarbonate is stimulated by PGE_2, NO, and some mucosal protective agents (Konturek et al., 1983a). Conversely, substances that inhibit the secretion of

bicarbonate, such as bile acids, indomethacin, tobacco smoking, and probably infection of HP will impair the integrity of mucosa (Hojgaard et al., 1996).

In addition, the mucosal defense is also supported by a hydrophobic epithelial lining, epidermal growth-factor regulated tissue regeneration and balance of gastric acid and alkali (Wallace, 1990; Konturek, 1990a).

Currently, drugs used for the treatment of gastric ulcer can be divided into two categories according to the mechanisms of action, namely the acid inhibitors/neutralizers and mucosal protective agents. Nevertheless, as mentioned previously, the mainstream for the management of peptic ulcer is to inhibit or neutralize gastric acid secretion by using histamine-2 receptor antagonists, proton pump inhibitors, anticholinergics and antacids. The effects of these types of antiulcer drugs are summarized in Figure 1.1.

Mucosal protective agents, on the other hand, can prevent the formation and development of ulcers by either directly or indirectly enhancing mucosal defenses. These so-called cytoprotective agents have being developed for this propose to prevent gastric damage without disturbance of normal gastric secretion (Robert, 1979; 1985). Such cytoprotective effects have been extensively studied in the gastrointestinal tract, liver (Stachura et al., 1981), and pancreas (Robert et al., 1983b).

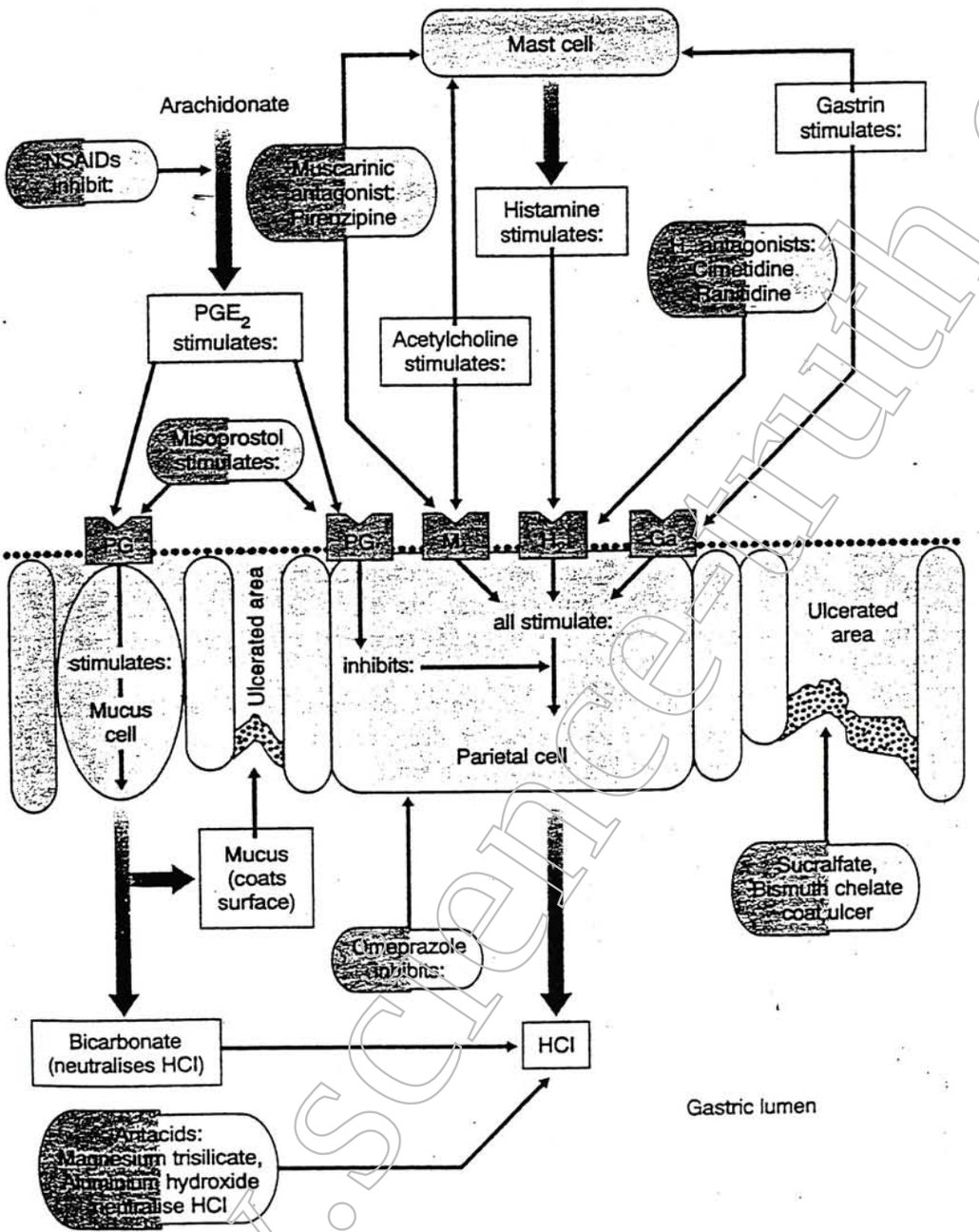


Figure 1.1 Effect of antiulcer agents (Galbraith et al., 1994)
The figure indicates the pathway for generation of gastric acid and the acting sites of antiulcer drugs. PG_1, M_1, H_2, and Ga represent four types of receptors in the membrane of the parietal cells.

It is thus well accepted that the gastric mucosal cytoprotection is associated with complex mechanisms including the mucus-bicarbonate secretion, level of mucosal sulfhydryls, mucosal blood flow, the rapid renewal of epithelial cell, etc. (Konturek, 1985). In the gastrointestinal mucosa, the metabolites of arachidonic acid, such as endoperoxides, hydroperoxides, prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) are important mediators involved in the ulceration process (Konturek et al., 1986), while PGs are associated with the cytoprotective effect (Robert, 1979), and TXs and LTs are related to the induction of ischemia and gastric lesions (Whittle et al., 1981; Peskar et al., 1986; Konturek et al., 1988).

When PGs are administrated at a lower antisecretory dose (<1%), they can protect the gastric mucosa against various necrotizing agents, e.g. absolute ethanol, 0.6 M HCl, 0.2 M NaOH, 25% NaCl and boiling water (Robert, 1979; Jacobson et al., 1976). In addition, mild irritation of these necrotizing agents can stimulate the synthesis of PGs in the gastric mucosa (Assouline et al., 1977). Prostaglandins can increase the amount of surfactant phospholipids and strengthen the hydrophobic barrier to prevent the diffusion of H^+ and other water-soluble irritants in the mucosa. Moreover, the protection of PGs is associated with the removal of back-diffusing hydrogen ions, an increase in GMBF and subsequent improvement of oxygen and nutrition supply (Leung et al., 1985).

The drugs used routinely for the treatment of gastrointestinal ulcers are summarized in Table 1.2.

Table 1.2 Clinically used therapeutic agents for the treatment of peptic ulcer

Effect	Type of drug	Drug	Side effects
Inhibition of gastric acid secretion	Histamine-2 receptor antagonists	Cimetidine Ranitidine Famotidine	Muscular pain, headache and dizziness, tiredness and mild gastrointestinal disturbance (e.g. diarrhoea, constipation and nausea), Inhibition of activities of gastrointestinal enzymes and the metabolism of certain drugs
	Proton pump inhibitors	Omeprazole	Diarrhoea, skin rashes, Headache
	Anticholinergic agents	Atropine Pirenzepine	Narrow therapeutic window Visual disturbances, dry mouth, urinary retention
	Prostaglandins	Misoprostol	Gastrointestinal disturbances, diarrhoea, nausea, dyspepsia, etc.
Neutralization of gastric acid	Antacids	Sodium bicarbonate Calcium carbonate Aluminum Hydroxide Magnesium Hydroxide	Hypersecretion; perforation Causing potential hypercalcemia Constipation, preventing absorption of Phosphate and other drugs, Osmotic laxative
Mucosal protective agent		Sucralfate, Aluminum Colloidal bismuth	Constipation Nausea, vomit, etc.

References: Kirsner, 1962; Fordtran et al., 1978; Gledhill, 1983; McGuigan, 1983; Meryn et al., 1983; Walan, 1984; Schunack, 1987; 1989; Wagstaff et al., 1988; Giercksky; 1989; Guerreiro et al., 1990; Anon, 1993b

From the information given in Table 1.2, it can been seen that the mainstream of peptic ulcer management lies in the inhibition of gastric secretion. The anti-secretory agents and antacids, however, possess certain unwanted side effects, such as hypertension and congestive heart failure (sodium bicarbonate), constipation (aluminum hydroxide and calcium carbonate), diarrhea (magnesium hydroxide), gastrointestinal discomfort, central nervous system disturbances (omeprazole), gynaecomastia and impotence (cimetidine), visual disturbances, dry mouth and urinary retention (pirenzepine), headaches, tiredness, dizziness and mild gastrointestinal disturbances including diarrhoea, constipation and nausea (ranitidine). Approximate 50% of ulcer patients experience the recurrence of peptic ulcer within 1 year after cessation of antisecretory therapy and antisecretory agents are not quite successful for the treatment of NSAIDs-induced gastric ulcer (Szabo, 1987). In addition, the acid-orientated therapeutic approach cannot explain why persons with high gastric acid output may not have any ulcer in the stomach and that the level of gastric acid secretion in most of ulcer patients is normal or below normal (Glavin et al., 1992). Furthermore, suppression of gastric acid secretion over a long period may cause bacterial colonization in the stomach. Therefore, therapies focused on enhancement of mucosal defense system should be further developed for the management of peptic ulcer.

Accordingly, the ideal antiulcer drugs should possess effects on both suppressing aggressive factors and strengthening gastric mucosal defenses. In order to achieve this therapeutic target and decrease ulcer recurrence, new drugs with multiple and superior healing effects should be developed.

Traditional Chinese medicines have been used for thousands of years for the treatment of various diseases including peptic ulcer. These natural remedies provide a rich resource for developing drug candidates. Despite many plants having been used for the treatment of gastrointestinal disorders, most of them have not been extensively studied both chemically and pharmacologically. In the present study, *Hippophae rhamnoides* L., a plant used for wound healing, was selected to evaluate its antiulcer activity.

Hippophae rhamnoides L. (Family: Elaeagnaceae) is a deciduous plant widely distributed in the north of China (Anon, 1977). The plant is traditionally used for wound healing and pain relief (Li, 1996) and the fruit juice has been developed as an health-drink in China and certain European countries. Phytochemical studies on Hr indicate that the plant contains vitamins (e.g. vitamins A, B1, C and E); essential oils (1,1-diethoxy-n-nonane, 1,1-diethoxy-n-tetradecane); fatty acids (e.g. palmaltic acid, oleic acid, linoleic acid, linolenic acid); phytosterols (e.g. β-sitosterol, ursolic acid, oleanolic acid); flavonoids (e.g. quercetin, isorhamnetin); amino acids, proteins, carbohydrates and mineral as major chemical components (Li, 1996; Xin, 1995; Gao, 1995).

Pharmacological studies on Hr suggest that the plant possesses various biological activities:

Effect on immune system

Administration of fruit juice of Hr into mice enhanced phagocytosis of macrophage and enhanced contents of lysozyme in serum (Zhang, 1989). In addition, treatment with the Hr flavonoid fraction (i.p. or s.c.) strengthened the phagocytosis of

macrophage cells in the abdominal lumen (Xu, 1987; Zhong, 1989). Pretreatment with the hexane fraction of Hr for 14 days in rats markedly increased the level of IgG, IgM, C_3 in serum (Wang, 1989).

Antineoplastic effect

Fruit juice of Hr and its hexane extract significantly inhibited the development of S_{180} sarcoma, B_{16} melanoma and P_{388} lymphocytic leukemia by blocking the synthesis of nitroso compounds in rats (Li, 1987). *In vitro* studies also demonstrated that Hr juice could arrest the growth of cancer cells including S_{180}, P_{388}, L_{1210} and human gastric cancer cell SGC-9901 (Zhang, 1990).

Effect on cardiovascular system

Fruit juice and the seed oil of Hr showed protective effect on the cardiovascular system, especially in improving the tolerance of hypoxia. The flavonoid fraction of Hr has been used for the treatment of angina pectoris in human as it can effectively improve the cardiovascular circulation (Zhang, 1987).

Effect on blood circulation

Treatment with an ethanol extract of Hr (7.5-30 mg/kg, i.v.) in rabbits markedly decreased the viscosity of total blood in a dose-dependent manner and significantly inhibited the formation of thrombosis (Bai, 1992). The seed oil of Hr was found to be able to inhibit platelet aggregation induced by collagen or ADP (Liu et al., 1988), decrease cholesterol level in serum and increase the level of high density lipoproteins (Chou, 1986).

Antioxidative activities

The flavonoid fraction of Hr has been proved to be able to scavenge oxygenic free radicals and hydroxyl free radicals formed in Fenton reaction (Ju, 1990). In addition, treatment with Hr oil (2.5mg/kg, i.p.) inhibited the formation of fatty liver by regulating the reaction rate of peroxidation and the content of vitamin E in tissues (Anon, 1993a). The seed oil of Hr was found to possess similar effect as vitamin E on the prevention of cellar membrane damages from high-lipid serum and increase of superoxide dismutase (SOD) activity (Wang, 1989). In addition, oral administration of Hr oil prevented the depletion of SOD and reduced-glutathione (GSH) induced by high dosage of vitamin D_3 (Cui, 1990).

Anti-inflammatory and wound healing action

An anti-inflammatory activity of Hr oil was found in a corn oil-induced inflammatory model. This was associated with decrease of swelling and inhibition of capillary permeability and inflammatory exudation. The Hr seed oil also showed potent healing effect on wounds induced by alkali solution in mice (Cao, 1987). Anti-inflammatory effect of Hr extract has been observed against chemical-induced eye burning (Nikulin et al., 1992). Furthermore, the Hr extract promoted healing processes in the experimental wounds and such an effect was associated with an intensive and rapid epithelization and tissue differentiation (Ianev et al., 1995). The presence of vitamins (e.g.: vitamin A, C, E) and minerals (e.g.: selenium, zinc, copper) in the Hr extract was believed to be responsible for the activity (Ianev et al., 1995).

Liver protection

The fruit juice of Hr has been found to be able to prevent the liver from the CCl_4- and acetaminophen-induced injuries with significant decrease of both malondialdehyde (MDA) and SGPT levels, and inhibition of the GSH depletion in the liver (Cheng et al., 1990; 1994).

Antiulcer effect

The antiulcer effects of Hr extract have been evaluated in reserpine-induced acute gastric lesions in rats and acetic acid-induced chronic ulcer in mice. Pretreatment with seed oil of Hr (5 ml/kg, i.g.) inhibited gastric lesions induced by reserpine (Zhou, 1986). In addition, *Hippophae* oil accelerated the healing process of gastrointestinal lesions that was associated with a decrease of MDA content (Liu et al., 1988).

Current pharmacological studies on Hr demonstrate that the plant is a promising candidate for developing preventive agents against the formation of peptic ulcers. Although the previous investigations indicate an antiulcer property of Hr, the studies are not extensive and the mechanisms of action have not been completely elucidated.

Aims of the present study

1 Evaluation of the antiulcer effect of *Hippophae rhamnoides* by various ulcer models including gastric acid-, chemical- and stress-induced acute lesion models as well as an acetic acid-induced chronic ulcer model.

2 Examination of both the preventive and healing effects of Hr against the ulcer models employed.

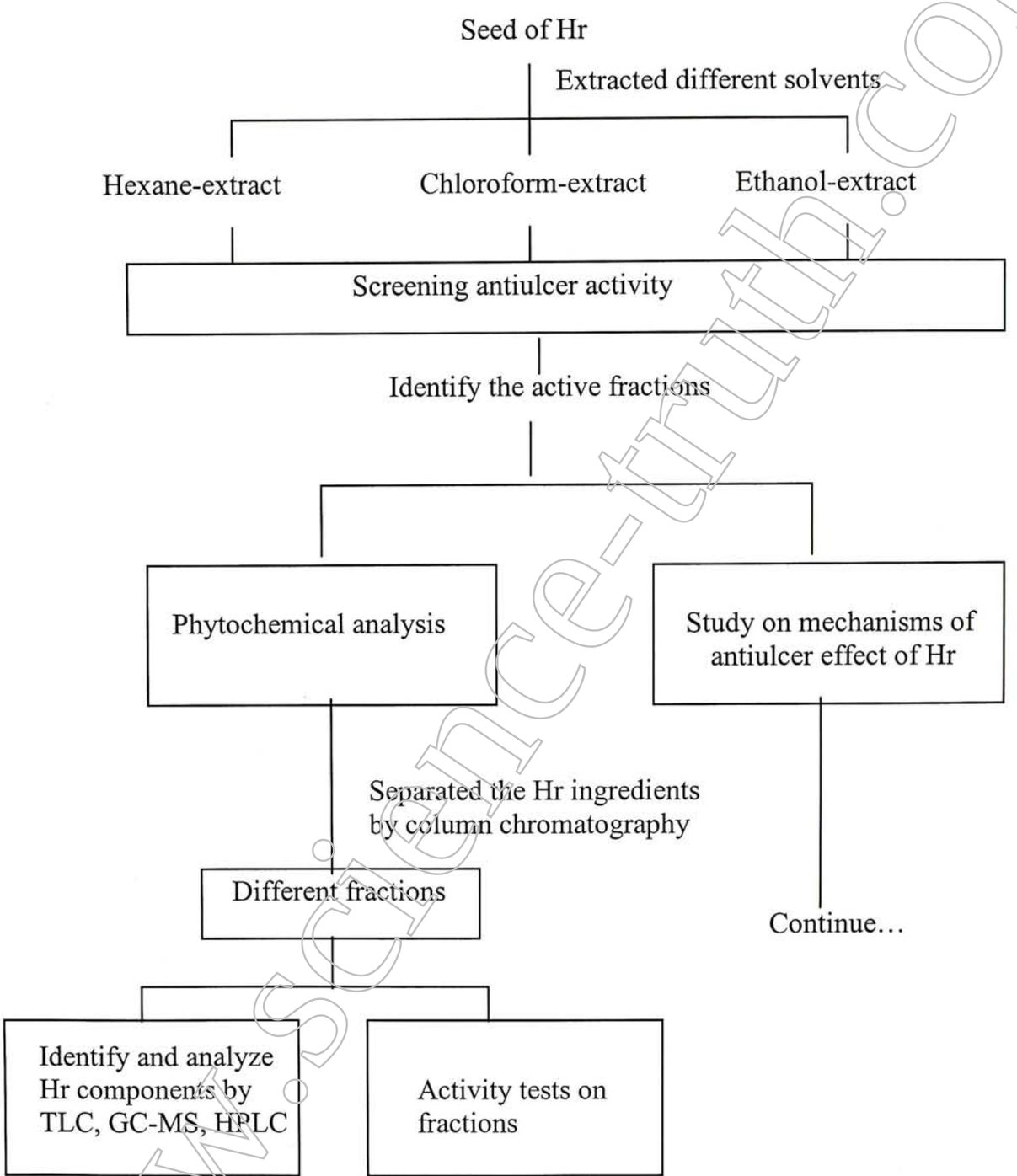
3 Study on mechanisms of action of Hr against ethanol-induced gastric lesions and investigation of the cytoprotective effect of this plant.

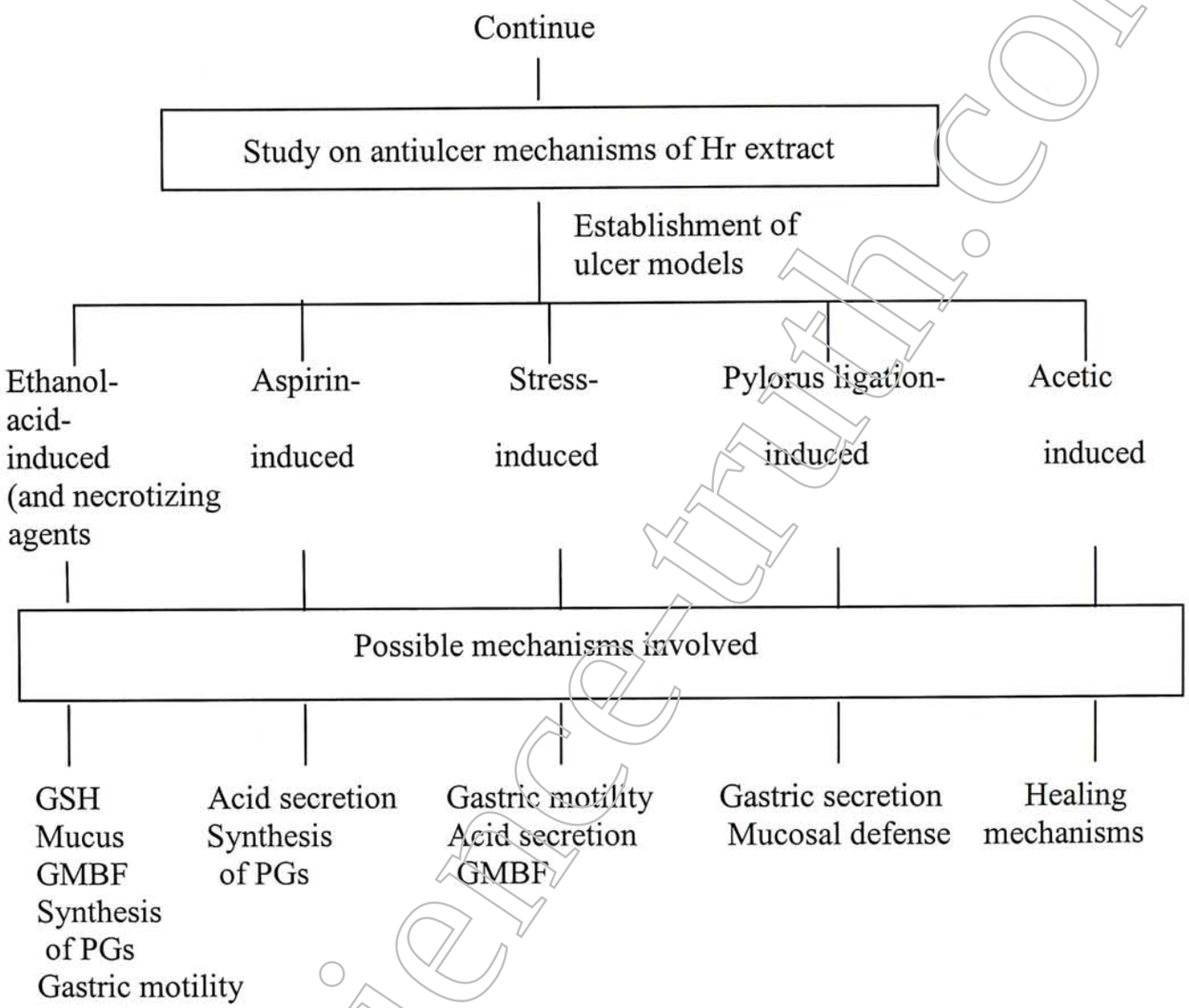
4 Assessment of antisecretory effect of *Hippophae rhamnoides* in pylorus ligation-induced gastric damage.

5 Identification and analysis of possible antiulcer components of *Hippophae rhamnoides*.

The detail experimental design is summarized in Figure 1.2.

Figure 1.2 Experimental design





Chapter 2

Evaluation of Antiulcer Effect Exhibited by *Hippophae rhamnoides* Using Different Ulcer Models

2.1 Introduction

The pathogenesis of gastrointestinal ulcers is so complex that no single mechanism can be employed to explain the total picture of ulcer formation adequately. Nonetheless, the study on the pathogenesis of various ulcers, such as chronic ulcer, drug-induced lesions, stress-induced lesions and relapse of ulcer, will deepen our understandings of the biochemical, functional and structural changes induced by ulcer formation. Currently, the most common approach in evaluation of therapeutic efficacy of potential antiulcer drugs is to establish different lesion models and investigate the effect of drug candidates against the models. Up to date, a number of gastric lesion models have been established and the most frequently used models are summarized in Table 2.1. Besides, there are some additional experimental lesion models available in the preclinical studies such as histamine- (Esplugues, 1982), platelet-activating factor (PAF)- (Escolar, 1989), hemorrhage shock- (Smith et al., 1987) and ischaema repercusion (Hernandez et al., 1987)-induced lesions.

In order to evaluate the antiulcer effect of *Hippophae* extract, six gastric ulcer models were employed in the present study including ethanol-, aspirin-, pylorus

ligation-, stress- and necrotizing agents (HCl, NaCl, NaOH)-induced acute gastric lesions as well as acetic acid-induced chronic ulcer model.

Table 2.1 Commonly used experimental gastric lesions models

Model	Method	Pathogenesis	Type of lesions and application
Ethanol-induced gastric lesions	Rats are fasted 24-48h Administration (0.5-2.0 ml, i.g.) of various concentrations of ethanol (35-100%), lesions are examined 0.5-2h later	Mucus secretion ↓ Intracellular mucin ↓ Bicarbonate secretion ↓ Non-protein sulfhydryl compound (SH) ↓ H^+ back-diffusion ↑ Na^+ transport ↓ Disturbance of GMBF Gastric motility ↓ Release of 5-hydroxytryptamine (5-HT)↑ Ischemia ↑ Transmucosal potential difference ↓ Vascular permeability ↑ Disturbance of PGs synthesis Leukotriene production ↑ Release of vasoactive products ↑	Lesions: linear haemorrhages on mucosa along the long axis of the stomach Application: study on cytoprotective mechanisms of drugs
NSAIDs-induced gastric lesions	Aspirin-induced: Rats are fasted 24h, oral administration of 150-200 mg/kg of aspirin buffered with HCl; lesion is examined 1-6h later Indomethacin-induced: s.c. 20-30mg/kg; lesion is examined 4-7 h later	Direct damage on mucosal cell Cell member permeability ↑ H^+ back-diffusion ↑ PGs synthesis ↓ Mucus and bicarbonate secretion ↓ Gastric acid secretion ↑ GMBF ↓	Lesions: multiple, small and dotted hemorrhagic lesions on mucosal segment Application: study drug especially NSAIDs-induced lesions

Stress-induced gastric lesions	Rats are fasted 24h, then are immersed in the water (21±1°C for 6-8 h or 4±1°C for 3h)	Motility ↑ Mucus secretion ↓ CNS Disorders Disturbance of micro-circulation Vascular permeability ↑ GMBF ↓ PGs synthesis ↓ H^+ back-diffusion ↑ production of free radicals ↑	Lesions: dotted or linear lesions on glandular segment Application: study lesion induced under stress
Pylorus ligation-induced gastric lesions	Rats fasted 48-72 h; then the pylorus is ligated; lesion is examined 2-18 h after ligation, gastric acid output is measured	Accumulation of gastric juice ↑ Stimulation of local nerves ↑ Disorder of CNS ↑ Disturbances of GMBF Gastric acidity ↑ Gastric secretion ↑	Lesions: multiple lesions on forestomach Application: study on gastric acid related damage and gastric secretion
Acetic acid-induced chronic gastric ulcer	Rats fasted 24 h before operation. Concentrated acetic acid is applied (100%, 1 min) or injected (0.015-0.15 ml, 10-40%) to gastric serosa. Ulcer is formed 3-5 days later.	Disturbance of GMBF around ulcer tissue Tissue damage ↑ Chemical-induced tissue necrosis	Lesions: only occur at the site of injection or application of acid; Lesions site is clear; the formation of ulcers requires several days. Application: Study on process of ulcer formation and ulcer healing Disadvantage: Perforation of the stomach; Large variation among individual animals

Reserpine-induced gastric lesions	Administration of reserpine (i.p. 5-10 mg/kg) to rats, lesion is examined 6-8h after	Release of 5-HT and histamine↑ Release of ACTH-sterols hormone↑ Disturbance of gastric secretion	Application: study drug-induced lesion
5-HT - induced gastric lesions	Rats are fasted 24h. After administration of 5-HT (s.c. 20 mg/kg), lesion is examined 12-20 h later	Vascular contraction↑ Local ischaemia ↑	Lesions: round lesions on gastric larger curvature Application: study on mechanism of acid secretion
Cortical hormone-induced gastric lesions	Hydroprednisone 4mg/rat for 4 days	Gastric acid and pepsin secretion ↑ Mucus secretion ↓ GMBF ↓ PGs synthesis ↓	Lesions: multiple lesions on glandular segment Application: study lesion induced under normal physiological conditions
Necrotizing agents-induced gastric lesions	Necrotizing agents: 0.6N HCl, 0.2N NaOH, 25% NaCl, Rats are fasted 24 h, administration of necrotizing agents (i.g. 1ml /rat); lesion is examined 1 h later	Direct damage on mucosal cell	Lesion: linear haemorrhages on mucosa along the long axis of the stomach Application: Study on cytoprotective effect of drug

* ↑: increased, ↓: decreased.

References: Shay et al., 1945; Brodie, 1966a; 1979; Takagi et al., 1969; Okabe et al., 1971; Dai et al., 1972; Guth, 1972; Ritchie, 1975; Yanv, 1978; Guth et al., 1979; Robert et al., 1979; Silen et al., 1981; Murakami et al., 1985; Szabo et al., 1985; Peskar, 1986; Szabo, 1987; Kitajima et al., 1989; Glavin et al., 1992

2.1.1 Ethanol-induced gastric lesions

The ethanol-induced gastric lesion model is widely used for the evaluation of the cytoprotective effects of drug candidates. It is believed that the pathogenesis of ethanol-induced gastric lesions is related to multiple factors including disturbance of GMBF and gastric motility, formation of free radicals, release of endogenous mediators, such as PGs, NO and histamine, induction of ischemia in tissues and injury of vascular vessels (Glavin et al., 1992). Therefore, the model can provide useful information on the mechanisms of drug action. In order to investigate cytoprotective effect of Hr seed extract, the ethanol-induced gastric lesion model was employed in the present study.

2.1.2 NSAIDs-induced gastric lesions

NSAIDs can induce severe adverse effect in the stomach, such as mucosal hemorrhage, bleeding, ulceration and perforation (Carson et al., 1987; Stodolnik et al., 1990; Trevethick et al., 1995). Pathogenesis of NSAIDs-induced gastric lesions is multifactorial. Under pH below 3.5, aspirin appears as a lipid soluble unionized molecule. The compound can perpetrate easily through cellular membranes. After absorption through the gastric mucosa, the molecule can release ionized salicylate and hydrogen ions that can cause cellular damage on the superficial mucosa. Subsequently, salicylate induces an increase of permeability of cell membrane that is associated with the deleterious influx of sodium, calcium and water leading to edematous swelling and death of the epithelia cell (Szabo, 1987). The lesions appear at the mucosal segment as multiple, small, dotted haemorrhages. The ulceration may relate to the decline in gastric mucosal potential difference due to an efflux of intracellular sodium into the gastric lumen and a back-diffusion of intraluminal acid

(Szabo, 1984). The model can be established with administration of acidified indomethacin or acetylsalicylic acid and the antiulcer effect of Hr extract was evaluated by this model.

2.1.3 Stress-induced gastric lesions

Chronic stress and anxiety can affect normal activity in nervous system. This may further influence physiological functions of the stomach and result in gastric mucosal erosions (Ritchie, 1975). The stress lesions may occur as upper gastrointestinal haemorrhages. Previous reports suggested that the disturbance of gastric mucosal microcirculation (Guth, 1972; Silen et al., 1981), abnormal gastric acid and pepsin secretion (Kitajima et al., 1989), hypermotility (Yanv, 1978) and disorders of the CNS maybe involved in the pathogenesis of stress lesions.

Several animal models of stress-induced gastric lesions have been established, such as cold stress-, swimming stress-, water immersion stress- and depression-induced lesions. As a high reproducibility, water immersion plus restraint is a commonly used model for stress-induced acute gastric lesions in rats (Murakami et al., 1985). This method was also employed in the present study to investigate the antiulcer effect of Hr extract.

2.1.4 Pylorus ligation-induced gastric lesions

The traditional clinical dictum of 'no acid-no ulcer' indicated the importance of gastric acid in the formation of peptic ulcer. The presence of acid is believed to be the major aggressive factor in the development of ulcers. In order to investigate the anti-secretion effect of drugs, the pylorus ligation-induced lesions model was firstly developed by Shay (Shay et al., 1945). This method is widely accepted nowadays for

evaluation of the anti-secretory agents. It is believed that the hypersecretion, disorders of CNS and disturbance of GMBF maybe mainly involved in the lesion formation (Brodie et al., 1966b). Therefore, this model was also established in the present study to investigate the antisecretory effect of Hr extract.

2.1.5 Acetic acid-induced chronic gastric ulcer

Acetic acid-induced chronic model of gastric ulcer is widely used to explore the healing effect of tested substances. The gastric ulcer can be induced by a single injection or applying acetic acid to the serosa of the stomach. After 2-3 days, the gastric lesions are formed, subsequently a spontaneous evolution and recovery of the ulcer can occur.

The mechanisms related to the pathogenesis of acetic acid-induced chronic gastric lesions are not fully understood. Previous studies suggested that the destruction of the lamina propria, the decrease in GMBF around ulcer tissue (Hirose et al., 1992) and direct irritation effects of the acid may be involved in the formation of the ulcer. Macroscopic and histological examination of the ulcer suggested the lesions resembled human peptic ulcers (Okabe et al., 1971a,b). As NSAIDs aggravate this type of damage and delay the healing process and antisecretory agents, such as cimetidine and omeprazole (Kamada et al., 1983; Okabe et al., 1977), accelerate the healing process, the endogenous prostaglandins, gastric acid, and collagenase maybe involved in the ulcer formation and development (Kollberg et al., 1984). The healing effect of Hr extract was evaluated with this model.

2.1.6 Necrotizing agents-induced lesion model

The presence of harmful foreign substances in the gastric lumen may become an exogenous pathogenic factor for ulceration. Several necrotizing agents including 0.6M HCl, 0.2N NaOH, and 25% NaCl are widely used to establish the exogenous substances-induced lesion models (Robert, 1979). However, the pathogenesis related to the different necrotizing agents-induced gastric lesions is still under investigation. The direct damage on mucosa cells and impairment of the gastric mocosal defense are believed to be the major causes for the induction of lesions. In the present study, the protective effect of Hr extract was tested against these models.

2.2 Materials and Methods

2.2.1 Plant materials

The seeds of *Hippophae rhamnoides* L. were collected in October 1996 at Jianping, Liaoning Province, China. (Figure 2.1)

2.2.2 Identification of the plant

The crude drug was identified via pharmacognostical approaches including macroscopic and microscopic examination. The identification was conducted by Professor Feng Rui-Zhi at the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences, Beijing. The plant was authenticated as *Hippophae rhamnoides* ssp. *sinensis*. L. and the vouch specimen was retained at IMPLAD.

2.2.3 Preparation of crude extract for animal studies

The seeds of *Hippophae rhamnoides* were powdered and soaked in hexane overnight at room temperature. The extraction solution was filtered and collected, then the residue was soaked with fresh hexane to continue the extraction until no more components could be obtained. All extraction solutions were combined and concentrated to dryness by rotatory evaporation at 40°C. The yield of this hexane-extract (Hr) was 8.36%.

The extracts were weighted and emulsified in a solution containing 1% Tween-80 (w/v). The vehicle solution was also used in the control group in different animal tests.

Figure 2.1 The plant of *Hippophae rhamnoides* L.

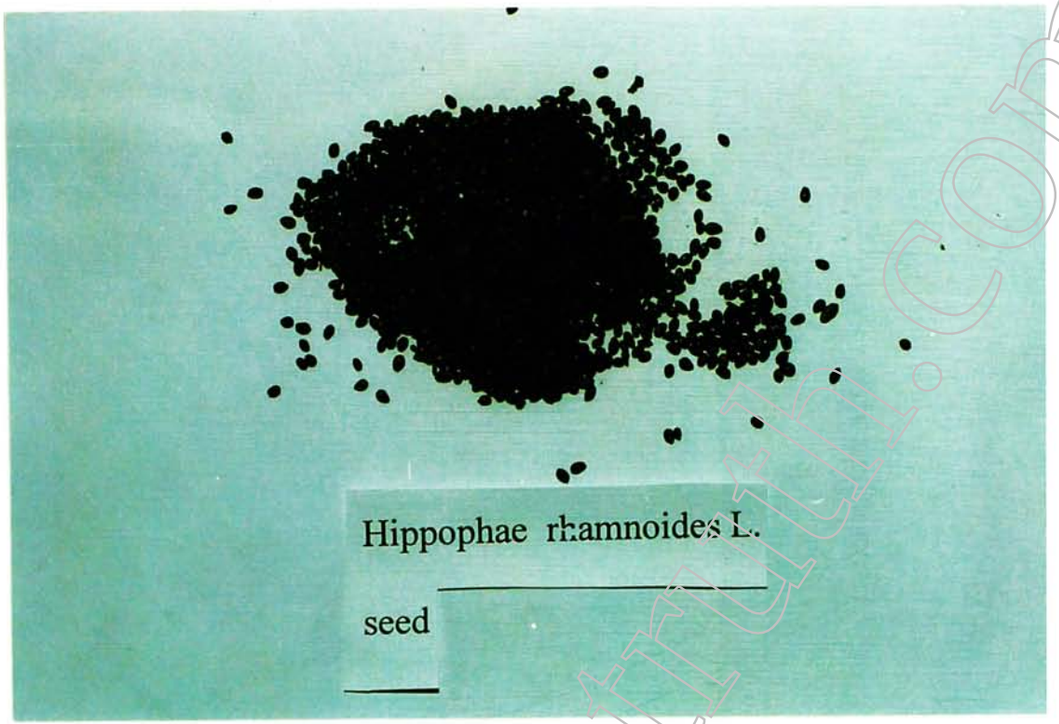


Figure 2.2 The seed of *Hippophae rhamnoides* L.

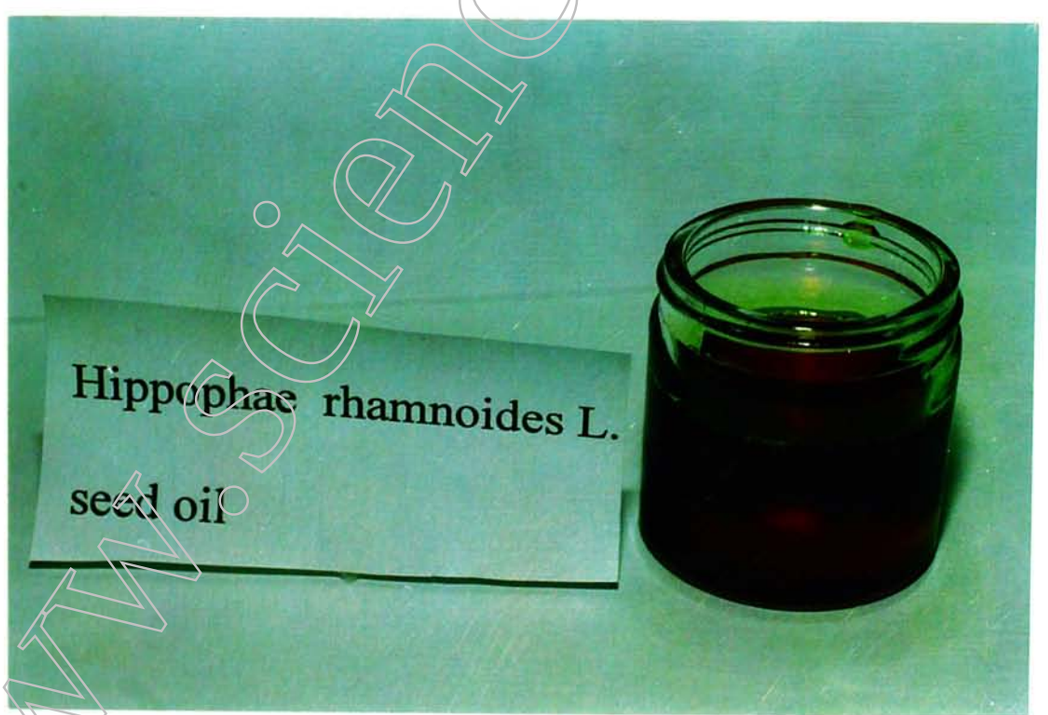


Figure 2.3 The hexane-extract of the seed of *Hippophae rhamnoides* L.

2.2.4 Experimental animals

Female Sprague-Dawley rats (210-230 g) were used for all the pharmacological tests. The animals were housed in an air-controlled room in which the temperature and relative humidity were maintained at 22 ± 1 °C and 70-75%, respectively. The rats were exposed to a daily cycle of light and dark. They were fed with a balanced pellet diet and given with tap water *ad. libitum*. The rats were fasted at least 24 h for the stress-induced acute lesion model and the acetic acid-induced chronic ulcer model. In the ethanol- and acidified aspirin-induced lesion models, the rats were previously fasted for 48h. In the test of pyloric ligation-induced gastric lesions, the rats were fasted for 60 h and were allowed free access to tap water until 2 h before the experiment.

2.2.5 Ethanol-induced gastric mucosal lesions

Acute gastric mucosal lesions were induced in starved rats by intragastrical (i.g.) administration of 60% ethanol (v/v) at dose of 6 ml/kg body weight. The Hr extract or vehicle was given to the rats 30 min before ethanol administration. The animals were sacrificed 30 min after the treatment with ethanol. The stomach was dissected and opened along the greater curvature. The gastric mucosa was examined for the degree of hemorrhages. The severity of gastric lesions was determined by measuring the area of injury using a grid transparency. The sum of the lesion areas was expressed as the lesion index (mm^2, LI). The percentage of protection of Hr was calculated by the following formula:

$$\text{Percentage of protection (\%)} = (LI_{control} - LI_{test}) / LI_{control} \times 100\%$$

2.2.6 Acidified aspirin-induced gastric lesions

Acidified aspirin (200 mg/kg in 0.15N HCl) was employed for establishment of the NSAIDs-induced gastric lesion model. Aspirin was suspended in 0.15N HCl solution containing 1% of Tween-80. The final concentration of aspirin was 50 mg/ml. The Hr extract or vehicle was given intragastrically 30 min before the aspirin administration. The animals were sacrificed 6 h after the administration of aspirin.

The removed stomachs were opened and rinsed with saline. The area of mucosal lesions was measured and the lesion index was calculated (Gyires, 1990).

2.2.7 Water immersion plus restraint-induced stress lesions model

The animals were fasted for 24 h with free access to water, then were administered of the Hr extract or vehicle solution. After 30 min, the rats were placed individually into metal stress-cages. The cages were then immersed into a water bath with temperature controlled at 24±1°C for 6 h. The surface of water was retained up to the xiphoid of rat. The rats were sacrificed 6 h after the water immersion and the gastric lesions were then measured and represented as lesion index. Cimetidine (100 mg/kg, i.g.) was used as positive control compound in this test

2.2.8 Pylorus ligation-induced gastric lesions

The pylorus ligation was performed according to the method described by Shay (Shay et al., 1945) with slight modification. Rats were fasted for 60 h with free access to a sucrose/salt solution (0.9%). After anesthetization, an incision was performed from the midline of rat abdomen. The pylorus was then ligated with a piece of thread. Extreme care was taken to prevent bleeding or hemorrhage induced blockage in the blood vessels. The Hr extract or vehicle was injected into the

duodenum immediately followed the ligation. The incision was then closed with sutures. After the surgery, the rats were allowed to recover from anesthesia, and were sacrificed batch to batch starting from 1 h to 8 h after the ligation.

The severity of gastric lesions was rated into five degrees according to the method reported by Barret (Barret et al., 1955):

0 = no visible lesions

1 = diameter of lesion is 1-3 mm, less than 10 lessons observed

2 = diameter of lesion is 1-3mm, more than 11 lessons observed

3 = diameter of lesion is 4-6mm, more than one lesson observed

4 = diameter of lesion is large than 7 mm, more than one lesson observed

5 = perforation of the gastric wall

The time-courses of the incidence and severity of gastric lesions, acidity, acid output, content of pepsin and protein were studied at 1, 2, 4, 6 and 8 h after the pylorus-ligation and gastric juice was collected at the specified time points. Before removal of the stomach, the end of the esophagus was closed with homeostatic forceps to avoid any loss of gastric contents. The collected gastric juice was centrifuged at 3000 r.p.m. for 15 min and the supernatant was harvested and stored at -80°C until further analysis. The methods for determination of output of gastric acid, acidity of gastric juice, and content of pepsin and protein are descried in the section 3.2.6, 3.2.8 and 3.2.9 of Chapter 3, respectively.

2.2.9 Acetic acid-induced chronic gastric ulcer

Chronic gastric ulcers were induced according to the method described by Okabe & Pfeifter (Okabe et al., 1971b) with slight modification. The rats were fasted for 24 h and anaesthetized. Acetic acid (0.05ml, 30%, v/v) was injected into the serosa of the stomach. After the simple surgery, the rat was then allowed to recover from anesthesia and to be fed with water and soft diet.

The Hr extract (0.3-0.6 g/kg) or vehicle was administrated intragastrically once daily starting from day 2 to day 14 after application of acetic acid. The rats were then sacrificed and the stomachs were removed to examine the gastric ulcers. Furthermore, the content of gastric mucus was measured following the procedure described in section 3.2.10 of Chapter 3.

In order to monitor the process of ulcer development, the rats were divided into six batches. At day 3, 5, 8, 11, 14 and 17 after the application of acetic acid, the rats were sacrificed batch by batch until the complete ulcer recovery was observed. The body weight of the rat and the size of the gastric ulcers were then determined.

2.2.10 Necrotizing agents-induced gastric lesions

Three necrotizing agents including 0.6M HCl, 0.2N NaOH, and 25% NaCl were employed independently to induce acute gastric lesions. The Hr extract or vehicle solution was administered (4 ml/kg, i.g.) 30 min before a necrotizing agent was given. The rats were sacrificed 30 min after treatment with necrotizing agent for examination and calculation of lesion index.

2.2.11 *Test of acute toxicity of Hr*

A preliminary test of acute toxicity of Hr extract was performed in mice. Mice were treated with Hr extract orally at a dose (24 g/kg) 40 times higher than the recommended effective dose (0.6 g/kg). The behaviors of the mice were monitored and recorded. One week after, the body weights of mice were measured and compared with the weights before the treatment. The mice were then sacrificed and their internal organs were removed for examination.

2.2.12 *Statistical analysis*

Statistical significant differences among Mean±SEM of each test group were assessed by analysis of variance (ANOVA) followed by Tukey's test. The significant level (α) was set at 0.05.

2.3 Results

2.3.1 Effect of Hr extract on ethanol-induced gastric lesions

Intragastrical administration of 60% ethanol induced severe linear haemorrhages on the mucosa along the long axis of the stomach (Figure 2.4.B). The Hr extract at doses ranging from 300-600 mg/kg (i.g.) significantly ($p<0.05$) decreased the formation of gastric lesions (Figures 2.4.C-2.4.D) in a dose-dependent manner (Table 2.2).

Table 2.2 Effect of Hr extract on ethanol-induced gastric lesions

Treatment	Dose of Hr extract (g/kg)	Lesion Index (mm^2) M ± SEM (n=12)	Percentage of protection (%)
Control	0	65.3±6.4	-
Hr extract	0.075	51.2±5.9	23
	0.15	46.8±5.3	30
	0.3	37.6±5.3*	45
	0.6	31.8±7.4**	55

Rats were pretreated intragastrically with the Hr extract or vehicle 30 min before administration of 60% ethanol (6 ml/kg, i.g.). The gastric lesions were measured 30 min after ethanol administration. Rats only received ethanol and vehicle solution without Hr treatment were used as the control. * $p<0.05$, ** $p<0.01$ when compared with the control. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

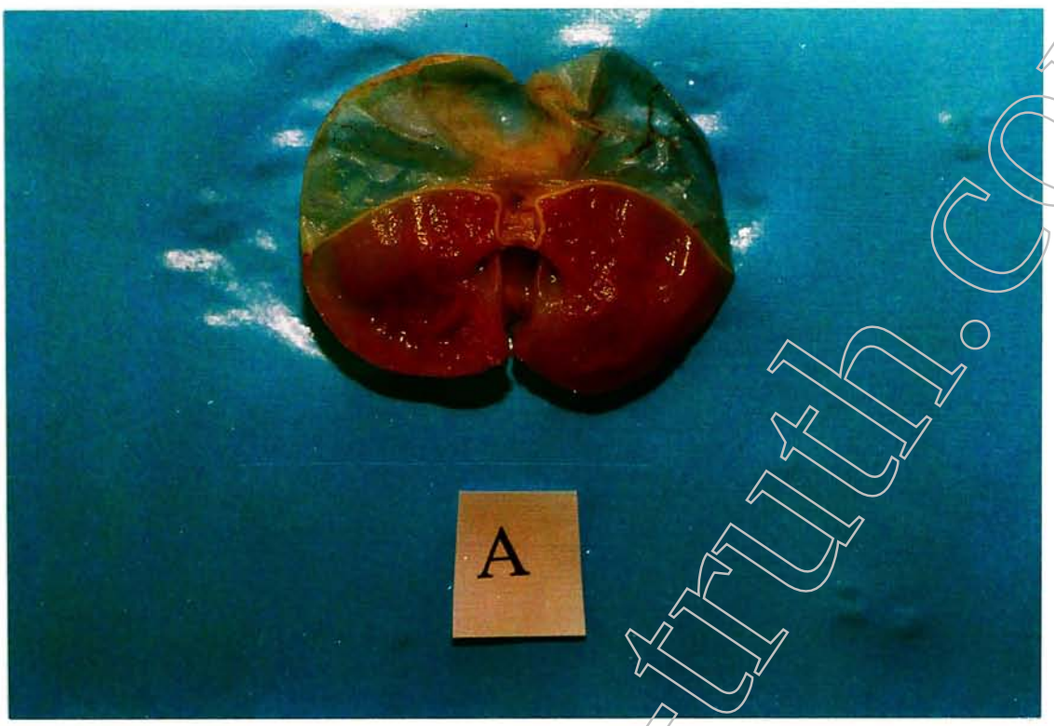


Figure 2.4.A Rat stomach without ethanol damage.

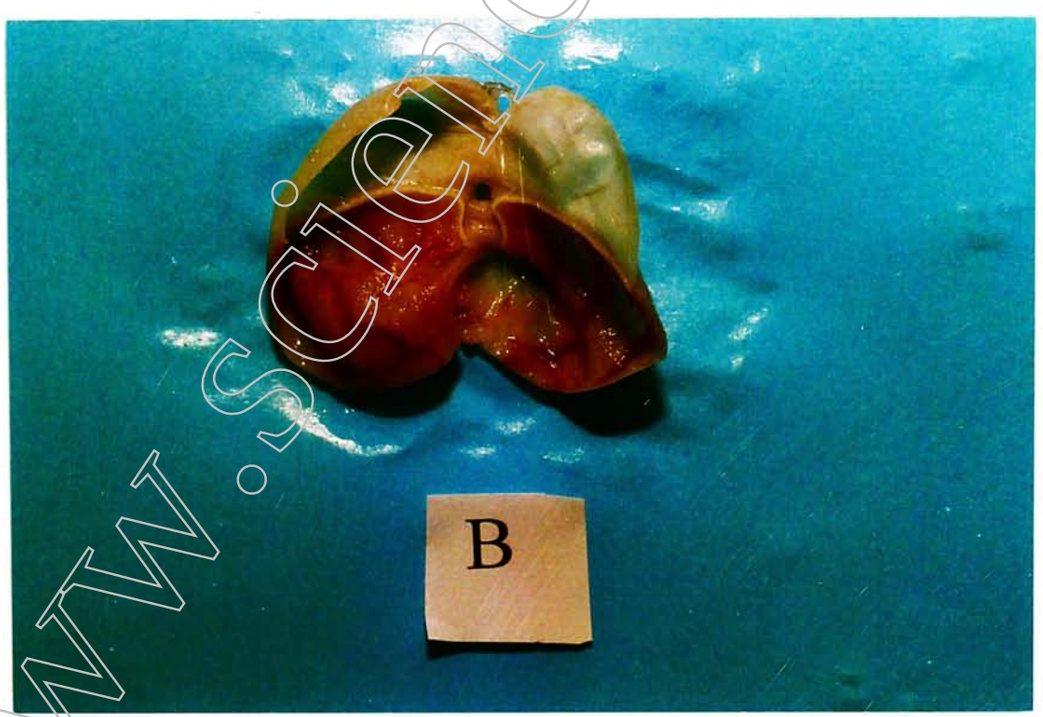


Figure 2.4.B Ethanol-induced gastric mucosal lesions. Rats were pretreated intragastrically with the vehicle 30 min before administration of 60% ethanol (6 ml/kg, i.g.). The gastric lesions were measured 30 min after ethanol administration.

Figure 2.4.C Gastric protection of Hr extract. Rats were pretreated intragastrically with Hr extract (0.3g/kg) 30 min before administration of 60% ethanol (6 ml/kg, i.g.). The gastric lesions were measured 30 min after ethanol administration.

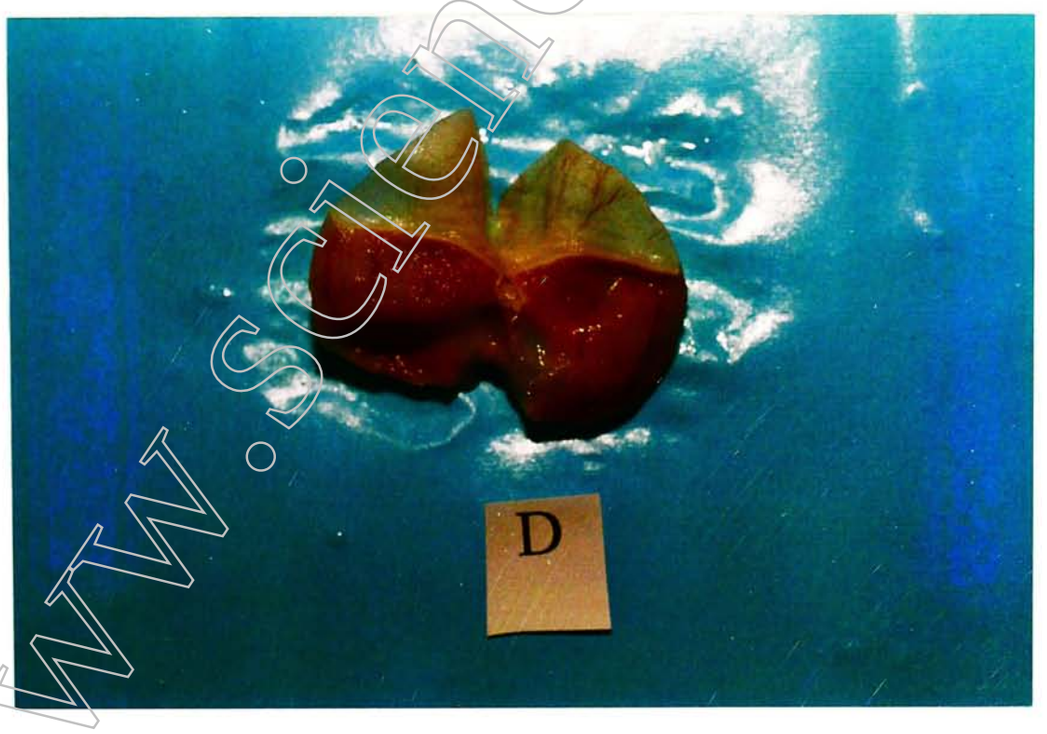


Figure 2.4.D Gastric protection of Hr extract. Rats were pretreated intragastrically with Hr extract (0.6g/kg) 30 min before administration of 60% ethanol (6 ml/kg, i.g.). The gastric lesions were measured 30 min after ethanol administration.

2.3.2 Effect of Hr extract on aspirin-induced gastric damage

Six hours after the administration of acidified aspirin (200 mg/kg, i.g.) to the rats, gastric lesions were induced in the glandular segment of stomach. Pretreatment with the Hr extract (0.3-0.6 g/kg) 30 min before aspirin dosing significantly decreased the severity of gastric damage. Interestingly, the rats only given aspirin without HCl buffer did not develop any lesions in the stomach. Results regarding this lesion model are given in Table 2.3.

Table 2.3 Effect of Hr extract on gastric lesions-induced by acidified aspirin

Treatment	Dose (g/kg) of Hr extract	Lesion Index (mm^2) M ± SEM (n=10)	Percentage of protection (%)
Control (aspirin/HCl)	0	10.0±1.2	-
Hr extract	0.3	5.1±1.1*	49
Hr extract	0.6	5.0±1.2*	50
Negative control (aspirin)	0	0	-

Rats were pretreated with the Hr extract or vehicle 30 min before administration of acidified aspirin (200 mg/kg-0.15N HCl, i.g.). The gastric lesions were measured 6h after aspirin dosing. Rats received acidified aspirin were represented as control (aspirin/HCl). Rats only received aspirin (200 mg/kg, i.g.) without HCl buffer were represented as negative control (aspirin). * $p<0.05$ when compared with the control (aspirin/HCl). The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

2.3.3 Effects of Hr extract on stress-induced gastric lesions

Gastric lesions were developed in the rats subjected to restraint with water immersion (24±1°C) after 6 h. Dotted hemorrhages were found in the glandular segment (Figure 2.5.A). Pretreatment with the Hr extract (0.3-0.6 g/kg, i.g.) significantly ($p<0.05$) reduced the gastric damage. The results are showed in Table 2.4 and Figure 2.5.B. In addition, β-sitosterol, a compound isolated from Hr extract, was tested with the stress model and the result was compared to that of cimetidine and Hr extract (Table 2.5).

Table 2.4 Effect of Hr extract on stress-induced gastric lesions

Treatment	Dose (g/kg) of Hr extract	Lesion Index (mm^2) M ± SEM (n=10)	Percentage of protection (%)
Control	0	10.9±1.3	-
Hr extract	0.3	7.0±0.9*	36
	0.6	4.9±0.8**	55

Rats were pretreated intragastrically with the Hr extract or vehicle 30 min before the water immersion at 24±1 °C. The gastric lesions were measured 6 h after the water immersion. Rats without Hr pretreatment were used as control. * $p<0.05$, ** $p<0.01$ when compared with the control. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

Table 2.5 Effect of Hr components on stress-induced gastric lesions

Treatment	Dose(mg/kg) of test compound	Lesion Index (mm^2) M ± SEM (n=8)	Percentage of protection (%)
Control	0	9.4±1.5	-
β-sitosterol	100	5.7±0.9	39
Cimetidine	100	3.1±1.1**	67
Hr extract	600	3.6±1.1*	62

Rats were pretreated intragastrically with the test samples or vehicle solution 30 min before the water immersion at 24±1 °C. The gastric lesions were measured 6 h after the water immersion. Rats without Hr pretreatment were used as the control. * $p<0.05$, ** $p<0.01$ when compared with the control. Cimetidine was employed as a positive control compound. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

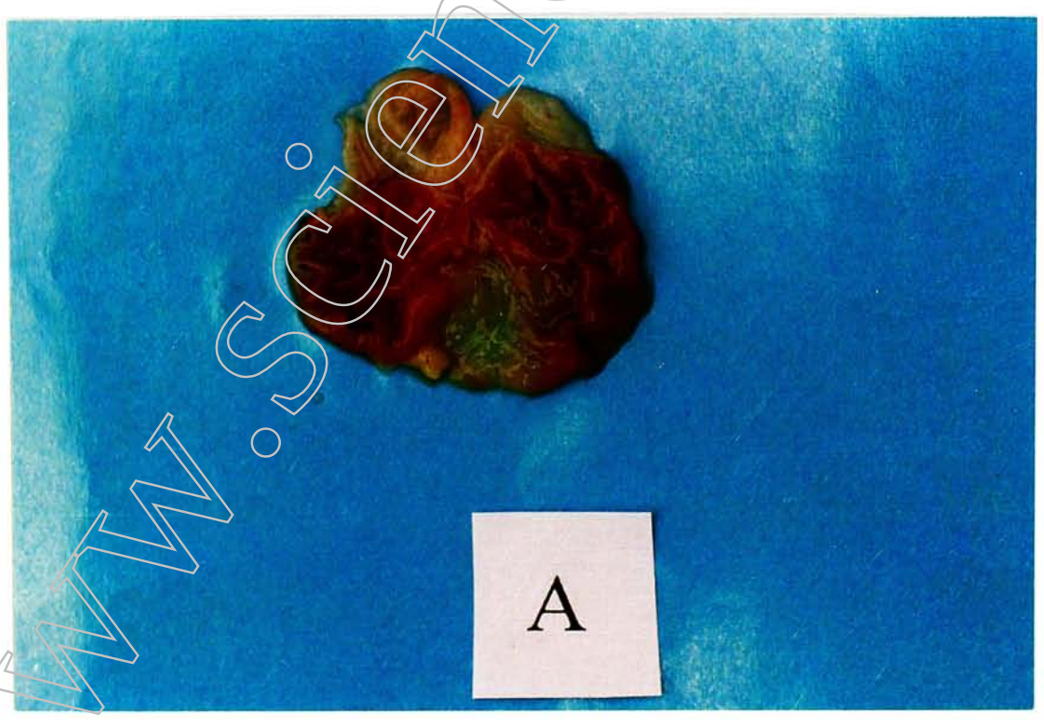


Figure 2.5.A Stress-induced gastric lesions. Rats were pretreated intragastrically with the vehicle 30 min before the water immersion at 24±1°C. The gastric lesions were measured 6 h after the water immersion.

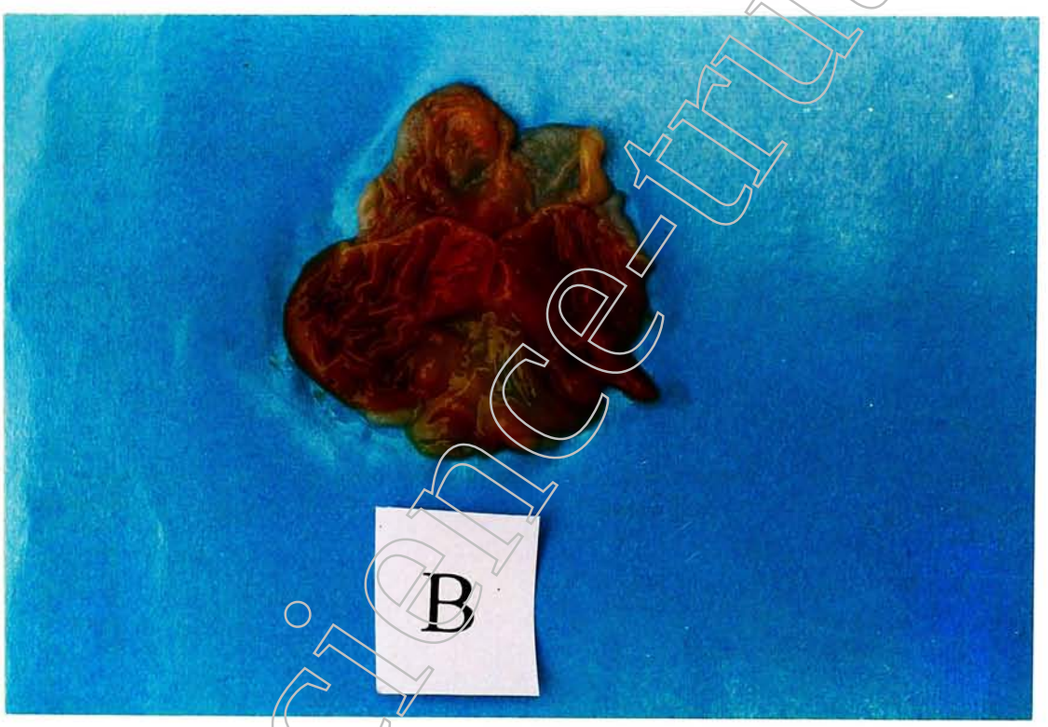


Figure 2.5.B Gastric protective effect of Hr extract. Rats were pretreated intragastrically with the Hr extract (0.6g/kg) 30 min before the water immersion at 24±1 °C. The gastric lesions were measured 6 h after the water immersion.

2.3.4 Effect of Hr extract on pylorus ligation-induced gastric injury

After the rat pylorus was ligated, a time-course study was performed to assess the lesion formation and the sequent pathological changes regarding the output of gastric acid, the acidity of gastric juice and the content of pepsin and protein in gastric juice. As shown in Figures 2.6.A-2.6.C and Table 2.6, the severity of gastric lesions was increased with time. The accumulation of gastric juice, the increase of acidity and pepsin content and the decrease of protein content of gastric juice were also observed. Lesions were found in the forestomach segment 2 h after the rat's pylorus was ligated and the incidence of ulceration increased to 100% 8 hours after the ligation.

Table 2.6 Time-course study on pathological changes in the stomach of pylorus-ligated rats (n=6)

Time (h)	Volume of gastric output (ml)	Acid output (n mol)	Acidity (m mol/l)	Content of pepsin (μg/ml)	Content of protein (μg/ml)	Lesion Index	Incidence (%)
1	1.05±0.14	24.6±7.2	22.65±3.92	161.8±23.7	7.88±0.63	0	0
2	2.40±0.49*	104.5±4.2	28.89±3.04	168.2±24.2	9.11±0.26	0.67±0.49	33
4	3.60±0.61**	176.8±32.3**	48.83±3.28**	233.0±28.4	7.96±1.43	1.17±0.60	50
6	7.41±0.69**	590.2±126.9**	77.90±10.23**	257.3±34.9	3.29±0.43**	2.17±0.54	83
8	7.92±1.2**	559.3±85.9**	72.01±5.50**	281.6±13.5**	2.51±0.45**	2.33±0.35*	100

All parameters are presented as mean ± SEM; *p<0.05, **p<0.01 when compared with the parameters measured at 1h after the ligation. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

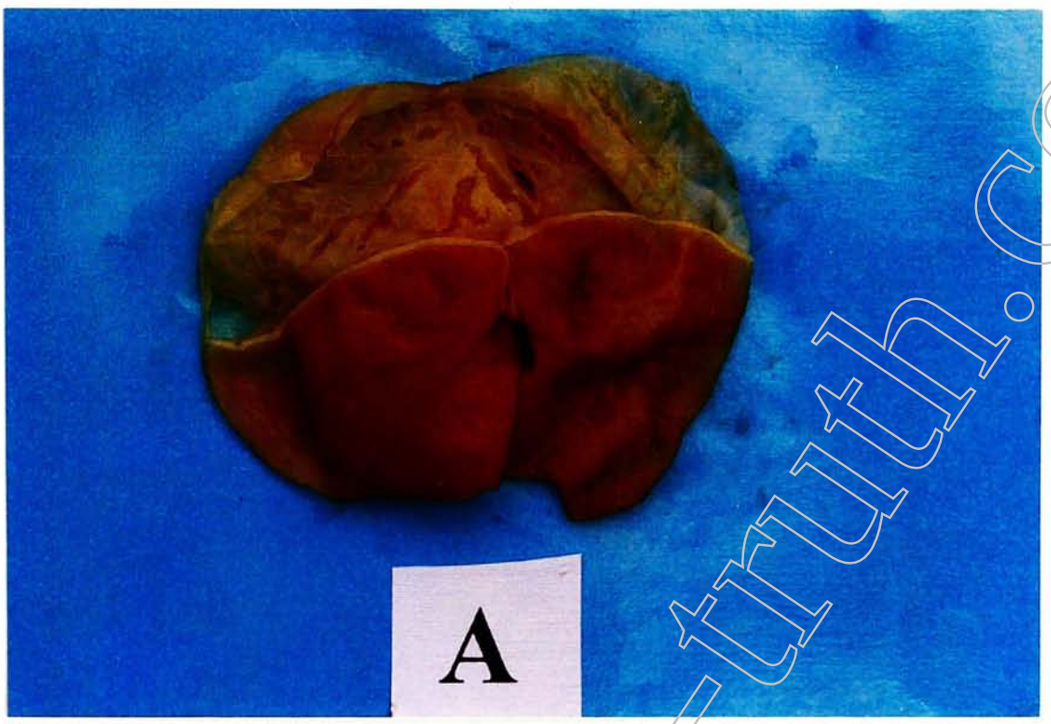


Figure 2.6.A Pylorus ligation-induced gastric lesions.
The lesions were examined 6 h after the pylorus ligation.

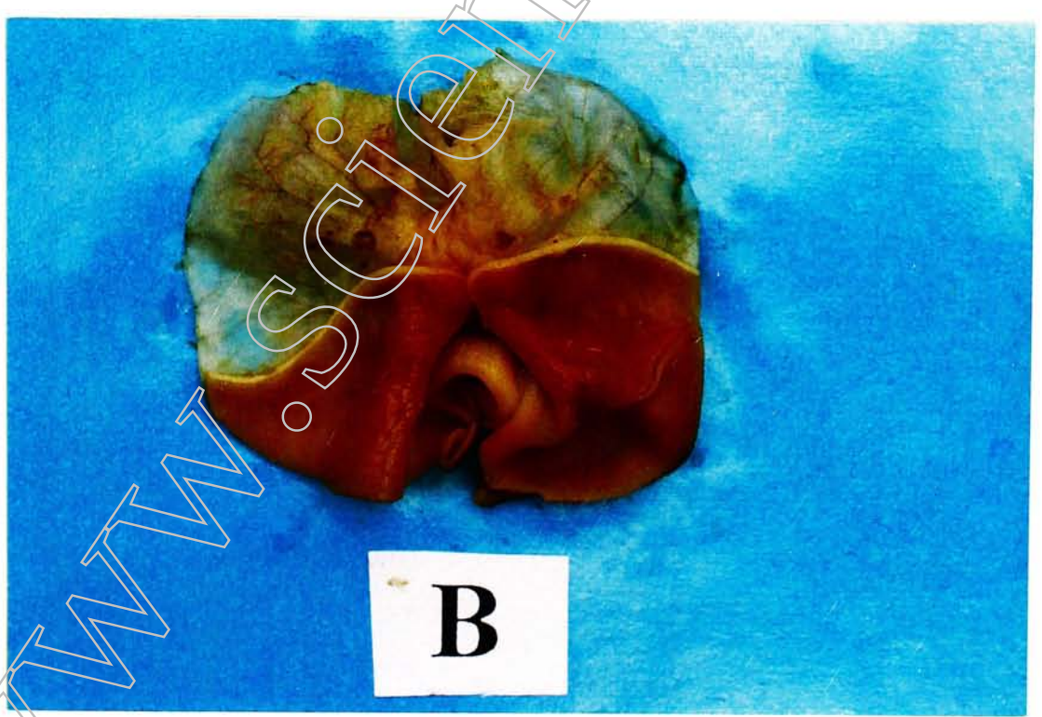


Figure 2.6.B Gastric protective effect of Hr extract.
Administration of Hr extract (i.d. 0.3g/kg) when the pylorus was ligated. The lesions were examined 6 h after the pylorus ligation.

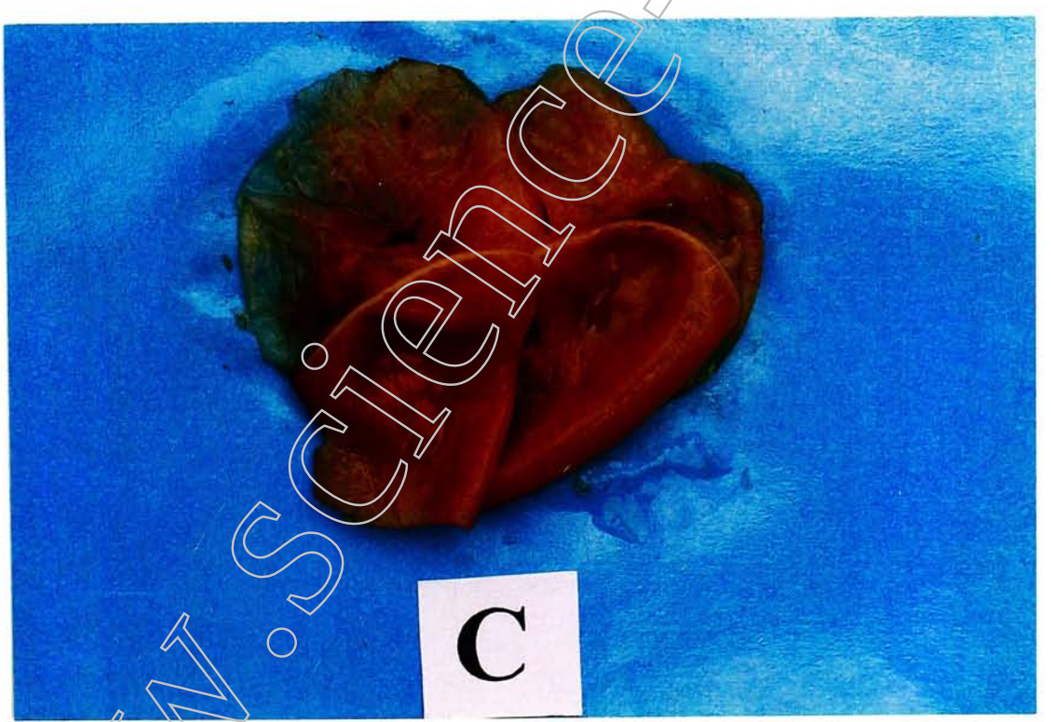


Figure 2.6.C Gastric protective effect of Hr extract.
Administration of Hr extract (i.d. 0.6g/kg) when the pylorus was ligated, the lesions were examined 6 h after the pylorus ligation.

The injection of Hr extract into the rat's duodenum significantly reduced the severity of lesions found in the forestomach segment 6 h after the pylorus ligation (Figure 2.6B-C.). In addition, β-sitosterol, a component isolated from the Hr extract, was tested with this ligation model in a parallel experiment with cimetidine as the positive control compound. As a result, cimetidine was found to be able to significantly inhibit the formation of the gastric lesions, whilst β-sitosterol only showed a 46% inhibition on gastric mucosal lesions that did not achieve a statistical significant level. The results are given in Table 2.7.

Table 2.7 Effect of Hr components on pylorus ligation-induced gastric lesions

Treatment	Dose(mg/kg) of test compound	Lesion Index M ± SEM (n=6)	Percentage of protection (%)
Control	0	2.17±0.70	-
β-sitosterol	100	1.17±0.6	46
Cimetidine	100	0*	100
Hr extract	600	0.33±0.33*	85

After the rat's pylorus was ligated, different test samples or vehicle were injected into duodenum immediately. Gastric lesions were measured 6h after the pylorus ligation. * $p<0.05$ when compared with the control group. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

Results shown in Table 2.7 indicated that the Hr extract was able to significantly reduce the mucosal damage induced by pylorus ligation. In order to verify the effect of Hr treatment, a study on measuring gastric acid output, the acidity, contents of pepsin and protein in the gastric juice were conducted at 6 h after the ligation. These results are given in Table 2.8.

Table 2.8 Study on pathological changes in the stomach of pylorus-ligated rats with treatment of Hr extract (n=17)

Treatment	Volume of gastric output (ml)	Acid output (n mol)	Acidity (m mol/l)	Content of pepsin (μg/ml)	Content of protein (μg/ml)	Lesion index	Percentage of protection (%)
Control	5.82±0.32	506.7±28.3	88.3±3.84	276.4±18.6	4.06±0.39	2.22±0.36	-
Hr extract							
300 mg/kg	5.92±0.44	454.6±29.4	70.1±3.13**	258.8±17.9	4.52±0.40	1.20±0.34*	46
600mg/kg	4.82±0.44	352.4±38.9*	69.9±4.76**	187.9±30.0*	4.27±0.66	1.21±0.18*	46

After the rat's pylorus was ligated, the Hr extract or vehicle was injected into the duodenum of rat immediately. All parameters were measured 6h after the pylorus ligation and presented as mean ± SEM; *$p<0.05$, **$p<0.01$ when compared with the control group. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

As shown in Table 2.8, Hr extract significantly decreased the acidity of gastric juice, content of pepsin and output of gastric acid when compared to the control group. Consequently, the gastric damage was apparently reduced. The content of protein and volume of gastric secretion, however, remained unchanged. These results suggest that Hr extract may possess an anti-gastric acid secretion effect.

2.3.5 Effect of Hr extract on acetic acid-induced chronic ulcer

Injection of acetic acid (0.05ml, 30%) into the serosa of the stomach induced gastric mucosal damage. The gastric injury was only confined at the injection site and the mucosal damage was associated with hemorrhage (Figure 2.9.A).

In order to investigate the process of ulcer development, gastric ulcer were examined continuously from day 3 to day 17 after acetic acid was applied (Figure 2.7). The body weight of rat was also examined at day 3, 5, 8, 11 and 14 after injection of acetic acid (Figure 2.8). Results indicated that the gastric ulcers were formed two or three days after the acid injection, subsequently the ulcer was developed and spontaneously recovered over two weeks. The mean value of ulcer index was found to be 1.3 ± 0.22 (mm^2) on day 3 and 0.094 ± 0.047 (mm^2) on day 17 ($p<0.01$).

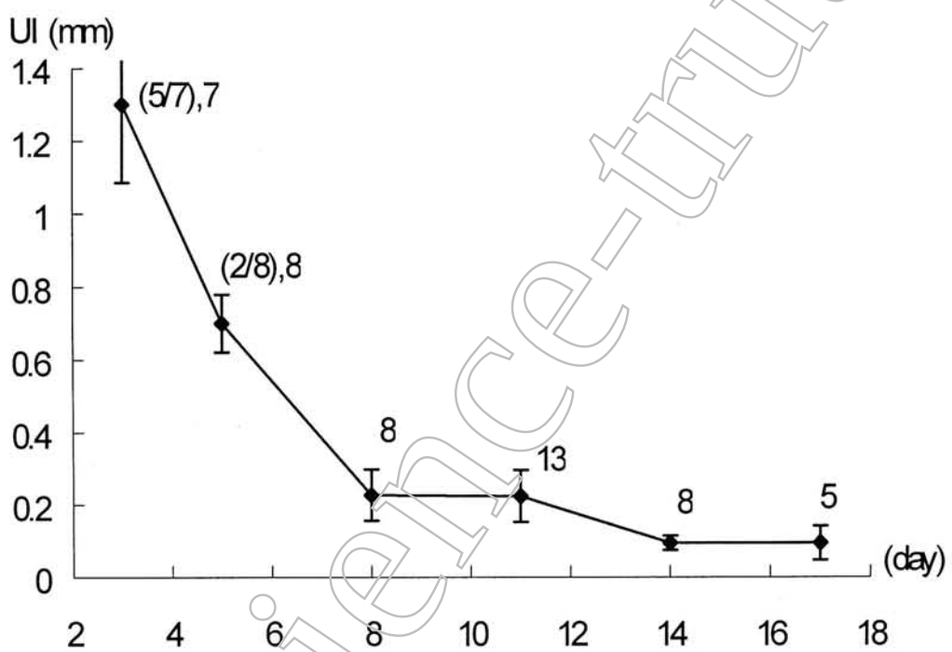


Figure 2.7 The spontaneous evolution and recovery of chronic gastric ulcers induced by acetic acid in rats.
Under anesthetization, acetic acid (0.05ml, 30%,v/v) was injected into the serosa of stomach. The areas (length and width) of gastric ulcers were examined at the specified days after the application of acetic acid. Each data-point represented mean ± SEM. n: the numbers of rats; (n/n): the incidence rate of gastric perforation.

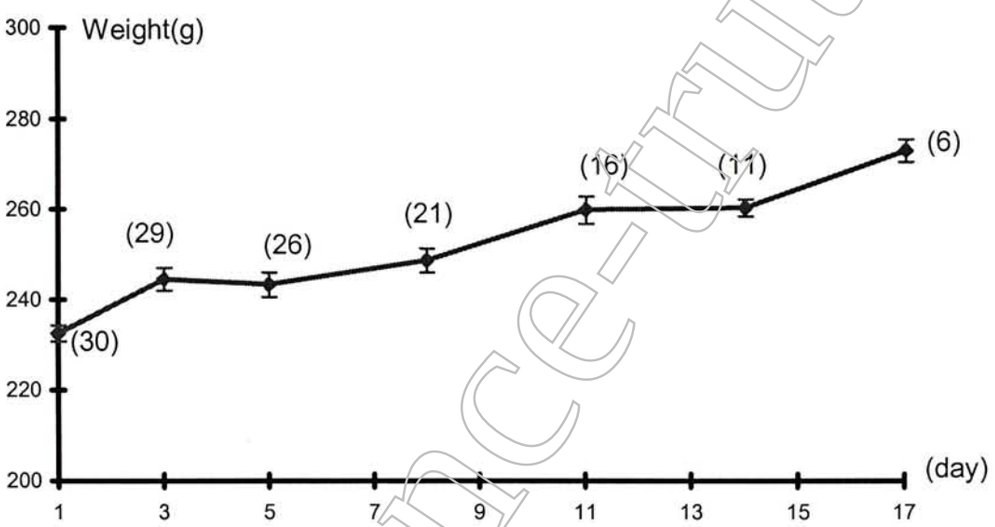


Figure 2.8 The time course of changes in body weight of rats.

After the application of acetic acid, the body weight of each rat was measured. Each data point represented as Mean ± SEM. (n): the number of rat

The healing effect of the Hr extract against acetic acid-induced ulcer formation and consequent changes in gastric mucus secretion were assessed independently. Administration of the Hr extract (0.3-0.6 g/kg) once daily for 12 days from day 2 after injection of acetic acid significantly accelerated the ulcer healing, which was associated with an increase of gastric mucus contents (Table 2.9). Such stimulation effect of Hr extract on gastric mucus secretion were also found in ethanol-induced gastric lesion model(Table 3.5) suggesting mucus secretion was an important factor in gastric mucosal defense and ulcer healing.

Table 2.9 Effect of the Hr extract on healing process against acetic acid-induced gastric damage (n=8)

Treatment	Dose of Hr extract (g/kg)	Ulcer Index (mm^2)	Healing Index (%)	Mucus Content (μg of Alcian blue / g wet weight of stomach)
Control	-	11.76±1.77	-	173.48 ±16.11
Hr extract	0.3	3.59±1.11***	70	218.78 ±26.87
	0.6	0.16±0.16***	99	259.78 ±22.34*

Acetic acid (0.05ml, 30%,v/v) was injected into the serosa of stomach. The Hr extract or vehicle was administered once daily from day 2 to day 14 after the application of acetic acid. Gastric lesions and mucus content were measured 30 min after the last administration of Hr. Healing index = $(UI_{control}-UI_{test}) / UI_{control} \times 100\%$.
* $p<0.05$; *** $p<0.001$ when compared with the control. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

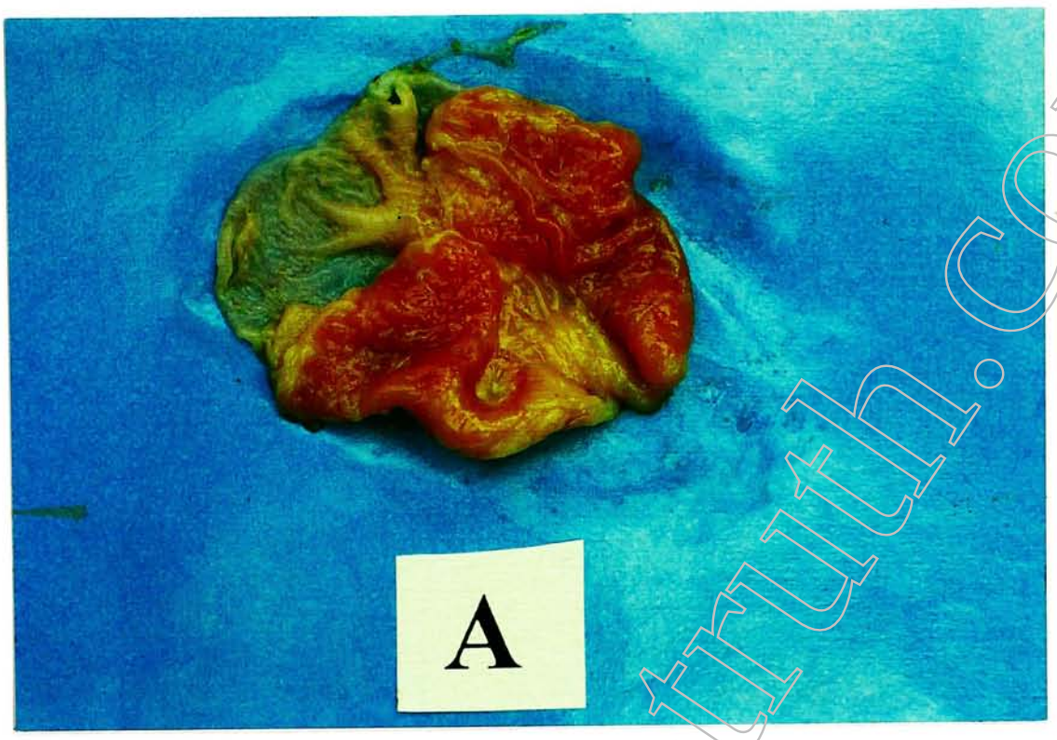


Figure 2.9.A Acetic acid-induced chronic gastric ulcer.
Acetic acid (0.05ml, 30%,v/v) was injected into the serosa of stomach. The area of gastric ulcer was examined at day 14 after the last administration of vehicle.

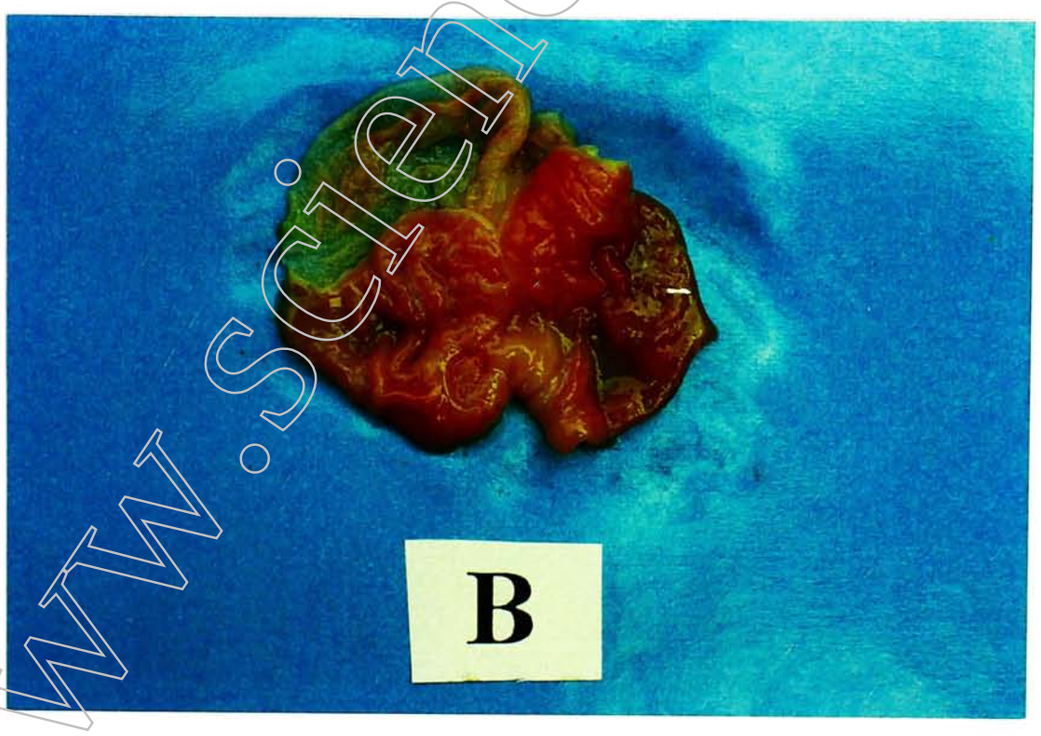


Figure 2.9.B Healing effect of Hr extract on acetic acid-induced gastric lesions.
The Hr extract (0.3g/kg) was administrated once daily from day 2 to day 14 after the application of acetic acid. Gastric ulcer was examined at the last day.

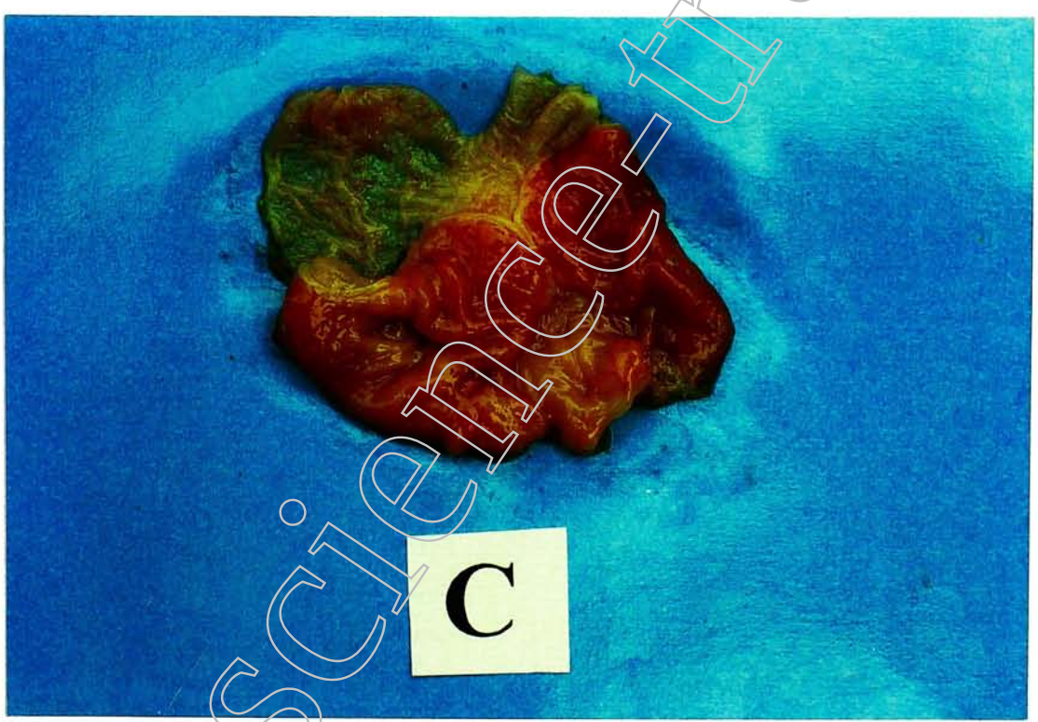


Figure 2.9.C Healing effect of Hr extract on acetic acid-induced gastric ulcer. The Hr extract (0.6g/kg) was administrated once daily from day 2 to day 14 after the application of acetic acid. Gastric ulcer was examined at the last day.

2.3.6 Effect of Hr extract on necrotizing agents-induced gastric damage

Three necrotizing agents, namely 0.6M HCl, 25% NaCl, and 0.2N NaOH, induced apparent gastric damage in rats. Oral administration of the Hr extract at dose of 0.6g/kg 30 min before the necrotizing agents dosing markedly inhibited 0.6M HCl- and 25% NaCl-induced gastric damage. However, the Hr extract did not show any gastric protective effects against 0.2N NaOH-induced gastric damage. The results are given in Table 2.10.

Table 2.10 Effect of Hr extract on necrotizing agents-induced gastric lesions

Treatment	Dose (g/kg) of Hr extract	Lesion Index (mm^2) M ± SEM (n=8)	Percentage of protection (%)
Control: 0.6M HCl	0	58.86±8.42	-
Hr extract	0.6	20.57±7.26*	60
Control: 0.2N NaOH	0	88.29±6.95	-
Hr extract	0.6	72.0±6.34	18
Control: 25% NaCl	0	77.38±9.21	-
Hr extract	0.3	60.43±4.60	22
	0.6	44.29±5.91*	43

Rats were pretreated intragastrically with the Hr extract or vehicle 30 min before the administration of necrotizing agents. The gastric lesions were measured 30 min after necrotizing agent administration. * $p<0.05$ when compared with the individual control. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

2.3.7 *Test of acute toxicity of Hr*

Mice receiving the Hr extract at a dose of 24g/kg did not show any abnormal behaviors during the one-week observation period. This dose is the maximum tolerated dose for the mice which is 40 times higher than the effective dose (0.6 g/kg) used in the present study. After the mice were sacrificed, the internal organs were examined. There was no visible damage found in the tissues indicating that Hr was not found toxic effect at this dose. Body weights of the mice were measured before and after the treatment. There was no significant change in the body weight compared with that of normal rats. The results are showed in Table 2.11. Further study on chronic toxicity in the rat will be conduced.

Table 2.11 Effect of Hr extract on body weight of mouse

Treatment		Body weight (g) Mean ± SEM (n=7)	
		Before treatment	After treatment
Normal	Male	25.6±1.2	32.2±1.3
	Female	20.8±0.7	25.6±1.2
With Hr treatment	Male	26.4±0.3	31.1±1.1
	Female	21.1±0.7	24.3±1.0

ICR mice were employed in this study. The mice received a single oral dose of Hr extract at 24 g/kg. The body weights were measured before and after the Hr treatment.

2.4 Discussion

Results obtained from the present study demonstrated that the hexane-extract of *Hippophae rhamnoides* seeds possessed anti-gastric ulcer effect against the six lesion models tested. Complicated mechanisms for the protective actions seem to be involved as both cytoprotective and antisecretory activities of Hr were observed against ethanol-, NSAIDs-, stress-, pyloric ligation-, acetic acid- and necrotizing agents-induced acute and chronic injury in either preventive or healing effects.

2.4.1 Cytoprotective effect of Hr against ethanol-induced lesions

In the present study, gastric lesions induced by 60% ethanol were found on glandular segment, the place that mainly provides mucosal defensive substances, such as mucus, PGs, GSH, bicarbonate, etc. The results indicate that decrease of the defensive function of gastric mucosa might be responsible for the ulceration. It was demonstrated in a previous study that ethanol-induced vascular damages can occur within 1-3 minutes after ethanol administration (Szabo, 1987). The injury was found in superficial and central capillaries and venules with an increase of vascular permeability and decrease of blood flow (Szabo, 1987). The vascular damage might cause hemorrhages and ischemia, and the tissue ischemia might induce the back-diffusion of H^+ through membrane permeation that may further lead to intramural acidosis and cell death (Peterson, 1995). On the other hand, the release of vasoactive substances, such as PGs, leukotrienes (LTs), platelet-activating factor (PAF) and other endogenous chemical mediators, may regulate and alter the GMBF (Sato et al., 1995).

The pretreatment of Hr extract 30 min before the administration of ethanol significantly protected the mucosa from the injury. This preventive effect of Hr was found in a dose dependent manner ranging from 75 to 600 mg/kg. As ethanol-induced

gastric damage involved a complex pathogenesis, further studies on the mechanism of protective action of Hr were conducted in the present study and this is discussed in Chapter 3.

2.4.2 Preventive effect of Hr on NSAIDs-induced gastric lesions

It is believed that NSAIDs, such as aspirin and indomethacin, induce gastrointestinal mucosal damage by at least two mechanisms, namely, PGs-dependent and PGs-independent mechanisms. As NSAIDs are able to inhibit cyclo-oxygenase activity in the gastrointestinal mucosa, the consequent biochemical and physiological disturbance in mucosa may become pathogenic factors to induce ulceration. In addition, the acidic molecules of NSAIDs may directly impair the integrity of the gastric mucosa, disturb the mucus layer and induce cytotoxic effects on parietal cells and mucosal cell membranes (Rainsford, 1989).

It was found in the present study that pretreatment of the Hr extract 30 min before acidified aspirin administration significantly inhibited the formation of gastric lesions. Interestingly, aspirin could not induce any lesion in the gastric mucosa without presence of HCl. The results suggest that the acidic condition is essential for inducing gastric lesions in this model, and the decrease of PGs levels by inhibition of its biosynthesis is insufficient to produce gastric injury. However the presence of aspirin, no doubt, increased the risk of gastric lesions. This finding is consistent with the previous studies on the aspirin and indomethacin-induced lesion model in which the presence of acid is also required (Konturek et al., 1981; Ueki et al., 1988). Accordingly, to manage NASID-induced gastric damage, the treatment only considering increase of content of PGs in the gastric mucosa may not be adequate and drugs for inhibition of gastric acid have to be employed (Prichard et al., 1988). Taking

this point into account, the protective effect of Hr extract may be related to either stimulation of PG biosynthesis or anti-gastric acid secretion or both effects.

2.4.3 Inhibitory effect of Hr on stress-induced lesions

It is believed that formation of stress-induced gastric lesions is associated with a series of pathological changes in the body including disturbances of gastric mucosal microcirculation (Guth, 1972; Ritchie et al., 1975; Silen et al., 1981;), hypermotility of the stomach (Yanv, 1978), and abnormal gastric acid and pepsin secretion (Kitajima et al., 1989). In addition, as a complex mechanism is involved in controlling release of neuron transmitters in the central nervous system (CNS), stress may affect the normal physiological functions of the CNS and these, in turn, may possibly contribute to the lesion formation (Henke, 1982).

In the present study, pretreatment of the Hr extract at dose ranging from 300 to 600 mg/kg significantly reduced the hemorrhages induced by stress and the extract given at dose of 600 mg/kg resulted in a 62% inhibition on the lesion formation (Table 2.4). Since stress can stimulate the vagus nerve and increase acid secretion (Haga et al., 1984), cimetidine (100 mg/kg, i.g.), a potent anti-acid secretion agent, was selected as a positive control in a parallel experiment for the treatment of the lesions. The compound was found to possess a 67% inhibition on the lesion development (Table 2.5) indicating gastric acid was possibly involved in the lesion formation in this model. As the Hr extract showed a similar effect as cimetidine, it is reasonable to assume that the extract also possesses an antisecretory activity. This assumption has been confirmed in the present study.

Study of β-sitosterol (100 mg/kg, i.g.), a component of Hr extract, on the stress-induced lesion formation indicated that, although the compound inhibited the

formation of gastric lesions by approximate 39%, the extent did not reach a significant level ($p>0.05$). Therefore, contributions of other components in the Hr extract cannot be ruled out.

In short, the inhibitory action of Hr extract on the stress-induced lesion formation may be associated with a decrease of gastric acid secretion, improvement of microcirculation in the mucosa and regulation of the gastric motility. Further studies (e.g. receptor binding assays) should be conducted to collect direct evidences for elucidation of mechanism of actions of Hr extract.

2.4.4 Inhibitory effect of Hr extract on pylorus ligation-induced gastric lesions

Gastric secretion, no doubt, has a close relationship with gastric lesion formation. Hydrochloric acid and pepsin are important aggressive factors in the pathogenesis of peptic ulcer. The present time-course study regarding the induction and development of gastric lesions in the ligation model indicated that the increased pepsin content and acidity of gastric juice were associated with an apparent development of gastric lesions six hours after the pylorus ligation (Table 2.6). The tissue damage was found mainly on the forestomach segment that differed from the lesion-pattern found in the other pathological models employed, as the lesions induced by ethanol, acidified aspirin and stress were mainly found in the glandular segment. These findings suggest that the forestomach and glandular segments are different in structure, therefore they have different defensive ability against the acid erosions. The treatment with Hr extract significantly reduced the lesions formation at doses ranging from 300 to 600 mg/kg (Table 2.8) suggesting an acid inhibition activity of Hr extract.

2.4.5 Healing effect of Hr extract on acetic acid-induced gastric ulcer

Under the present experimental conditions, chronic gastric damage was developed and aggravated within 3-5 days after the application of acetic acid. The spontaneous ulcer recovery occurred starting from day 5 to day 17 post-operation. The daily treatment of Hr extract for 12 days markedly accelerated the ulcer-healing process that accompanied substantial increase of gastric mucus content (Table 2.9). It was demonstrated that the adherent mucus layer not only can protect mechanical damage induced by foreign substances but also can provide an unstirred layer to prevent diffusion of H^+ into deeper layer of mucosa. Such an effect is mainly due to the neutralization of gastric acid (Miller, 1983) and inhibition of pepsin activity by maintaining mucusal pH level higher than pH 3 (Levey et al., 1954).

The findings in this experiment suggest that Hr extract may possess activities on tissue regeneration, acid inhibition and the stimulation of mucus secretion. These activities maybe, at least partly, contribute to the healing effect of Hr and other mechanisms, if any, should be further investigated with some up-to-date experimental techniques.

2.4.6 Protective effect of Hr extract against necrotizing agents-induced gastric damage

A number of necrotizing agents, such as 60% ethanol, 0.6 M HCl, 25% NaCl, and 0.2 M NaOH, can be used to induce gastric lesions and the models established with these agents can be employed to evaluate the cytoprotective effect of drug candidates (Robert, 1979). Since these harmful substances possess different irritation effects on gastric mucosa, the pathogenesis of the tissue damage may vary according to the properties of the irritants. It was observed in the present study that the Hr extract markedly inhibited gastric lesions induced by 60% ethanol (Table 2.2), 0.6 M HCl and 25% NaCl, but nor 0.2M NaOH (Table 2.10). The results indicate that Hr extract possesses a cytoprotective effect against selected harmful substances especially acid related necrotizing agents and agents that directly impair the cell membrane (e.g. ethanol and NaCl). However, as the defensive system in the mucosa may be inefficient against attack from alkaline solution, the enhancement of the defensive function of the stomach by Hr extract at dose of 600 mg/kg may be not adequate against the NaOH-induced damage.

2.4.7 Summary

In the present study, Hr extract produced significant gastric protective effects against the gastric damage models tested. The effects may be associated with promoting the secretion of mucus in the gastric mucosa (e.g. in acetic acid-induced chronic gastric ulcer); inhibiting acid and pepsin secretion (e.g. in pylorus ligation-, stress-, NSAIDs- and acetic acid-induced gastric lesion models); accelerating tissue regeneration (chronic ulcer model); improving mucosal microcirculation (stress- and

NSAIDs-induced gastric lesion models); changing content of prostaglandins in the gastric mucosa; (NSAIDs-induced gastric lesion model) and cytoprotection (ethanol model). The activity of Hr extract seems to be related to both preventing the stomach from attack of aggressive factors and strengthening the mucosal defense system. Therefore, the extract is a promising candidate for development of antiulcer agent. Further studies are required to elucidate the mechanisms of action and identify the active plant ingredients.

Chapter 3

Study on the Cytoprotective Effect of *Hippophae rhamnoides* against Ethanol-Induced Gastric Damage

3.1 Introduction

Cytoprotection is defined as an ability of some agents to protect cellular membranes and inhibit formation of gastric mucosal lesions induced by a variety of necrotizing agents without inhibiting normal gastric acid secretion; and adaptive cytoprotection is believed to be regulated by the endogenous PGs (Robert, 1979; 1985). Cytoprotective phenomenon has been extensively studied in the gastrointestinal tract, liver (Stachura et al., 1981), and pancreas (Robert et al., 1983a).

Previous studies suggest that the mechanisms related to cytoprotection are complex and the main factors involved in gastric cytoprotection are given in Table 3.1 (Moody et al., 1978; Miller et al., 1979; Lichtenberg et al., 1983; Peskar, 1986; Konturek, 1990b).

Table 3.1 The major factors related to gastric cytoprotection

Gastric mucus secretion ↑
Bicarbonate secretion ↑
Mucus secretion ↑
Surface-active phospholipids ↑
Gastric mucosal barrier ↑
Gastric mucosal flood flow ↑
Gastric motility ↓
Scavenging free radicals
Leukotriene production ↓
Release of protective mediates ↑ (e.g.: prostaglandins; non-protein sulfhydryl compounds; epidermal growth factor; NO)
Normalizing vagus function

↑: increased. ↓: decreased.

There are several endogenous substances in the gastrointestinal mucosa that may relate to either cytoprotection or ulceration. For instances, the metabolites of arachidonic acid such as endoperoxides, hydroperoxides, PGs, thromboxanes (TXs), and leukotrienes (LTs) possess different functions in the stomach. Experimental evidences suggest that PGs may show cytoprotective activity (Robert, 1976), whilst TXs and LTs are the mediators in the process of ischemia and tissue damage in the gastric mucosa (Peskar et al., 1986). In addition, cytoprotection may also be induced by certain agents such as glutathione and capsaicin that show different mechanisms from the prostanoids (Konturek et al., 1986).

There are several cytoprotective agents that are clinically available for the treatment of peptic ulcer, such as sucralfate and colloidal bismuth preparations. These drugs exert both healing and preventive effects against acute mucosal lesions induced by ethanol and aspirin. Sucralfate and bismuth can be precipitated and polymerized at gastric surface to form a viscous gel or bind selectively to the gastrointestinal epithelium and to stimulate release of PGs (Konturek et al., 1987). These

gastroprotective effects can be partially reduced by pretreatment with indomethacin, an inhibitor of PGs synthesis. Certain antacids such as $Al(OH)_3$, were suggested to be able to stimulate the synthesis of PGs in gastric mucosa, thus they can be considered as cytoprotective agents (Konturek et al., 1986).

Ethanol-induced gastric mucosal lesions are widely studied and established as a model to evaluate mechanism of cytoprotective agents. The ethanol-induced gastric lesions involve multiple pathogenic factors that are summarized in Table 2.1. The most significant factors related to the pathogenesis of ethanol-induced gastric lesions are the decrease in mucus and bicarbonate secretion, depletion of non-protein sulfhydryl compounds in gastric mucosa, alternation of GMBF and gastric motility, production of free radicals, release of endogenous mediators (e.g. histamine, 5-hydroxytryptamine), induction of ischemia in tissues and injury of vascular vessels (Glavin et al., 1992). Therefore, studies with this model can provide useful information on the evaluation of the cytoprotective effects of drug candidates.

3.2 Materials and Methods

3.2.1 Chemicals and Instruments

The following chemicals were purchased commercially and used in the present study:

Alcian Blue 8GX (Sigma)

Acetic acid (BDH)

Borontrifluoride methanol solution(BF3) (Sigma)

Calcium Chloride (Sigma)

Cupric sulphate (Merck)

Diethyl Ether (BDH)

5,5' Dithiobis-(2-nitrobenzoic acid) (Sigma)

EDTA (Sigma)

Ethanol (Merck)

Folin & Ciccalten phenol reagent (Sigma)

Formalin (BDH)

Glutathione (Sigma)

Haemoglobin (BDH))

Hydrochloric Acid (Merck)

Indomethacin (Sigma)

Linoleic acid (18:2n-6, LA) (Sigma)

Linolenic acid (18:3n-3, LNA) (Sigma)

Arachidonic acid (20:4n-6, AA) (Sigma)

Magnesium chloride (BDH)

Methanol (BDH)

Pepsin (Sigma)

Pentobarbitone sodium (Sigma)

PGE2 immunoassay kit (R&D Systems Production)

Protein standard (Sigma)

Sodium carbonate (Merck)

Sodium chloride (Sigma)

Sodium hydroxide (Merck)

Sodium tartrate (Merck)

Sucrose (Sigma)

Sulphuric acid (BDH)

α-tocopherol (Sigma)

γ-tocopherol (Sigma)

Trichloroacetic acid (Sigma)

The following instruments were employed in the present study:

UV-visible spectrophotometer (Beckman DU 650, USA)

pH meter (Hanna)

Laser Doppler flow meter (PeriFlux PF_3)

Centrifuge (Centurion 6000 Series)

Rotatory evaporator (BUCHI R-114)

Homogenizer (ULTRA-TURRAX T25)

GC (HP 5980 Series II)

GC-MS (Finnigan Mat GCQ)

HPLC (Hewlett Packard series 1050)

Autotitration system (Titration Manager TIM 900)

3.2.2 Test on effects of different concentrations of ethanol on gastric mucosa

Female Sprague-Dawley rats (210-230 g) were used for all the pharmacological tests in this section. Rats were fasted for 48 h before experiments. Five concentrations (20%, 40%, 60%, 80%, 100%, v/v) of ethanol were given to rats intragastrically (i.g.) at dose of 6 ml/kg. The animals were sacrificed 30 min after the treatment of ethanol. The stomach was dissected and opened along the greater curvature. The gastric damage was examined and the severity of gastric lesions was determined as the lesion index (mm^2).

3.2.3 *Examination of the gastric protective effect of Hr extract by different routes of administration*

The Hr extract or vehicle was given to the fasted rat 30 min before administration of ethanol (60%, 6 ml/kg, i.g.) via three independent routes including intragastrical, intraperitoneal and subcutaneous administration. The animals were sacrificed 30 min after the ethanol treatment. The gastric mucosa was examined for gastric ulcer and the severity of gastric lesions was represented as the lesion index (mm^2).

3.2.4 *Study on relationship between gastric protective effect of Hr extract and endogenous PGs*

Indomethacin is an inhibitor of the biosynthesis of endogenous prostaglandins (PGs). This compound was employed in the present study to evaluate relationships between endogenous PGs and the cytoprotective effects of Hr extract. Rats were pretreated subcutaneously with indomethacin at a dose of 5 mg/kg 30 min before administration of the Hr extract (300-600 mg/kg, i.g.) or vehicle. Ethanol was then given (60% v/v, 6ml/kg, i.g.) 60 min after indomethacin administration. The rats were sacrificed 30 min after the treatment of ethanol and the gastric lesions were examined and represented as lesion index.

3.2.5 *Measurement of gastric mucosal blood flow (GMBF)*

The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). To keep the animal warm, heating lamps were used. The abdomen of rat was opened from a midline incision. The proximal esophagus and duodenum were ligated respectively. Care was taken to avoid bleeding or blockage of blood vessels.

The stomach was opened along the greater curvature, then carefully drawn up through a central oval aperture and spread out on the perspex platform (Mersereau et al., 1973). An ex-vivo stomach chamber was set up to secure the stomach to the platform with the mucosa facing upward.

After the chamber was prepared, the stomach was washed with deionized water for three times before the measurement of GMBF. GMBF in the rat was measured with a laser Doppler flow-meter. As the signal obtained from the laser Doppler is represented as electrical units, the data of GMBF are expressed as relative values calculated as a difference between reading at a specified time and reading of basal GMBF.

After a 30-min stabilizing period, the first GMBF data was recorded and taken as the basal value. The GMBF was measured every 15 min for 75 min. Either deionized water (1.5ml, in control group) or 60% ethanol (1.5ml) was added into the stomach for incubation. The incubation solution was collected after the GMBF reading had been taken, and the fresh solution was filled again for next incubation. The collected chamber incubation solutions was used for analysis of gastric acidity and volume of the gastric secretion.

In order to investigate effect of the Hr extract on GMBF in rats with the treatment of ethanol, the rats were pretreated with Hr extract (0.075-0.15 g/kg, i.p.) 30

min before measurement of the basal GMBF. The rest of the experimental procedure was the same as those described above.

3.2.6 Measurement of gastric secretion and acidity in gastric juice

The total volume of gastric juice collected from the stomach was determined using a measuring cylinder with a scale of 0.10 ml.

For examining the acidity of the gastric juice, 0.2 ml of collected solution was diluted 10 times with deionized water (pH 7.4). Acidity of each test sample was determined with an autotitration system and NaOH (0.01N) was used as the titration agent. The end-point of titration was set at pH 7.4 (Misher et al., 1969) and acidity was represented as mmol/l of H^+.

3.2.7 Measurement of gastric emptying rate

Gastric emptying rate of the rat was determined by measuring the elimination rate of resin pellets (Rohm and Haas Amberlite ion exchange resin IRC-50, 1 mm in diameter) from the stomach into the small intestine during a 30-min period (Brodie, 1966b). To investigate the effect of Hr extract, rats were pretreated with either the extract or vehicle (1 ml of 1% Tween-80) intragastrically and twenty of resin pellets were administrated with vehicle or ethanol (60%, 6 ml/kg, i.g.) 30 min later. The rats were sacrificed 30 min after the resins were given. Before removal of the stomach, the end of esophagus and pylorus were occluded with homeostatic forceps to prevent any loss of the pellets. The number of pellets remaining in the stomach was counted and the rate of gastric emptying was calculated with the following formula.

Gastric emptying rate (%) = [(20- number of resin remained) / 20] × 100%

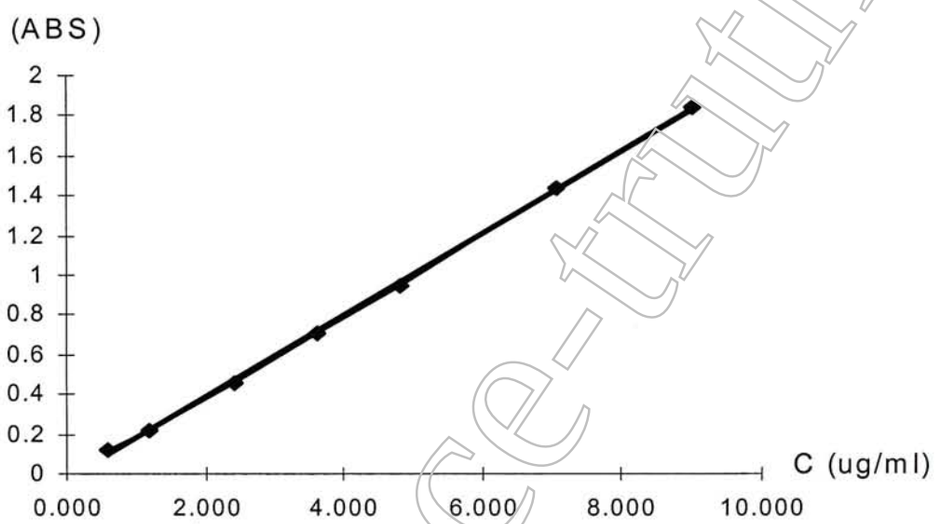
The result was expressed as the percentage of pellets expelled. The gastric lesions induced by ethanol were examined at the same time and expressed as lesion index.

3.2.8 Measurement of pepsin content in the gastric juice

The pepsin content in gastric lumen was determined according to the method described by Berstad (Berstad, 1975) with modification. Briefly, the bovine haemoglobin (2.5% w/v, 2ml) was used as substrate and mixed with HCl (0.3M, 0.5ml) and 0.1 ml gastric juice collected. The solution was incubated at 25°C for 10 min, trichloroacetic acid (0.3M, 5ml) was then added immediately to terminate the reaction. The precipitates formed were filtered, and optical density of the resultant solution was measured with an UV-spectrophotometer at 280 nm.

A series of standard solutions of pepsin were prepared freshly with different concentration (0.6, 1.2, 2.4, 3.6, 4.8, 7.1, 9.0 μg/ml). The UV absorbance of each solution was determined and linearity of the calibration curve was found over 0-10 μg/ml. The pepsin content in the collected gastric juice was then measured with the UV-spectrophotometer and assessed from the standard curve (Figure 3.1). The content of pepsin was expressed as μg /ml gastric juice.

Figure 3.1 The calibration curve for measuring pepsin content in the gastric juice



ABS represents the absorbance of test samples measured at 280nm with an UV-spectrophotometer. Concentrations of pepsin in the collected samples were calculated according to the following formula:

Concentration of pepsin (μg /ml) = 4.8569×(ABS reading) + 0.1323

Linearity of the calibration curve was reflected by the correlation coefficient (R^2=0.9994). Adequacy of the assay was supported by the 1.59 % coefficient of variation for the variability of interday assay.

3.2.9 Measurement of protein content in the gastric juice

The protein content in gastric juice was determined according to the method reported by Lowry (Lowry et al., 1951). Briefly, a series of stock solutions were prepared as follows:

Reagent A: dissolving 10 g of sodium carbonate in 100 ml of sodium hydroxide (0.5N)

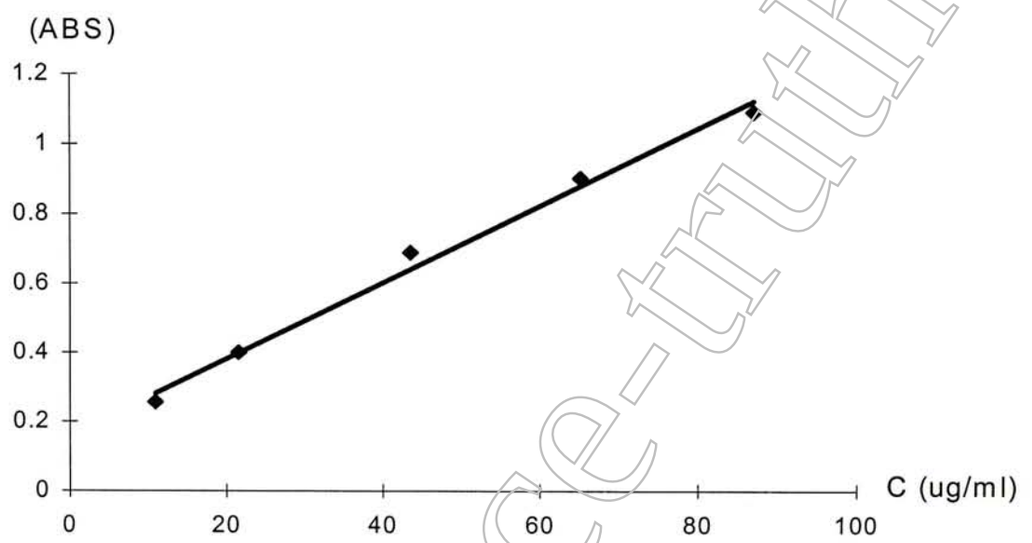
Reagent B: dissolving 1 g of cupric sulphate in 100 ml deionized water

Reagent C: dissolving 2 g of sodium tartrate in 100 ml deionlized water

Before determination, a solution was freshly prepared by mixing 100 ml of reagent A with 1 ml of reagent B and 1 ml of reagent C. Gastric juice (0.1 ml) was added into 5 ml of the mixed solution and incubated for 15 min at room temperature. The freshly prepared Folin and Ciccalten reagent (0.5 ml, 2N) was then added to the incubation solution and mixed well. This solution was incubated for 45 min at room temperature and the absorbance of this solution was measured at 750 nm with an UV spectrophotometer. The protein content was calculated from a standard curve and expressed as μg /ml gastric juice.

For preparation of standard curve for measurement of protein content, a series of standard solution of protein were prepared freshly with different concentrations (10-90 μg/ml). The absorbance of each solution was determined at 750nm (Figure 3.2).

Figure 3.2 The calibration curve for measuring protein content in the gastric juice



ABS represents the absorbance of test samples measured 750nm with an UV-visible spectrophotometer. Concentrations of protein in the collected samples were calculated according to the following formula:

$$\text{Protein content } (\mu g/ml) = 86.71 \times (\text{ABS reading}) - 10.197$$

Linearity of the calibration curve was reflected by the correlation coefficient (R^2=0.9921). Adequacy of the assay was supported by the 2.15 % coefficient of variation for the variability of interday assay.

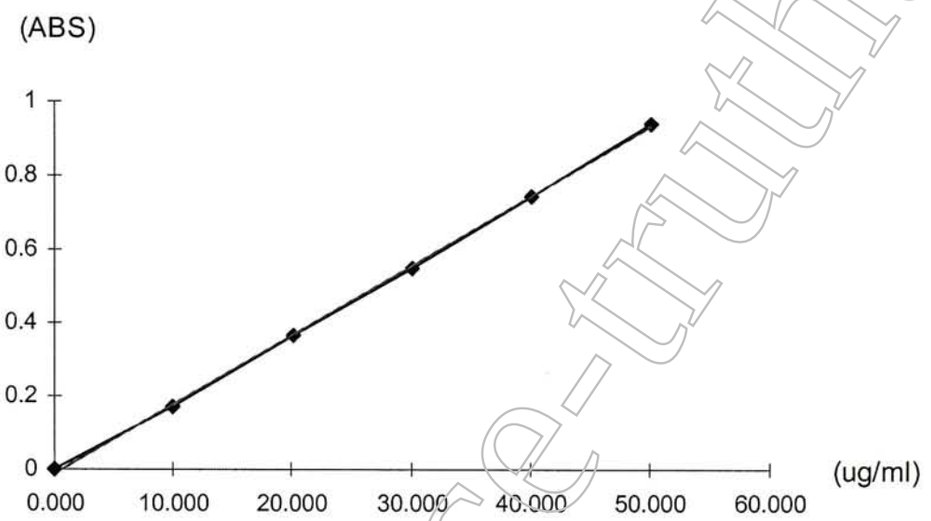
3.2.10 Measurement of mucus content on the gastric wall

The mucus content in the gastric wall was measured according to the method established by Corne (Corne et al., 1974) with sight modification. The Alcian buffer solution was prepared by dissolving 0.1% Alcian blue 8GX in 0.16 M sucrose buffered with 0.05 M sodium acetate and then adjusted to pH 5.8 with 1 N HCl. After removal of rat stomach, the glandular segment was weighed and soaked immediately in 10 ml of the Alcian blue solution for 2h. Uncomplexed dye was removed by two successive incubations with 10 ml of 0.25 M sucrose each time. The time for the incubations was 15 min and 45 min, respectively. Dye complexed with mucus was extracted with 10 ml of 0.5 M magnesium chloride, then shaken vigorously for a total of 2 hrs at 15-min intervals. The blue extract (5 ml) was taken and mixed with an equal volume of diethyl ether. The resultant emulsion was centrifuged at 3000g for 10 min. The aqueous layer was collected and its absorbance was measured at 580 nm. The quantity of Alcian blue extracted was estimated according to the standard curve. The content of mucus was expressed as μg (Alcian blue)/g (wet glandular tissue).

Standard solutions of Alcian blue were prepared in different concentrations (10, 20, 30, 40, 50 μg/ml) to establish a calibration curve (Figure 3.3).

To investigate the effect of Hr extract, rats were pretreated with either the extract or vehicle (1 ml of 1% Tween-80) intragastrically, ethanol (60%, 6 ml/kg, i.g.) was then given 30 min later. The rats were sacrificed 30 min after the ethanol administration and the stomach was removed for examination of mucus. In this study, carbenoxolone (100 mg/kg, i.g.) was employed as the positive control compound.

Figure 3.3 The calibration curve for measuring mucus content in the gastric mucosa



ABS represents the absorbance of test samples measured at 580nm with an UV-visible spectrophotometer. Concentrations of Alcian blue in the collected samples were calculated according to the following formula:

Concentration of Alcian blue (μg /ml) = 53.204 × (ABS reading) + 0.5597

Linearity of the calibration curve was reflected by the correlation coefficient (R^2=0.9995). Adequacy of the assay was supported by the 1.72 % coefficient of variation for the variability of interday assay.

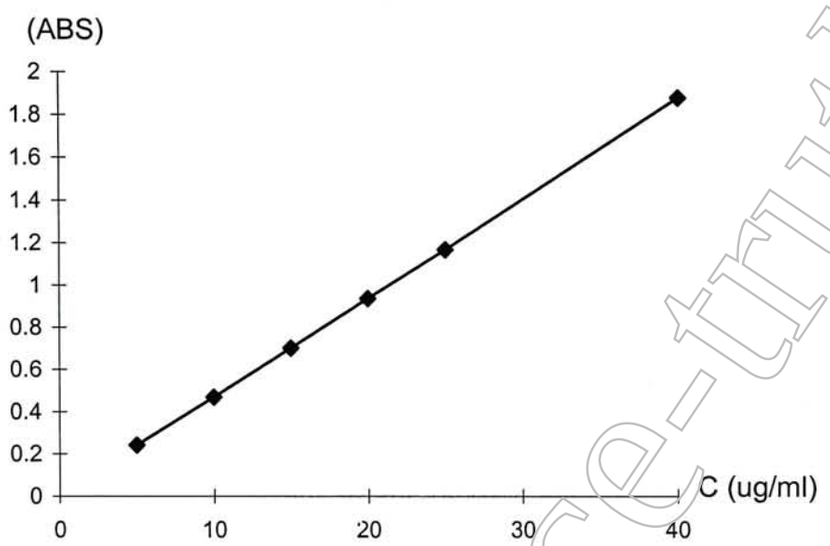
3.2.11 Measurement of GSH content in the gastric mucosa

The reduced glutathione (GSH) present in the gastric mucosa was measured using the method reported by Sedlak (Sedlak et al., 1968) with slight modification. DTNB (5,5'-dithiobis-2-nitro-benzoic acid, 0.01M) was prepared by dissolving 99 mg of DTNB in 25 ml of absolute methanol. Tris-buffer (0.4 M, pH 8.9) was prepared by dissolving 48.4 g of Tris-base in 100 ml of deionized water buffered with 0.2 M ethylenediaminetetraacetic acid-sodium (EDTA-Na_2). The final volume was made up to 1000 ml with deionized water and the pH was adjusted to 8.9 by the addition of HCl (1 N). Stock solution of glutathione (GSH, 10^{-3} M) was dissolved in 0.02 M EDTA-Na_2.

The glandular part of the rat stomach was weighed and homogenized with 0.02 M EDTA-Na_2 at 0°C. The homogenate was mixed with 5 ml of 0.02 M EDTA-Na_2, 4.0 ml of deionized water and 1.0 ml of 50% trichloroacetic acid in a test tube. The solution was vortexed for 10-15 min and then centrifuged at approximately 3000g for 15 min. The supernatant (2ml) was collected and mixed with 4.0 ml of 0.4 M Tris-buffer (pH 8.9) and 0.1 ml of DTNB solution. The absorbance of the sample was measured at 412 nm with an UV-spectrophotometer within 5 min after the addition of DTNB. Solution without homogenate was used as a blank.

Standard solutions of GSH were prepared in different concentrations (5, 10, 15, 20, 25, 40 µg /ml) and were used for establishment of the standard curve (Figure 3.4).

Figure 3.4 The calibration curve for measurement of GSH content in the gastric mucosa



ABS represents the absorbance of test samples measured at 412 nm with an UV-visible spectrophotometer. Concentration of GSH in the collected sample was calculated according to the following formula:

$$\text{Content of GSH } (\mu g/ml) = 21.387 \times (\text{ABS reading}) - 0.0512$$

Linearity of the calibration curve was reflected by the excellent correlation coefficient ($R^2 = 1$). Adequacy of the assay was supported by the 1.0 % coefficient of variation for the variability of interday assay.

3.2.12 Measurement of PGE_2 content in gastric mucosa

The measurement of prostaglandin E_2 (PGE_2) is based on the competitive binding between the PGE_2 presented in tissues and a fixed amount of alkaline phosphate-labeled PGE_2 for the binding sites on mouse monoclonal antibody. During an incubation period, the mouse monoclonal antibodies bound to the gold anti-mouse antibody coated onto a microplate. After a wash, the excess conjugate and unbound sample were removed. A substrate solution was then added to the wells to determine the activity of bound enzyme by a color reaction. The reaction was terminated by adding a stop-reagent. The absorbance of samples was recorded with an UV-spectrometer at 405 nm. As the intensity of color generated was inversely proportional to the concentration of PGE_2 in the test sample. The content of PGE_2 was estimated according to a calibration curve.

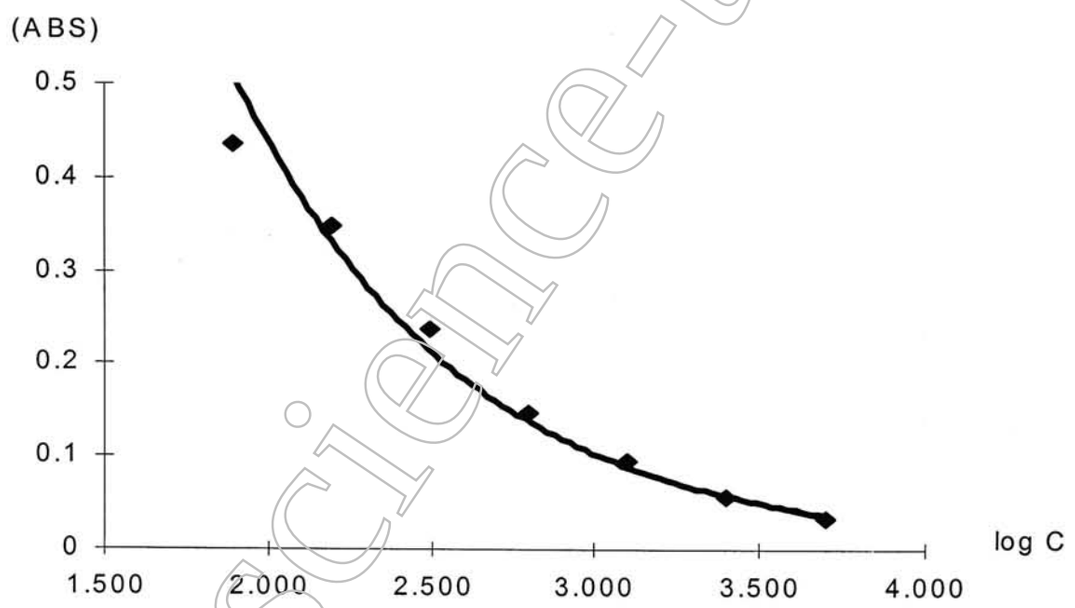
In order to measure the content of PGE_2 in the gastric mucosa, the stomach was removed after the rat was sacrificed. The stomach was placed on an ice-cold glass plate, then the glandular mucosa was carefully scraped off and frozen immediately with liquid nitrogen. The mucosa samples were stored at -80°C until assayed.

To determine the content of PGE_2 in the gastric mucosa, the sample was weighed and added to a buffer solution containing 100 mM NaCl, 1mM $CaCl_2$, 1mg/ml D-glucose and 28 mM indomethacin. The indomethacin was used for inhibition of biosynthesis process of PGs during the testing period. The sample was homogenized for 20 sec in an ice-bath, then was centrifuged at 10,000 r.p.m. for 20 min at 4 °C. The supernatant was collected and 10-time diluted with a buffer solution used for measuring protein content in the mucosa (Read et al., 1981). The content of PGE_2 was analyzed with an immunoassay kit (PGE_2 Immunoassay kit) and was

calculated according to a standard curve of PGE_2. The result was expressed as PGE_2 pg/mg protein of gastric mucosa.

Standard solutions of PGE_2 were freshly prepared in different concentrations (78, 156, 313, 625, 1250, 2500, 5000 pg/ml) to establish the calibration curve (Figure 3.5).

Figure 3.5 The calculation curve for measuring PGE_2 content in the gastric mucosa



ABS represents the absorbance of each test solution recorded at 405 nm. A non-linear calibration curve was obtained. The logarithm concentrations of PGE_2 in the collected samples were calculated according to the following formula:

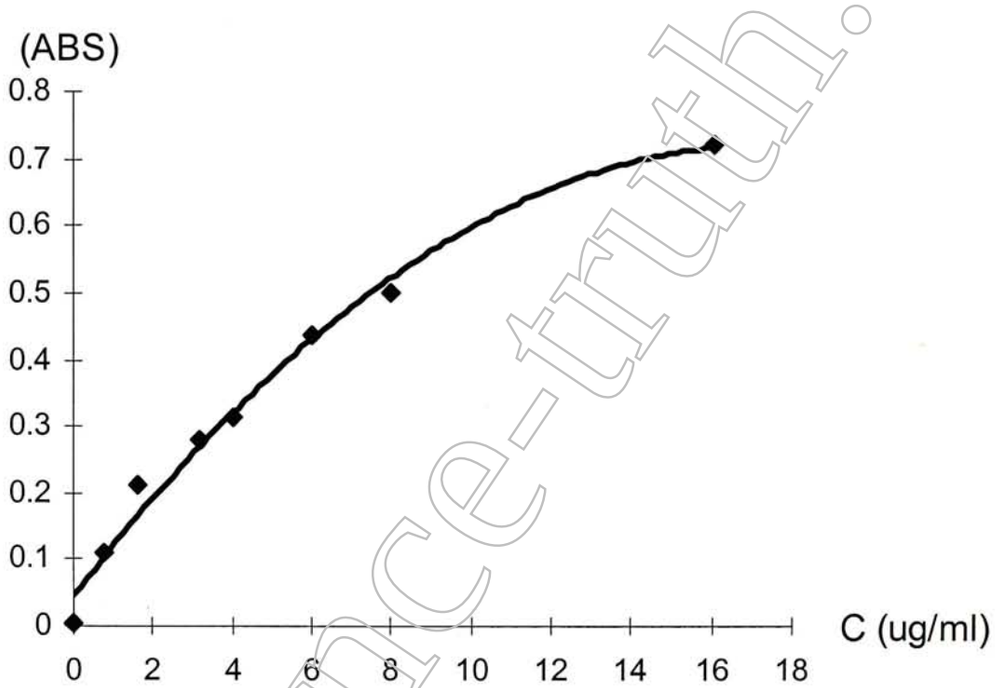
$$\log C\ (pg/ml) = -0.2879 \log (ABS\ reading) + 1.4437 \qquad R^2 = 0.9891$$

3.2.13 Determination of protein content in gastric mucosa

The protein content of gastric mucosa was determined according to the method reported by Read (Read et al., 1981). A dye solution was prepared by adding 0.01% Serva blue G (Serva, Germany) into 1.6 M H_3PO_4/0.8M ethanol. The stock solution of dye (30ml) was mixed with 80 ml of H_3PO_4 (16 M) and 37.5 ml of absolute ethanol, then diluted up to 1000ml with deionized water. The solution was filtered and stored for later use. For testing protein concentration, 20 µl of test sample was diluted to 1 ml with saline, and mixed with 1.5 ml of dye solution. After 4-min incubation, the absorbance of the solution was measured with a spectrophotometer at 595 nm.

The standard curve of protein was measured in a concentration range of 0-25 µg protein /ml and was used for calculation of the protein concentration of mucosa.

Figure 3.6 Calculation curve for measuring protein content in the gastric mucosa



ABS represents the absorbance of test samples measured at 595nm. Concentrations of protein in the collected samples were calculated according to the following formula:

Protein concentration ($\mu g/ml$) = $36.44 \times (ABS)^2 + 2.96 \times (ABS) + 0.07$ $R^2 = 0.9973$

3.3 Results

3.3.1 Test on gastric lesions induced by different concentrations of ethanol

Gastric damage induced by ethanol at different concentrations was evaluated in rats. The results are given in Table 3.2. According to this observation, 60% ethanol was selected to induce gastric lesions in the related experiments.

Table 3.2 Gastric damage induced with ethanol at different concentrations

Ethanol concentration (v/v, %)	Number of rats	Lesion index (mm^2) Mean ± SEM
20	11	2.7 ±1.4
40	12	41.1± 5.3
60	11	63.6 ±9.6
80	11	108.8±18.4
100	5	218.0±13.9

Ethanol (6 ml/kg, different concentrations as above) was given to rats intragastrically 30 min before the rats were sacrificed.

3.3.2 Effect of Hr extract on ethanol-induced gastric damage by different routes of administration

The protective effect of Hr extract was compared with different routes of administration including intragastric, intraperitoneal and subcutaneous administration. The results are shown in Table 3.3.

Table 3.3 Comparison of protective effect of Hr extract against ethanol-induced gastric injury with different routes of administration

Treatment	Dose of Hr (g/kg)	Administration route	Lesion index (mm^2) Mean ± SEM (n=12)	Percentage of protection (%)
Control	-	i.g.	65.25±6.35	
Hr extract	0.075	i.g.	51.17±5.89	23
	0.15	i.g.	46.83±5.34	30
	0.3	i.g.	37.58±5.34*	42
	0.6	i.g.	31.75±7.38**	55
Control	-	i.p.	84.09±5.96	
Hr extract	0.037	i.p.	57.10±9.43	32
	0.075	i.p.	52.33±9.25*	38
	0.15	i.p.	49.80±10.89*	41
	0.3	i.p.	49.00±7.61*	42
Control	-	s.c.	98.2±17.4	
Hr extract	0.15	s.c.	85.2±10.2	13

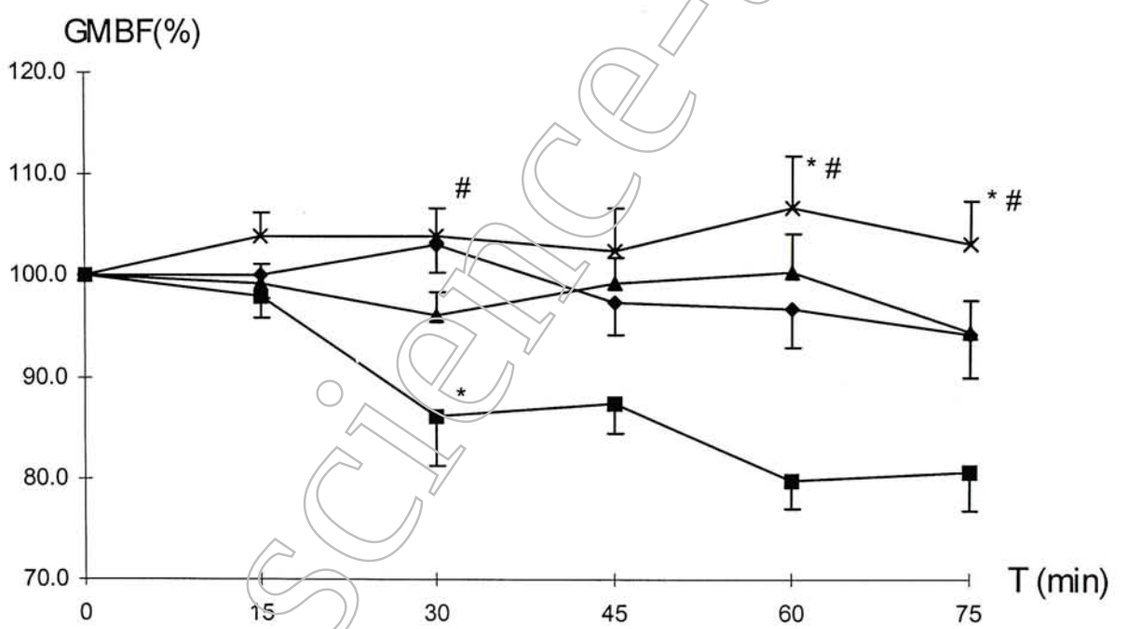
Pretreatment with the Hr extract via different routes of administration 30 min before administration of ethanol (60%, 6ml/kg, i.g.). The gastric lesions in rats were measured 30 min after treatment of ethanol. *$p<0.05$, **$p<0.01$, compared with the related controls. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

The results showed that pretreatment with Hr extract to rats via intragastric or intraperitoneal administration 30 min before ethanol (60%) administration significantly reduced the formation of gastric lesions in a dose-dependent manner. However, with subcutaneous administration, Hr extract (0.15g/kg) did not show such gastric protective effect.

3.3.3 Effect of Hr extract on GMBF and output of gastric acid

The alterations of gastric blood flow in the presence of ethanol with or without the Hr treatment are demonstrated in Figure 3.7.

Figure 3.7 Effect of the Hr Extract on Gastric Mucosal Blood Flow

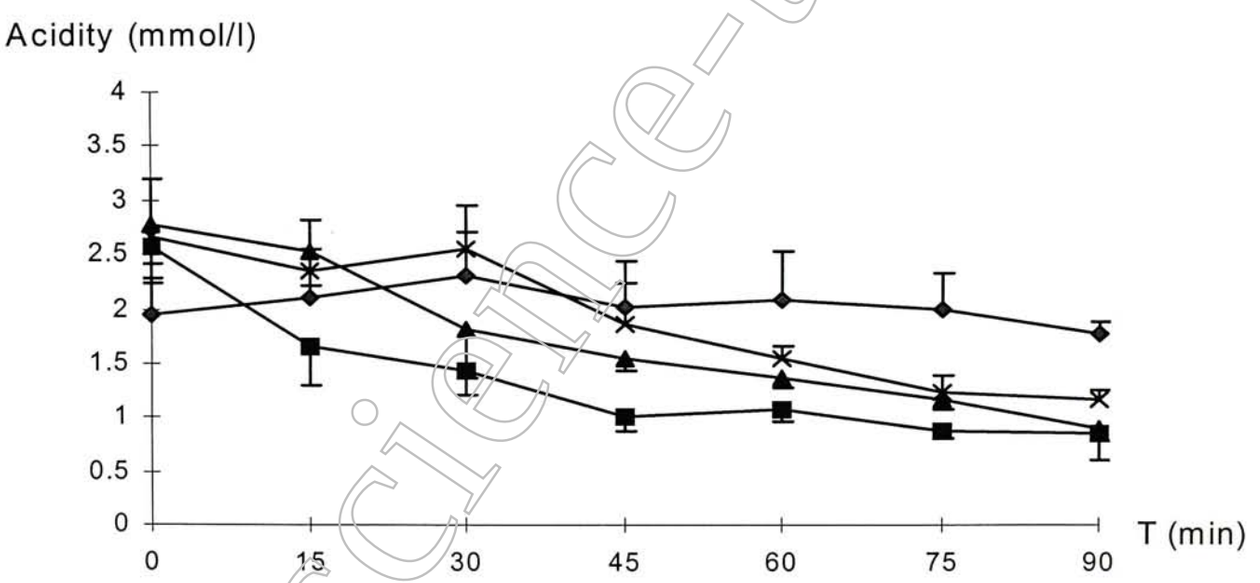


The GMBF in rats was measured with a laser doppler meter. Seven rats were tested in each group. The rats were pretreated with Hr extract (0.075-0.15 g/kg, i.p.) or vehicle 30 min before the measurement of basal GMBF, 60% ethanol (1.5ml) was then added to an ex-vivo chamber for incubation. Rats without Hr and ethanol treatments were used as the control. ♦: control; ▮: with ethanol treatment alone; ▲: with both ethanol and the Hr (0.075 g/kg) treatments; ✕: with both ethanol and the Hr (0.15 g/kg) treatments.
* $p<0.05$ compared with the control (♦), # $p<0.05$ compared to the group (▮) with ethanol but without the Hr treatment. The Tukey's test followed analysis of variance was used for the statistical test.

It is shown in Figure 3.7 that administration of Hr extract significantly inhibited the ethanol-induced decrease in GMBF 45 min after the basal GMBF taking. Moreover, the Hr extract at dose of 0.15 g/kg (i.p.) increased the GMBF significantly at the time from 60 to 75 min after the basal GMBF taking compared to the control rats and the rats only with ethanol treatment.

The acidity of the gastric juice of rat was monitored at the same time as the GMBF measurement. The results are presented in Figure 3.8. It was found that the Hr treatment did not affect gastric acid secretion in ethanol treated rats significantly.

Figure 3.8 Effect of the Hr extract on the acidity of gastric juice



The acidity of the gastric juice was measured with an autotitration system. Seven rats were tested in each group. The rats were pretreated with Hr extract (0.075-0.15 g/kg, i.p.) or vehicle 30 min before the measurement of basal GMBF, 60% ethanol (1.5ml) was then added to an ex-vivo chamber for incubation. The incubation solution was collected after the GMBF was measured at each time interval. Rats without Hr and ethanol treatments were used as the control. ♦: control; ■: with ethanol treatment alone; ▲: with both ethanol and the Hr (0.075 g/kg) treatments; ✕: with both ethanol and the Hr (0.15 g/kg) treatments. The Tukey's test followed analysis of variance was used for the statistical test.

3.3.4 Effect of Hr extract on gastric emptying rate

It was observed in the present study that administration of 60% ethanol into the rat stomach induced severe damage on the gastric mucosa. Such damage was associated with a significant decrease ($p<0.001$) in gastric emptying rate (Table 3.4). The results suggest that reduction of gastric motility may be involved in the pathogenesis of ethanol-induced gastric lesions. Administration of Hr extract prevented the decrease of gastric emptying rate in a dose dependent manner. In addition, the Hr treatment did not affect the gastric emptying rate of resin pellets in the normal rats (Table 3.4).

Table 3.4 Effect of Hr extract on gastric emptying rate in rats

Treatment	Dose of Hr extract (g/kg)	Gastric emptying rate (%) M ± SEM (n=12)	Lesion index (mm^2)
Control (without ethanol)	-	57.47±3.17	0
Hr extract (without ethanol)	0.3	49.50±5.60	0
	0.6	52.50±4.24	0
Control (with ethanol)	-	32.08±4.24$^{###}$	65.3±6.4
Hr extract (with ethanol)	0.075	38.75±5.37	51.2±5.9
	0.15	43.75±4.7	46.8±5.3
	0.3	44.58±2.08*	37.6±5.3*
	0.6	49.58±3.17*	31.8±7.4**

Hr extract was administered 30 min before the resin pellets given with or without 60% ethanol. Rats were sacrificed 30 min after ethanol administration and the pellets remained in the stomach was counted to calculate the rate of gastric empty.
* $p<0.05$, compared with control group (with ethanol); $^{###}$ $p<0.001$ compared to the control group without the ethanol treatment. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

3.3.5 Effect of Hr extract on gastric mucus

It has been shown in this study (Table 3.5) that pretreatment with Hr extract at doses ranging from 300 to 600 mg/kg (i.g.) significantly enhanced the secretion of gastric mucus compared with the control. The effect was found to be similar to that of carbenoxolone (100 mg/kg, i.g), the positive control compound employed.

Table 3.5 Effects of Hr extract on gastric mucus secretion

Treatment	Dose (g/kg)	Mucus (μg/g tissue) M±SEM (n=10)
Control	-	40.52 ± 2.49
Hr extract	0.3	66.92± 11.74*
	0.6	74.20±14.22*
Carbenoxolone	0.1	63.68±4.05**

Pretreatment rats with Hr extract or vehicle 30 min before administration of ethanol (60%, 6ml/kg, i.g.). Content of mucus in gastric wall was measured 30 min after treatment of ethanol. Carbenoxolone was used as positive control.
*$p<0.05$, **$p<0.01$ when compared with the control group. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

3.3.6 Effect of Hr extract on gastric GSH content

The present study showed that intragastrical administration of 60% ethanol induced severe gastric damage that was associated with a significant decrease of GSH level in the gastric mucosa ($p<0.001$) compared with that of normal rats. The results are given in Table 3.6. The findings suggest that depletion of GSH contents may be involved in the pathogenesis of gastric lesions induced by ethanol. Pretreatment with Hr extract (0.3-0.6 g/kg) significantly reduced the depletion of GSH in the gastric mucosa and showed gastric protective activity.

Table 3.6 Effect of Hr extract on gastric mucosal GSH level in the rats administered with ethanol

Treatment	Content of GSH in the gastric mucosa (μmol / g wet tissue) M±SEM (n=8)
Control (with ethanol)	1.258 ± 0.052
Hr extract 0.3g/kg	1.538 ± 0.040*
Hr extract 0.6g/kg	1.514 ± 0.038*
Normal rats (without ethanol)	1.914 ± 0.108***

Pretreatment with Hr extract solutions (i.g.) 30 min before administration of ethanol (60%, 6ml/kg, i.g.). The rats were sacrificed 30 min after the ethanol treatment and the gastric glandular segment was collected for measuring GSH content.
*: $p<0.05$, ***: $p<0.001$ when compared with the control rats with ethanol treatment. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

3.3.7 Influence of Hr extract on endogenous prostanglandin-E_2

The effects of the Hr extract and linolenic acid, a component of Hr extract, on the content of PGE_2 in the gastric mucosa were investigated (Table 3.7). Results indicated that PGE_2 level in the mucosa of normal rats was not changed markedly after the rats were fasted for 48h. Administration of ethanol (60%, 6ml/kg) significantly increased the gastric mucosal PGE_2 levels ($p<0.01$) compared with the normal group. However, pretreatment with Hr extract and linolenic acid did not significantly reduce the increased PGs induced by ethanol treatment.

Table 3.7 Influence of Hr on content of endogenous PGE_2 in the gastric mucosa

Treatment	Content of PGE_2 in gastric mucosa (pg /mg protein) M±SEM (n=8)
Control (with ethanol)	494.8± 84.1
Hr extract 600 mg/kg (with ethanol)	325.2± 49.0
Linolenic acid 240 mg/kg (with ethanol)	320.2± 33.7
Normal rats (without ethanol, not fasted)	175.6± 27.6**
Normal rats (without ethanol, fasted for 48 h)	169.5± 49.4**

Pretreatment with Hr extract (i.g.) 30 min before administration of ethanol (60%, 6ml/kg, i.g.). The rats were sacrificed 30 min after treatment of ethanol. The gastric glandular mucosa was scraped off and the content of PGE_2 was measured with a immunoassay kit. The protein content in the gastric mucosa was measured using a dye-complex method. **: $p<0.01$, compared with the control rats with ethanol treatment. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

3.3.8 Antagonistic effect of indomethacin on the gastric protection of Hr extract

Subcutaneously administered indomethacin at a dose of 5 mg/kg did not induce any visible damage in the gastric mucosa of rat. However, pretreatment with indomethacin reduced the protective effect of Hr extract against the ethanol-induced lesions (Table 3.8).

Table 3.8 Effect of indomethacin on gastric protective effect of Hr extract

Treatment	Lesion index (mm^2) M±SEM (n=10) With indomethacin	Percentage of protection (%)	Lesion index (mm^2) M±SEM(n=10) Without Indomethacin	Percentage of protection (%)
Control	85.60±6.38	-	84.09±5.96	-
Hr extract (0.3g/kg)	67.88± 7.02	25	58.57± 4.49*	36
Hr extract (0.6g/kg)	55.50± 8.24*	35	33.31± 4.80***	61

Pretreatment with indomethacin (5mg/kg, s.c.) 30 min before administration of Hr extract or vehicle (4ml/kg, i.g.). Ethanol was then given (60% v/v, 6ml/kg, i.g.) to rats 60 min after the indomethacin administration. The rats were sacrificed 30 min after the treatment of ethanol to measure gastric damage induced. ***: $p<0.001$, *: $p<0.05$ when compared with the control group. The Tukey's test followed one-way analysis of variance (ANOVA) was used for the statistical test.

3.4 Discussion`

3.4.1 Formation of gastric lesions induced by ethanol at different concentrations

The damaging effects of ethanol at different concentrations on the gastric mucosa were examined in this study (Table 3.2). Results showed that 20% ethanol failed to induce gastric lesions, but the color of mucosa was slightly changed to red. Ethanol at concentrations of 40-60% induced visible mucosal lesions along the long axis of the stomach and 80-100 % ethanol induced severe linear hemorrhagic lesions. As a previous time-course study demonstrated that ethanol (concentration > 40%) could induce gastric lesions within 1-3 min and the lesion could be further developed in a short time (Szabo, 1987), a 30-min interval for the induction of gastric lesions was applied in the present study. After several tests in the present study, it was found that the 60% ethanol-induced gastric lesions showed a high reproducibility, thus this concentration was selected in all the experiments related to the ethanol-induced gastric lesions in this study.

3.4.2 Different routes of administration

In the present study, intragastrical (0.3-0.6 g/kg) and intraperitoneal (0.075-0.3 g/kg) administrations of the Hr extract significantly reduced gastric damage induced by 60% ethanol (Table 3.3). The results suggest that the Hr extract may have both local (via i.g. route) and systemic (via i.p. route) effect on the stomach protection. However, subcutaneous administration of the Hr extract at concentration of 0.15 g/kg failed to inhibit the formation of gastric lesions (Table 3.3).

The difference between the Hr effects produced via intraperitoneal and subcutaneous routes of administration under the same dosing regime (0.15 g/kg)

suggest that there is a difference between the absorption or disposition of the Hr components when the compounds are given via these two routes. As the lipophilic property of the Hr components, they should be rapidly absorbed and widely distributed into subcutaneous tissues but slowly released into systemic circulation. Therefore the difference between the composition of the peritoneum and subcutaneous tissues may be the reason to cause such difference in the Hr activity when the extract is given with these two routes. To further confirm this assumption, the dose and pretreament time of Hr extract should be increased when it is administered via the subcutaneous route.

3.4.3 Roles of GMBF, gastric acidity and acid output in the formation of gastric lesions

It is believed that the gastric mucusal blood flow plays an important role in maintenance mucosa integrity (Miller, 1983). Sufficient blood flow can provide adequate supply of oxygen and nutrients to mucosa, and can help in the removal of harmful substance or maintenance of a physiological pH in the gastric mucosa. As previous studies have suggested that increase of GMBF prevented the formation of gastric lesions (Moody et al., 1978; Jacobson, 1985; Shorrock et al., 1988); accordingly, any decrease in GMBF may cause ischaemia in the tissue that may lead to gastric mucosal lesions (Kamada et al., 1983). In addition, when gastric tissues are damaged, sufficient gastric blood flow can assist in the maintenance of normal cellular functions and accelerate ulcer healing process (Jacobson, 1985). Thus, to investigate the effects of Hr extract on GMBF may provide useful information regarding the mechanism(s) of antiulcer action.

In the present study, a significant decrease in GMBF was observed 15 min after the application of ethanol onto the surface of the gastric mucosa in the ex-vivo chamber (Figure 3.7) which was associated with severe gastric mucosal lesions. As ethanol can induce direct damage mucosal tissues (Liu et al., 1995), the release of vasoactive substances (e.g. leukotrienes and platelet-activating factor), alternation of gastric mucosal microcirculation, increase of vascular permeability; induction of blood stasis and tissue ischaemia may occur (Szabo, 1987). The tissue ischaemia can cause the back-diffusion of H^+ ions through membrane permeation and further induce intramural acidosis and cell death (Peterson, 1995). In addition, the release of vasoactive substances such as prostaglandins, leukotrienes, PAF and other endogenous chemical mediators may regulate and alter the GMBF (Sato et al., 1995).

Pretreatment with Hr extract (0.15 g/kg) intraperitoneally 30 min before ethanol application successfully kept the GMBF at the basal physiological level (Figures 3.7) and the gastric acidity did not alter significantly (Figure 3.8). These findings indicate that the Hr extract may exert its protective effect by maintaining sufficient gastric blood flow as well as mucosal integrity. The evidence collected from the present study suggests that in ethanol-induced gastric injury, gastric acid can only be considered as an aggravating-factor in the lesion formation. Acid can induce cell damage simply when the integrity of gastric mucosa has been impaired by ethanol. This assumption can be supported with a test using cimetidine as pretreatment agent against ethanol-induced lesions. Cimetidine, an inhibitor of gastric acid secretion, successfully reduced stress and pylorus ligation-induced mucosal damage (Tables 2.4 and 2.7), but failed to prevent the lesions formation induced by ethanol (Table 4.11) suggesting gastric acid is not the principal pathogenic factor in the ethanol model. These findings agree with the previous report (Kuwayama et al., 1987) and further

demonstrated that the protective effect of Hr on the ethanol-induced lesions more focuses on maintenance of mucosal integrity rather than inhibition of gastric secretion. Therefore, Hr can be considered as a cytoprotective agent.

3.4.4 Effects of Hr extract on gastric motility

Normal gastric motility is important to maintain physiological function of the stomach. As irritants in the stomach may accelerate or reduce gastric motility and cause digestive system disorders, measurement of gastric emptying rate is frequently employed in the evaluation of stomach functions. In the present study, the role of Hr extract on gastric emptying rate was examined in both normal rats and the rats treated with ethanol.

The results showed that ethanol delayed gastric emptying rate significantly and this was accompanied with severe gastric lesions. At gastric protective doses (0.3-0.6g/kg), Hr extract did not affect the gastric emptying rate in normal rats, but it markedly prevented the ethanol-induced reduction in gastric emptying rate in a dose-related manner (Table 3.4). The findings suggest that changes in gastric emptying rate may correlate with the generation of gastric lesions induced by ethanol. As the reduction of gastric emptying capacity induced by ethanol was associated with the irritation of alcohol in the stomach, the contribution of Hr extract in the normalizing gastric motility may be of benefit in the protection of the stomach.

It is interesting to notice that Hr extract possesses a regulatory effect on gastric motility, i.e., maintaining motility in normal rats, but modifying pathogenic changes in the gastric emptying capacity. Such an effect may be generated by the multiple components of Hr extract and the regulative action of Hr on gastric motility may involve complex mechanism(s) of action.

3.4.5 Effect of Hr extract on gastric mucus

It is believed that the mucus and the bicarbonate ions consist of a mucous-bicarbonate barrier coated on the surface of the mucosa. The formed unstirred mucus layer generates a pH gradient that maintains a neutral covering on the surface of epithelium and prevents gastric epithelium from digestion with acid and pepsin (Hemstrom et al., 1984). It was found in this study that rats treated with ethanol suffered a depletion of gastric mucus. Such reduction in mucus could be prevented with pretreatment with Hr extract (Table 3.5). These findings suggest that the cytoprotective effect of Hr extract may be associated with the stimulation of mucus secretion, because a mucus coating with adequate thickness can help in the prevention of cells being attacked by pepsin, gastric acid, bacteria and various toxins. Furthermore, as mucus plays an important role in the repair process of superficial damage of tissues, the activities of Hr may also be accompanied with accelerating tissue repair and increasing cellular restitution.

3.4.6 Effect of Hr extract on gastric GSH content

Glutathione is believed to be the most abundant sulfhydryl compound in cells. It is a physiological antioxidant usually in a reduced form (GSH) (Bhattacharya, 1955). GSH is found to be responsible for various intracellular activities including maintaining the integrity of the biological membrane (Szabo et al., 1981), preventing mucosa from damage by harmful oxidative species (Reed et al., 1980) and scavenging free radicals (Takeuchi et al., 1993). Thus, glutathione can be considered as a "cytoprotective agent".

It was found in the present study that the administration of ethanol to rats significantly reduced the content of GSH by approximately 35% in the gastric mucosa (Table 3.6). The results are consistent with the findings that ethanol-induced gastric lesions accompanied a significant decrease of nonprotein sulfhydryl compounds in gastric mucosa, especially in GSH levels in the rat and human (Szabo et al., 1981; Szabo, 1984; Loguercio et al., 1991). Subsequently, the depletion of gastric mucosal GSH induced severe gastric ulceration (Boyd et al., 1979; Boyd, 1981) and patients with peptic ulcer usually have lower gastric glutathione levels (Hirokawa et al., 1995). As ethanol-induced tissue damage accompanies the release of superoxide free radicals and oxidation of low density lipoproteins (Szelenyi et al., 1988), agents that can stimulate the generation of sulfhydryl compounds may help in scavenging free radicals, and consequently prevent damage of cell membranes induced by lipid peroxidation (Chan et al., 1989).

The activity of the Hr extract in the stimulation of GSH secretion has been demonstrated in this study. Pretreatment with Hr extract to the rats before the ethanol treatment markedly inhibited the reduction of GSH induced by ethanol and decreased the lesion formation. The finding suggests that the gastric protective action of Hr extract may correlate with enhancing stomach functions in scavenging free radicals. As oxygen radicals and the reactive oxygen metabolites are involved in the pathogenesis of ethanol- and NSAID-induced gastric mucosal lesions, the relationship between depletion of GSH and the gastric lesions has been extensively studied (Hahm et al., 1997; Pihan et al., 1987; Szabo et al., 1981). In addition, as Guardia suggested (Guardia et al., 1994) that sulfhydryl compounds play an important role in stimulating secretion of gastric mucus, the findings in this study further confirm the

cytoprotective effect of Hr may be partially attributed to the stimulation of GSH and mucus generation in the gastric mucosa.

3.4.7 Effect of Hr extract on endogenous prostaglandins

Prostaglandins have been found to possess cytoprotective actions including maintaining integrity of gastric mucosa, inhibiting secretion of acid and pepsin and promoting secretion of mucus, GSH and bicarbonate (Bolton, 1976; Robert, 1976; 1979; 1983; Gatner, 1979; Kauffman, 1980a; Miller, 1983). Its effects on the increase in GMBF, and alteration of gastric motility and vascular permeability have also been demonstrated (Glavin et al., 1992). To investigate the involvement of PGs in the protective effect of Hr, indomethacin, a potent PG biosynthesis inhibitor (Whittle, 1981), was used in the present study.

It has been shown in the previous studies that indomethacin at a dosage of 300 mg/kg (i.g.) can induce severe gastric and duodenal mucosal lesions (Ueki et al., 1988), but at lower dosage the compound can only inhibit gastric prostaglandin synthesis without induction of tissue damage (Okada et al., 1989). Findings in the present study indicated that subcutaneous administration of indomethacin (5mg/kg) did not increase the gastric lesions in the rats treated with ethanol (Table 3.8). However, the protective effect of Hr extract at doses of 0.3 and 0.6g/kg (i.g.) was reduced 31% and 43%, respectively, when indomethacin was given 30 min before the Hr administration. The findings suggest that the antiulcer effect of Hr may be partially mediated by endogenous prostaglandins.

In order to further investigate changes in concentration of PGs in the gastric mucosa after the Hr treatment, a direct measurement of the PGE_2 content in the glandular mucosa of stomach was conducted (Table 3.7). Results indicated that

administration of 60% ethanol to the rat stomach significantly increased PGE_2 content. The result agreed with the previous findings reported by Konturek (Konturek et al., 1983b). Pretreatment with Hr extract to the rats with the ethanol treatment inhibited the enhanced PGE_2 level. The results suggest that administration of Hr can affect the PGE_2 level in the glandular mucosa, and the unsaturated fatty acids of Hr, such as linolenic acid, showed the similar effect as Hr extract. However, as different types of PGs may be present in the mucosa, the relationship between PGs content and protective effect of Hr seems not straightforward. A complex mechanism including both PGs-dependent and PGs-independent pathway may thus be involved in the gastric protection of Hr.

3.4.8 Summary

The findings in this study indicate that ethanol-induced lesion model can be used successfully to examine the cytoprotective effect of Hr extract. The collected evidence suggests that underlying mechanisms of the gastric protective effect of Hr are complicated. The mechanisms may be associated with improvement of mucosal blood flow, modification of gastric motility, stimulation of mucus secretion, increase of GSH generation, protection of mucusal integrity, increase of free radical scavenging, neutralization of gastric acid by increasing the thickness of mucus layer and acceleration of tissue repair. The effect of Hr on endogenous prostaglandins is not conclusive. Further experiments are needed to reveal other mechanism(s), if any, involved in the cytoprotective effect of Hr extract.

Chapter 4

Study on plant constituents of *Hippophae rhamnoides*

4.1 Introduction

Traditional Chinese medicines (TCM) have been used for centuries for the treatment of gastrointestinal disorders. This is a rich resource for the development of antiulcer agents. In the last two decades, research on plant medicines has been extensively increased and plant-origin functional foods are being rapidly developed and marketed. In addition, pharmacological and chemical studies on plant components can provide important information for drug development, especially in developing lead compounds with new chemical structures. In this study, *Hippophae rhamnoides* was further studied chemically and pharmacologically to search for its antiulcer components.

4.2 Materials and Methods

4.2.1 Plant Materials

The seeds of *Hippophae rhamnoides* L. were collected in October 1996 at Jianping, Liaoning Province, China.

4.2.2 Plant Extraction

Hr seed were powdered and soaked in hexane overnight. The extraction solution was filtered and collected, the residue was soaked with fresh hexane to continue the extraction until no more components could be obtained. The extraction solutions were combined together and concentrated to dryness by an evaporator at 40°C. The yield of hexane-extract of Hr (h-extract) was 8.36%.

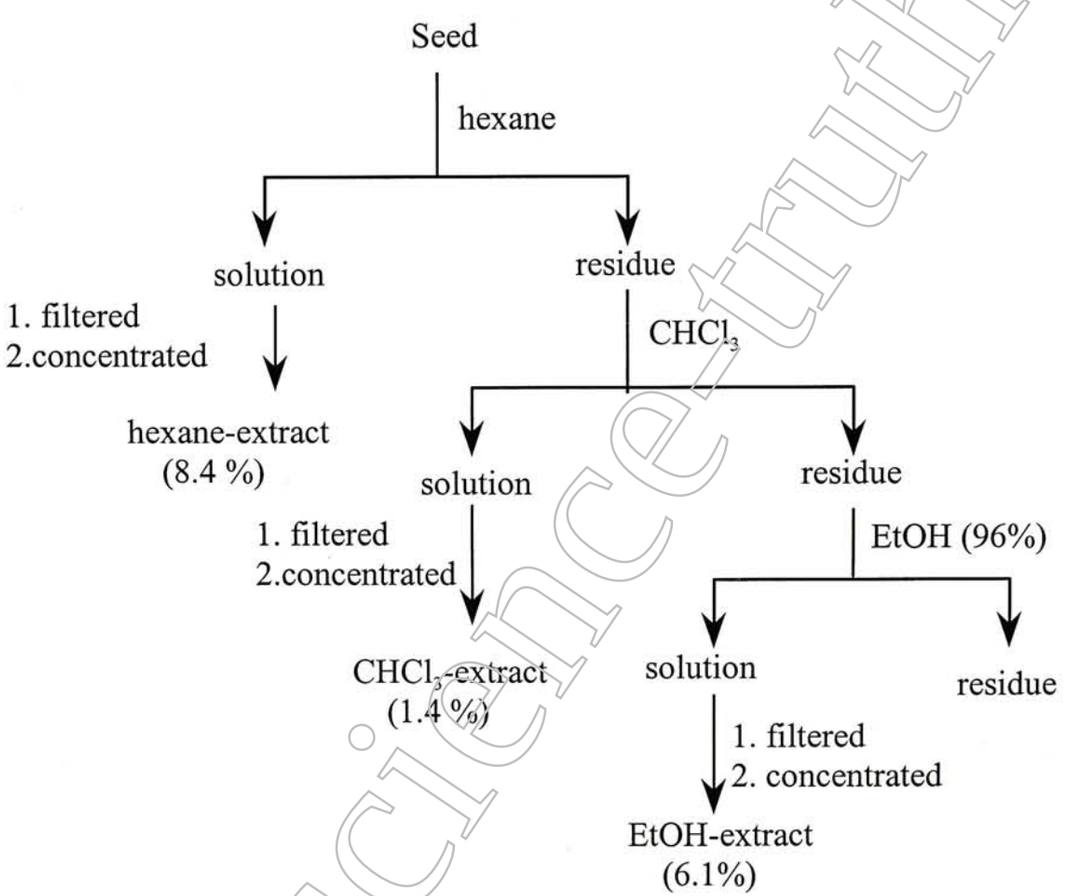
The remained residue was dried at room temperature to remove any solvent absorbed. The dried residue was soaked with chloroform for 8h and the resultant solution was filtered. The extraction process was repeated two more times and the extraction solutions were combined and concentrated to dryness at 40°C. The yield of $CHCl_3$-extract (c-extract) was 1.44%.

After the $CHCl_3$ extraction, any remaining residue was dried at room temperature and further extracted with ethanol for three times. At each time, the residue was soaked with ethanol for 8 hours and the extraction solutions were collected and filtered. The combined extraction solution was concentrated to dryness at 60°C and the yield of this ethanol-extract (e-extract) was 6.06%.

This separation scheme is described in Figure 4.1.

The hexane, chloroform and ethanol extracts obtained were examined for their antiulcer activities against ethanol-induced gastric damage.

Figure 4.1 Fractionation Scheme of *Hippophae rhamnoides*



4.2.3 Fractionation of hexane-extract by column chromatography

Column chromatography was employed for further separation of the hexane-extract. The stationary phase of column chromatography (ϕ2.5cm×40 cm) was silica gel and the ratio of extract sample vis silica gel was (1:10, w/w). The sample was initially eluted with hexane, then the polarity of the mobile phase was gradually increased by adding chloroform (2%, 5%, 10%, 20%, 40%, 50%). The elutes were collected every 25 ml and concentrated to examine the chemical components by TLC. The fractions with similar components were combined together which were further tested for the antiulcer activity.

The collected fractions from the column chromatography were examined for the ingredients contained with thin layer chromatography (TLC). The concentrated samples were applied to TLC plates (silica gel, F_{254}) and developed with different solvent systems including chloroform-hexane (2:3), chloroform-hexane (1:1), chloroform and chloroform-acetone (20:1) in the order of increase of polarity.

After the development, the plate was dried to evaporate the remaining solvents. The samples were examined by two detection methods, i.e., observed directly under an UV-lamp at 254nm/365nm; and sprayed with vanillin-sulphuric acid reagent, then heated at 110°C for 10 min. After spraying the detection reagent and heating the TLC plate, the plant ingredients showed light blue to dark purple colours. The fractions containing similar components were combined and four fractions were finally generated from the hexane-extract, namely fraction-0, fraction-1, fraction-2 and fraction-3. The yields of these four fractions were 0.8%, 47.5%, 1.1% and 0.3%, respectively. The activaties of the fractions 0-3 of Hr were examined against ethanol-induced lesion formation.

4.2.4 Phytochemical identification and analysis of vitamin content of Hr extract

Preliminary identification and measurement of vitamin content of Hr was performed via TLC and HPLC analysis. Vitamin A, vitamin C and vitamin E (α-tocopherol and γ-tocopherol) were employed as standard compounds for comparison.

4.2.4.1 Identification of vitamin A and vitamin C in the Hr extract by TLC

The Hr extract was applied to TLC plates (silica gel) and developed with several solvent systems independently. The standard compounds of vitamins A and C were spotted on the same plate for comparison. The solvent systems employed for separation of Hr extract were:

(1) Chloroform-ethyl acetate (20:1)

(2) Chloroform-acetone (20:1)

(3) Toluene

(4) Toluene-ethyl acetate (97:3)

(5) Methanol

(6) Chloroform- ethyl acetate (10:1)

After the plate was developed, it was dried at room temperature to remove any remaining solvents. The samples were examined by two detection methods, i.e., observed directly under an UV-lamp at 254nm/365nm; and sprayed with the specified detection reagents. The R_f values of the separated compounds were compared with standards in the different solvent systems.

Spraying reagents:

(1) Vanillin-sulphuric acid reagent:

Solution A: 5% Ethanolic sulphuric acid

Solution B: 1% Ethanolic vanillin

The solutions A and B were freshly mixed at a ratio of 1:1 (v/v) before use. After the spraying, the TLC plates were heated at 110°C for 10 min and the colour and R_f values of individual components were observed and recorded.

(2) I_2 reagent:

I_2 (0.5%) was dissolved in chloroform and sprayed on TLC plates. The colours and R_f values of the test components were observed and recorded.

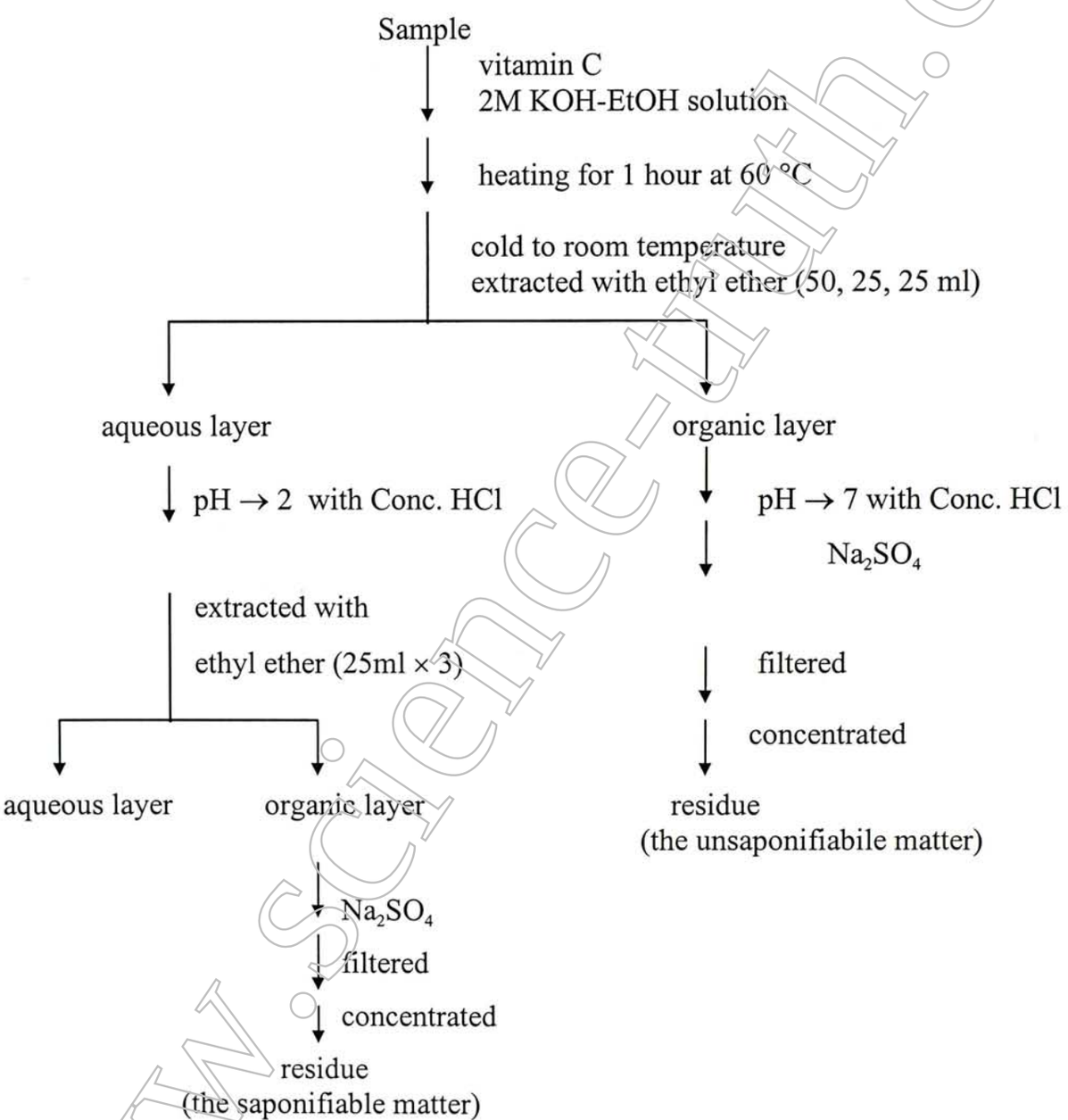
4.2.4.2 Identification of vitamin E (α-tocopherol and γ-tocopherol) by HPLC

For determination of α-tocopherol and γ-tocopherol content in Hr extract with HPLC, the test sample was firstly processed to obtain an unsaponifiable preparation. In brief, the Hr extract (4g) was mixed with 2g of vitamin C in a round flask. The function of vitamin C is to prevent any oxidation of the sample during the saponification. A potassium hydroxide solution (50ml, 2M, dissolved in ethanol) was added into the flask that was equipped with a reflex condenser. The mixture was heated at 60 °C for 1 hour with a magnetic stirrer. After reaction, the mixture was allowed to cool down to room temperature and 100 ml of water was added through a separating funnel. The aqueous solution was extracted with ethyl ether using a volume of 50, 25, 25 ml, respectively for three times. The organic layer was collected to prepare the unsaponifiable matter. The remained aqueous solution was concentrated to obtain the saponifiable matter.

The collected ethyl ether solution was washed three times with 3 × 50 ml of water to neutralise the alkali, then 5g of anhydrous Na_2SO_4 was added for the removal of water remained in the sample. After filtering off the solid substances, the solution was evaporated to dryness. A total of 0.311 g of the unsaponifiable matter was obtained from the 4 g of Hr extract. This unsaponifiable preparation was used for identification and

analysis of vitamin E (α-tocopherol and γ-tocopherol) as well as phytosterols. The detailed procedure is described in Figure 4.2.

Figure 4.2 Preparation of the saponifiable and unsaponifiable matters of Hr

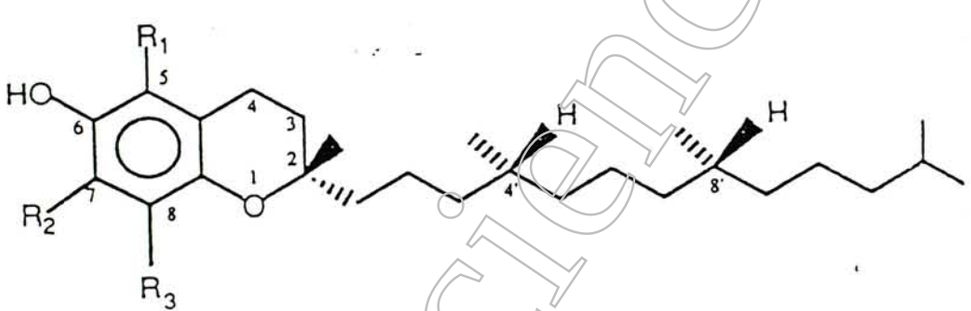


The α-tocopherol and γ-tocopherol were employed as standards for identification of the presence of vitamin E in the Hr extract. The prepared unsaponifiable matter of the Hr extract was analysed with HPLC with methanol as

mobile phase. The separation was performed on a reverse phase C_{18} column (250mm × 4mm, i.d.) and the flow rate of mobile phase was set at 1.0 ml/min at room temperature. Samples (10 μL) was injected into the column and detected with an UV detector at 280 nm.

Vitamin E, in fact, is a collective term for tocopherols and tocotrienols, a group of analogues with potent antioxidative activity. The structures derive from 6-chromanol and the structural difference of the analogue lies on degree of the saturation in the respective isoprenoid side chains (Figure 4.3).

Figure 4.3 The structures of vitamin E



	R1	R2	R3
α- tocopherol	CH3	CH3	CH3
β- tocopherol	CH3	H	CH3
γ-tocopherol	H	CH3	CH3
δ-tocopherol	H	H	CH3

α-Tocopherol and γ-tocopherol were identified according to the following methods: (1) The peaks of α-tocopherol and γ-tocopherol in the HPLC chromatogram were identified by comparing the retention times of the vitamin E standards with those of the corresponding peaks in the Hr samples. (2) Recording ratio changes in the peak areas of the test sample between α-tocopherol and γ-tocopherol (α/γ) with or without addition of the standard compounds were recorded. (3) Comparing the UV-spectrum of the corresponding peaks in the Hr extract with those of vitamin E standards.

4.2.4.3 Analyses of the content of α-tocopherol in the Hr extract

The relative content of α-tocopherol in the Hr extract was analyzed by reverse phase HPLC. The conditions were described in section 4.2.4.2

The peak of α-tocopherol in the extract sample was identified by comparing the retention time of peaks (Figures 4.4-4.5), the UV-spectrum (190nm-400nm) between of the sample and α-tocopherol standard; and the changes in the ratio of peak area (A_{280}nm/A_{254}nm) when the detection wavelength was shifted (Table 4.1).

Table 4.1 Identification of α-tocopherol in the Hr extract

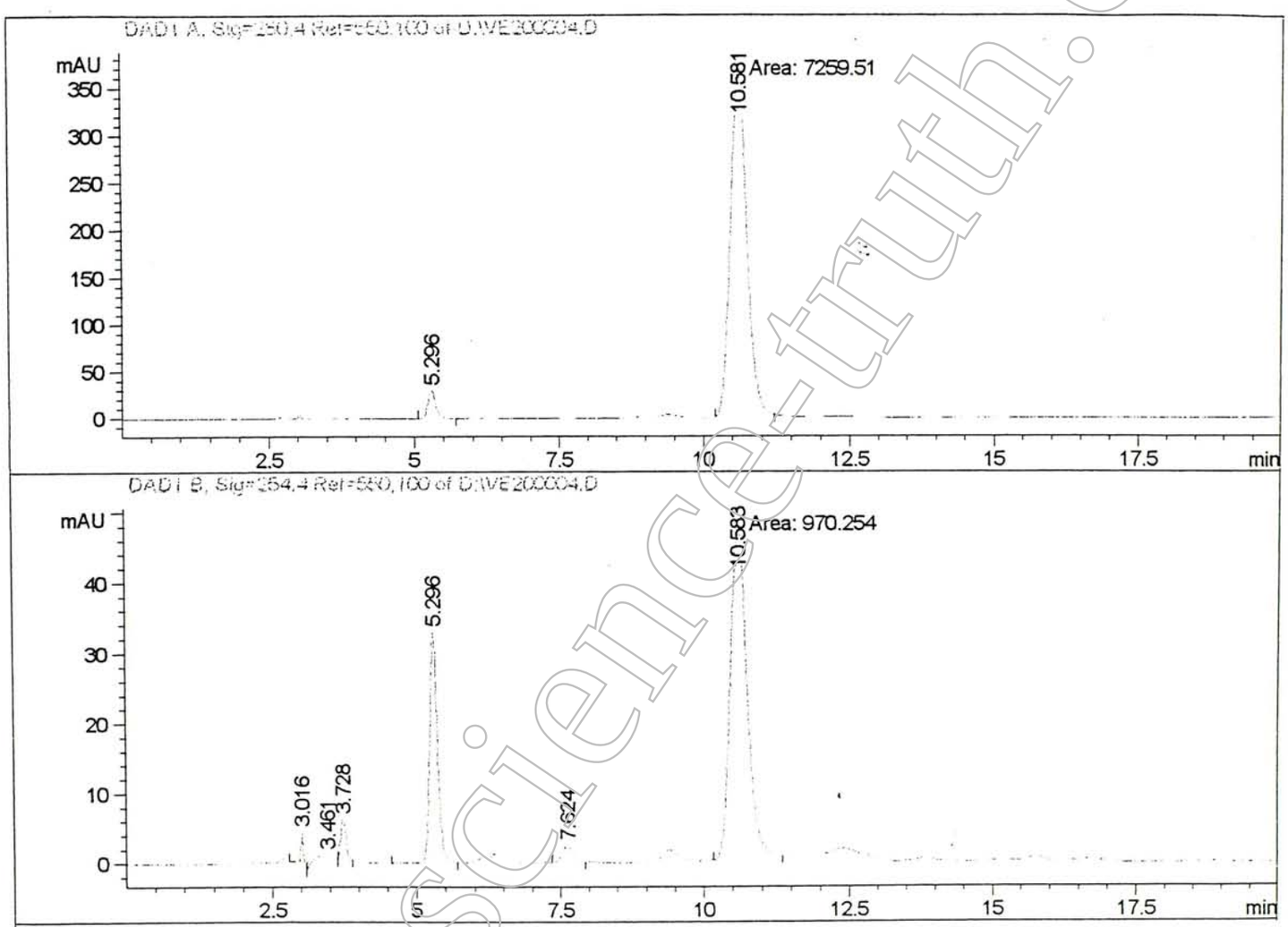
Detection λ	280 nm		254 nm		ratio of
	RT (min)	Area (mAU)	RT (min)	Area (mAU)	A_{280}/A_{254}
α-tocopherol	10.581	7259.51	10.583	970.25	7.48
sample peak1	10.557	243.09	10.561	32.66	7.44

The relative content of α-tocopherol was calculated according to the formula:

$$\text{α-tocopherol content } C\ \%\ (\text{mg/ml}) = [(A_{sample} \times C_{standard})/A_{standard}] \times 100\%$$

A: Area of the peak; C: concentration

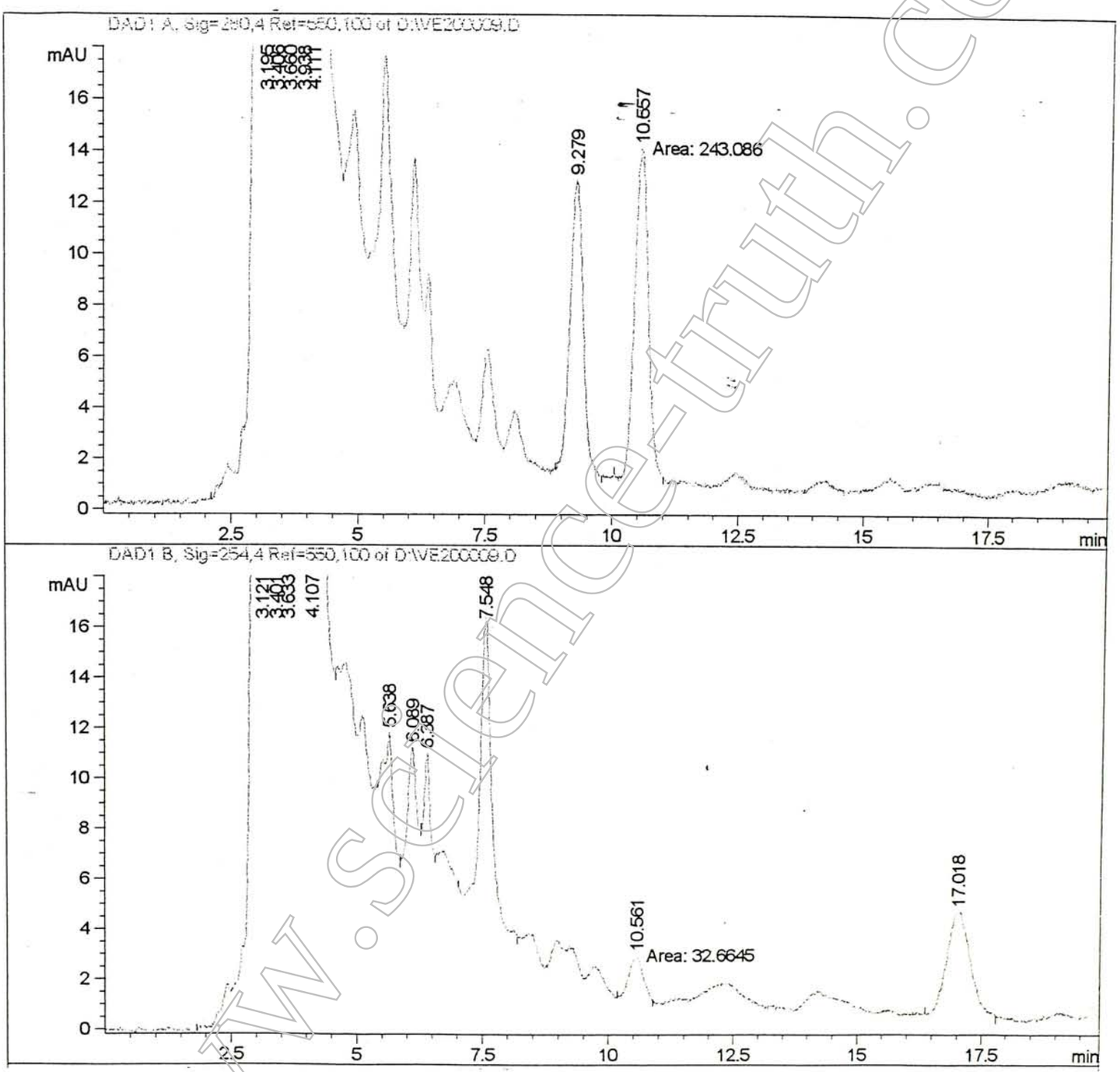
Figure 4.4 HPLC chromatography of α-tocopherol



A: Detected at 280 nm.

B: Detected at 254 nm.

Figure 4.5 HPLC chromatography of Hr extract



A: Detected at 280 nm.

B: Detected at 254 nm.

4.2.4.4 Identification and analysis of fatty acid in the Hr fractions

4.2.4.4.1 Esterification of fatty acids

In order to analyse the composition of fatty acids in the Hr extract by GC-MS, the saponifiable matter and the fatty acid standards were required to be esterfied, thus all the fatty acids were converted to the related fatty acid methyl esters (FAME).

Esterification was performed according to the method described by Bordier (Bordier et al., 1996) using heptadecanoic acid (17:0) as internal standard. In brief, 20 mg of fatty acid standard or the Hr extract and 1 mg of internal standard were mixed in a test tube. Borontrifluoride (BF_3) methanol solution (2 ml) and 1 ml of toluene were added into the mixture and flushed them with nitrogen for 10 seconds for removal of oxygen. The tube was closed immediately and the mixture was heated at 90 °C for 45 min. After the reaction, the solution was allowed to cool down to room temperature and hexane (3 ml) with water (1 ml) were then added and mixed vigorously. The mixture was centrifuged at 1000g for 5 min. The organic layer was transferred to a new tube and dried with nitrogen to remove solvent. The residue was dissolved with 1 ml of hexane and used for the analysis of fatty acid composition by GC-MS.

4.2.4.4.2 Isolation and identification of FAME by GC-MS

The prepared esterified samples were injected into a GC-MS system (Finnigan Mat GCQ) for analysis. The separation was performed on a capillary column (SP-2380, 30 m × 0.25 mm i.d.) coated with a film containing 90%-biscyanopropyl-10%-cyanopropylphenylsilocane (0.20 mm in thickness). The injection temperature was 280 °C and the volume of the injection was 1 µl. After an isothermal period of 3 min at 100 °C, the oven temperature was increased to 200 °C at a rate of 10°C/min and retained at

200°C for 4 min. The temperature of the GC-MS interface was 280°C. The detection was performed with a mass detector with both the electron ionization (EI) mode and chemical ionization (CI) mode at 70 eV. The test samples were scanned from 60 to 450 atomic mass units (amu) at 1 scan/sec.

4.2.4.5 Quantitative analysis of composition and relative content of fatty acid in the Hr fractions

The saponifiable matter prepared previously was analysed for fatty acids in the Hr extract. After the saponification, acidity of the aqueous portion was adjusted to pH 2 with concentrated HCl. The solution was extracted with ethyl ether for three times (25 ml × 3). The ethyl ether layer was collected and 10g of anhydrous Na_2SO_4 was added to remove the remaining water. After filtering, the solution was evaporated to dryness and the yield of the saponifiable matter was 1.75%. The preparation scheme is described in Figure 4.2.

Fatty acid methyl esters were analyzed on a flexible silica capillary column (SP 2560; 100 ×0.25 mm, i.d.; Supelco, Inc.) with a gas-liquid chromatograph (HP 5980 Series II) equipped with a flame-ionization detector and an automated injector. Column temperatures were increased from 250 to 300°C at a rate of 1°C/min. Hydrogen was used as the carrier gas at a high pressure of 15 psi.

The retention time of each standard fatty acid was used for identification of composition of the Hr extract. The peak area of the standard fatty acid was used to calibrate the relative content of the components.

Hexadecanoic acid (C 16:0), hexadec-9-enoic acid (C 16:1 n7), heptadecanoic acid (17:0), octadecanoic acid (C 18:0), octadec-9-enoic acid (C 18:1 n9), octadec-11-enoic acid (C 18:1 n7), octadeca-9,12-dienoic acid (C18:2 n6), eicosanoic acid (C20:1

n9), octadeca-9,12,15-trienoic acid (C18:3 n3), etc. were employed as standard compounds (provided by Pref. Z.Y.Chen, Department of Biochemistry, The Chinese University of Hong Kong).

4.3 Results

4.3.1 Phytochemical analysis and identification

4.3.1.1 Identification of vitamin A

For examination of vitamin A, the developed TLC plate was observed under an UV-lamp at 365nm. Vitamin A possesses a yellow-green fluorescence against a dark background. In addition, with the detection of vanillin-sulphuric acid reagent, vitamin A appeared as a dark purple spot on the TLC plate. Compared with the vitamin A standard, the presence of vitamin A in the Hr extract was examined. The results were showed in Table 4.2.

Table 4.2 Examination of vitamin A with TLC

Solvent systems	(1)	(2)	(3)	(4)
R_f: V_A standard	0.79	0.83	0.69	0.85
R_f: a component of Hr extract	0.78	0.82	0.69	0.85

After development of the TLC plate with one of the following solvent systems, the plate was examined under UV-lamp and sprayed with vanillin-sulphuric acid reagent. Solvent system: (1) chloroform-ethyl acetate (20:1); (2) chloroform-acetone (20:1); (3) toluene; (4) toluene-ethyl acetate (97:3).

The results indicated that there was a compound in the Hr extract having the similar chromatographic behaviours to that of vitamin A standard when it was examined with different solvent systems on TLC. The fluorescence colour of the spot was the same as that of vitamin A when detected with both UV (365nm) and the spray reagent. These findings suggested the presence of vitamin A in the Hr extract.

4.3.1.2 Identification of vitamin C

For determination of vitamin C, the developed TLC plate was observed under an UV-lamp at 254 nm. Vitamin C appeared as a dark spot against the bright-green fluorescence background of the TLC. The plate was then sprayed with a $CHCl_3$ solution containing 5% I_2 and vitamin C showed a dark-brown colour immediately. In addition, with detection of the vanillin-sulphuric acid reagent, vitamin C appeared as a light-purple spot in the TLC plate. There was a component in the Hr extract that showed the same chromatographic behaviours to vitamin C when the TLC plate was developed with different solvent systems. The colours of this component were the same to that of vitamin C detected by both UV detection and the spray reagent. Compared with the standard compound, the presence of vitamin C in the Hr extract is confirmed in this study. The results of TLC examination are given in Table 4.3.

Table 4.3 Examination of presence of vitamin C in Hr extract with TLC

Solvent system	chloroform-ethyl acetate (10:1)	methanol
R_f: V_C standard	0.63	0.80
R_f: Hr extract	0.63	0.81
Detection Method	1. UV examination at 254 nm 2. Spray reagent: 5% $I_2/CHCl_3$	1. UV examination at 254 nm 2. Spray reagent: vanillin-sulphuric acid

After development of the TLC plate with one of the solvent systems, the plate was examined under UV-lamp (254 nm) and detected with spraying-reagent.

4.3.1.3 Identification of α-tocopherol and γ-tocopherol

Identification of α-tocopherol and γ-tocopherol in the Hr extract was performed. The relative content of α-tocopherol in the Hr extract was measured by reverse phase HPLC under the conditions described previously. The peaks of α-tocopherol and γ-tocopherol in the extract sample were identified by comparing the retention time and the UV-spectrum (190nm-400nm) of the peak with that of α-tocopherol and γ-tocopherol standard, separately. Comparison of the percentage changes in the peak area of corresponding peaks with the change of detection wavelengths (λ_{280}nm; λ_{254}nm) was also performed (Table 4.4).

Table 4.4 Identification of α-tocopherol and γ-tocopherol in the Hr extract

sample	Retention Time (min)	
	α-	γ-
α-tocopherol standard	6.34	
γ-tocopherol standard		5.57
The unsaponifiable matter	6.40	5.58
α-tocopherol +Hr	6.36	5.55
γ-tocopherol + Hr	6.32	5.51

The identification of α-tocopherol and γ-tocopherol in the Hr extract were performed by HPLC.

These results indicated that two components of Hr possessd the similar retention times as those of α-tocopherol and γ-tocopherol standards. Furthermore, the changes in the ratio of peak area (A_α/A_γ) of the corresponding peaks in the Hr extract were observed after the addition of α-tocopherol and γ-tocopherol standards. These findings suggested the presence of α-tocopherol and γ-tocopherol in the Hr extract.

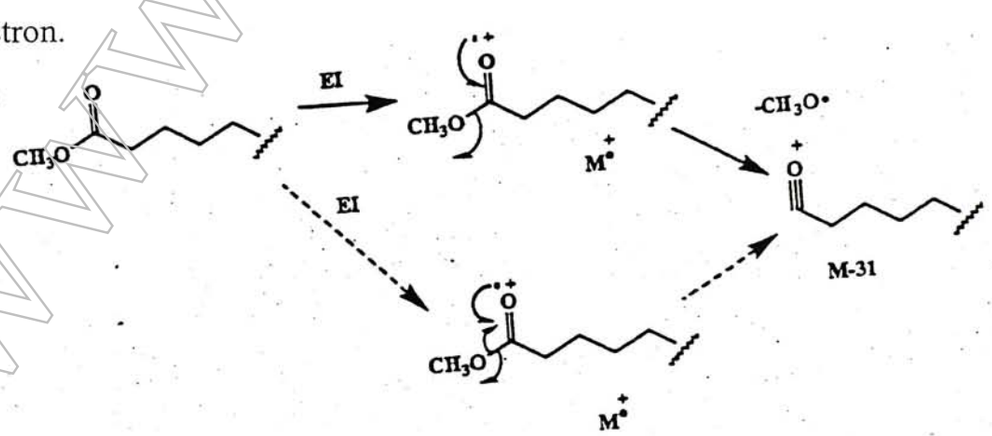
4.3.1.4 Quantitative Analysis of α-tocopherol content in the Hr extract

The content of α-tocopherol in the Hr extract was analyzed quantitatively with reverse-phase HPLC. Data obtained from this experiment indicated that the unsaponifiable matter of Hr contained 0.08% (w/w) α-tocopherol. As percentage of the unsaponifiable matter in the Hr extract was 3.2%, the content of α-tocopherol in Hr extract was 0.0026%.

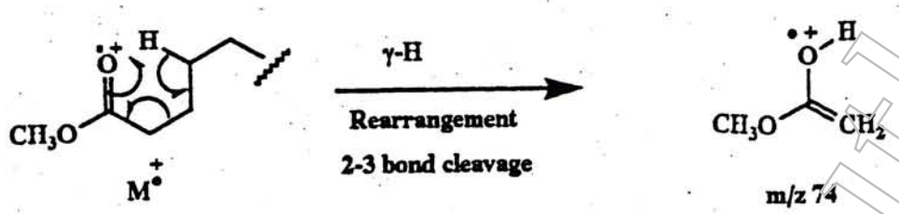
4.3.1.5 Identification of composition of fatty acid in the Hr extract

EI and CI mass detectors were employed for identification of fatty acid methyl ester (FAME) compositions of Hr. In addition, comparing the retention times of Hr ingredients in the GC profile with those of the fatty acid standards was performed.

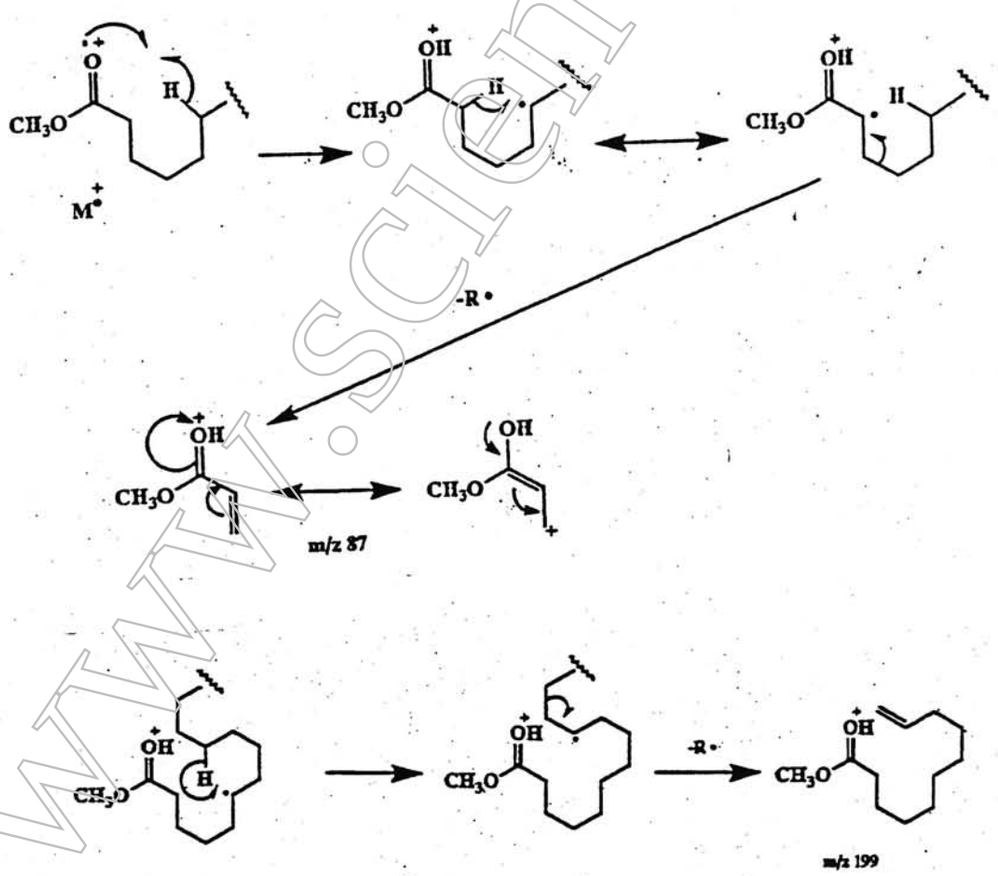
The fragmentation pattern of fatty acids in the mass spectra possesses important value for the structural identification. Electron ionization of a fatty acid with saturated linear side chain usually generates a series of ion-fractions with 14 mass unit difference. The molecular ion ($M^{\cdot+}$) is an odd electron species occurring at an even mass (McLafferty, 1980). The presence of a molecular ion is a valuable feature for identification of FAME. In addition, a characteristic ion of fatty acid at lower abundance usually occurs at M-31 mass unit. The electron covalent bond between two atoms can be broken that leads to each atom with a single unpaired electron.

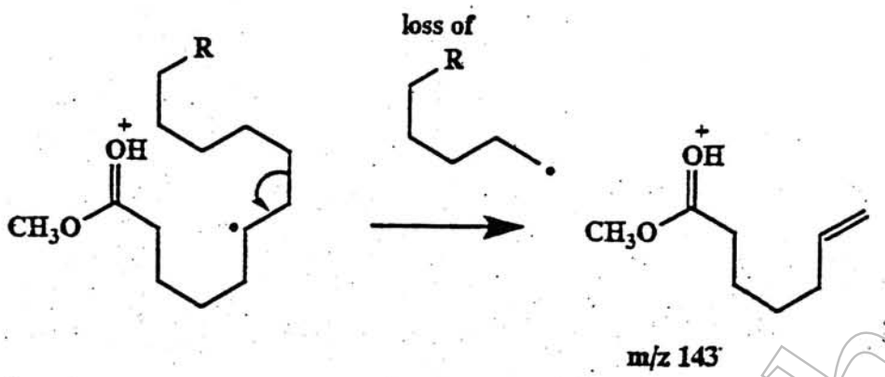


The most abundant ion presenting at m/z 74 is another valuable feature for identification of straight chain FAME. The generation of this ion involves a hydrogen-transfer (γH-transfer) followed by cleavage of the carbon-carbon bond with two carbons removed by the β-cleavage.



The other abundant ions in the FAME mass spectra are at m/z 87, 101, 115, 129, 143, 157, 171, 185 and 199 (Spiteller et al., 1980).





The most abundant ion presented in CI mass spectra of FAME is $(M+H)^+$ that is the most valuable feature for identification of straight chain FAME. In addition, the ion corresponding to the loss of methanol usually also presents in CI mass spectra.

The saponifiable matter of Hr was examined by GC-MS detected with EI and CI mass detectors. Mass spectra of major Hr components in the saponifiable matter were obtained to identify composition of fatty acids. Eleven major peaks were observed and identification results indicated the presence of fatty acids in the Hr extract.

Peak1: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 242 with fragment ions at 211(M^+-$CH_3O^{\cdot}$), 199 [CH_3OC (OH)$(CH_2)_8CHCH_2^+$], 143 [$CH_3OC(OH)(CH_2)_4CHCH_2^+$], 87 ($CH_3OC(OH)CHCH_2^+$) and 74 [$CH_3OC$ (OH) $(CH_2)^{\cdot+}$]. The MS spectrum suggested that the compound had a structure similar as tetradecanoic acid (14:0).

Peak 2: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 270 and fragment ions at 238 (M^+-CH_3OH), 199 [CH_3OC (OH)$(CH_2)_8CHCH_2^+$], 143 [$CH_3OC(OH)(CH_2)_4CHCH_2^+$], 87 [$CH_3OC(OH)CHCH_2^+$] and 74 [$CH_3OC$ (OH)

$(CH_2)^{\cdot+}$]. Its CIMS spectrum exhibited a molecular ion $(M+H)^+$ peak at m/z 271 and fragment ions at 311 (M^++$C_3H_5^{\cdot}$), 299(M^++$C_2H_5^{\cdot}$), 255, 237, 219, and 199. The MS spectrum suggested that the compound was hexadecanoic acid (16:0). Its retention time in GC was the same as that of hexadecanoic acid standard (21.896 min).

Peak 3: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 268 and fragment ions at 236 (M^+-CH_3OH). Its CIMS spectrum showed a molecular ion $(M+H)^+$ peak at m/z 269 and fragment ions at 297(M^++$C_2H_5^{\cdot}$), 237 (M^+-CH_3OH), and 219. The MS spectrum suggested that the compound was hexadec-9-enoic acid (16:1). Its retention time in GC was the same as that of hexadec-9-enoic acid standard (23.453 min).

Peak 4: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 298 and fragment ions at 266 (M^+-CH_3OH), 199 [$CH_3OC(OH)(CH_2)_8CHCH_2^+$], 143 [$CH_3OC(OH)(CH_2)_4CHCH_2^+$], 87 [$CH_3OC(OH)CHCH_2^+$] and 74 [$CH_3OC$ (OH) $(CH_2)^{\cdot+}$]. Its CIMS spectrum exhibited a molecular ion $(M+H)^+$ peak at m/z 299 and fragment ions at 339 (M^++$C_3H_5^{\cdot}$), 327(M^++$C_2H_5^{\cdot}$), 265, 247, 199, and 143. The MS spectrum suggested that the compound was octadecanoic acid (18:0). Its retention time in GC was the same as that of octadecanoic acid standard (26.372 min).

Peak 5: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 296 and fragment ions at 264 (M^+-CH_3OH). Its CIMS spectrum exhibited a molecular ion $M+H)^+$ peak at m/z 297 and fragment ions at 337 (M^++$C_3H_5^{\cdot}$), 325(M^++$C_2H_5^{\cdot}$), 265(M^+-CH_3OH), 247, 199, and 149. The MS spectrum suggested that the compound

was octadec-9-enoic acid (18:1). Its retention time in GC was the same as that of octadec-9-enoic acid standard (28.206 min).

Peak 6: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 294 and fragment ions at 262(M^+-CH_3OH), 245, 220, 191, 178, 164, 149, 110, 95 and 81. Its CIMS spectrum exhibited a molecular ion $(M+H)^+$ peak at m/z 295 and fragment ions at 335(M^++$C_3H_5^{\cdot}$), 323(M^++$C_2H_5^{\cdot}$), 245, 199, and 149. The MS spectrum suggested that the compound was octadeca-9,12-dienoic acid (18:2). Its retention time in GC was the same as that of octadeca-9,12-dienoic acid standard (31.128 min).

Peak 7: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 266 and fragment ions at 234(M^+-CH_3OH), 254, 205, 192, 177, and 164. The MS spectrum suggested that the compound had a structure similar to palmitic acid (16:2).

Peak 8: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 292 and fragment ions at 260(M^+-CH_3OH), 236, 250, 208, 166, 149, 135, and 124. Its CIMS spectrum exhibited a molecular ion $(M+H)^+$ peak at m/z 293 and fragment ions at 333(M^++$C_3H_5^{\cdot}$), 321(M^++$C_2H_5^{\cdot}$), 261, 149 and 109. The MS spectrum suggested that the compound was octadeca-9,12,15-trienoic acid (18:3). Its retention time in GC was the same as the standard of octadeca-9,12,15-trienoic acid (34.795 min).

Peak 9: The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 326 and fragment ions at 297, 283, 255, 199 [$CH_3COC(OH)(CH_2)_8CHCH_2^+$], 143 [$CH_3OC(OH)(CH_2)_4CHCH_2^+$], 87 [$CH_3OC(OH)CHCH_2^+$] and 75. The MS spectrum

suggested that the compound was arachidic acid (20:0). Its retention time in GC was the same as that of arachidic acid standard (32.427 min).

Peak 10 The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 324 and fragment ions at 292 (M^+-CH_3OH), 275, 263, 250, 236,152 and 95. The MS spectrum suggested that the compound was eicosanoic acid (20:1). Its retention time in GC was the same as that of eicosanoic acid standard (34.287min).

Peak 11 The EIMS spectrum exhibited a molecular ion ($M^{\cdot+}$) peak at m/z 354 and fragment ions at 322 (M^+-CH_3OH), 199 [$CH_3OC(OH)(CH_2)_8CHCH_2^+$], 143 [$CH_3OC(OH)(CH_2)_4CHCH_2^+$], 87 [$CH_3OC(OH)CHCH_2^+$] and 75. The MS spectrum suggested that the compound had a similar structure as docosanoic acid (22:0).

4.3.1.6 Analysis of relative content of fatty acids in the Hr extract

The composition of fatty acid of Hr extract was quantitatively analyzed with GC. Results showed that the major fatty acids present in Hr extract were: hexadecanoic acid (C16:0), hexadec-7-enoic acid (C16:1), octadecanoic acid (C18:0), octadec-9-enoic acid (C18:1), octadeca-9,12-dienoic acid (C18:2), eicosanoic acid (C20:1) and octadeca-9,12,15-trienoic acid (C18:3), etc. The relative amounts of these fatty acids in the hexane-extract of Hr and its fractions are shown in Table 4.5.

Table 4.5 The relative content of fatty acids in the Hr fractions

Fatty acid	Retention Time (min)	Relative content (%) of fatty acid in fraction-0	Relative content (%) of fatty acid in fraction-1	Relative content (%) of fatty acid in fraction-2	Relative content (%) of fatty acid in fraction-3	Relative content (%) of fatty acid in the Hr extract
Hexadecanoic acid C 16:0	21.896	21.21	8.85	15.52	21.54	8.65
Hexadec-9-enoic acid C 16:1 n7	23.453	1.31	0.57	0	3.11	Trace
octadecanoic acid C 18:0	26.372	3.86	2.40	3.64	5.28	2.25
octadec-9-enoic acid C 18:1 n9	28.206	14.14	18.55	23.37	23.66	17.75
octadec-11-enoic acid C 18:1 n7	28.432	3.27	2.28	4.24	4.70	2.26
octadeca-9,12-dienoic acid C 18:2 n6	31.128	27.49	37.58	30.01	13.02	37.24
arachidic acid C 20:0	32.427	3.95	0	0	0	Trace
eicosanoic acid C20:1 n9	34.287	0	0	0	0	Trace
brassidic acid C 22:1 n9	34.531	0.38	0	0	0	Trace
octadeca-9,12,15-trienoic acid C18:3 n3	34.795	3.79	28.59	13.54	5.20	29.66
Trans-13-docosenoic acid C 22:1 n7	35.483	0.54	0	0	0	Trace
Others		19.57	1.18	9.67	23.48	2.20

- The composition of fatty acid in the different Hr extracts were analyzed by GC.

4.3.1.7 Identification of phytosterols of Hr by GC-MS

EI mass spectra were used for identification of *phytosterols*. The presence of aampesterol, β-sitosterol and stigmasterol were suggested by the GC-MS data.

Campesterol: Its EIMS spectrum exhibited a molecular ion peak ($M^{+\cdot}$) at m/z 400 and fragment ions at 385 (M^+-CH_3), 382 (M^+-H_2O), 367 (M^+-CH_3-H_2O), 315 (M^+-H_2O-C_2H_5), 289 (M^+-H_2O-C_7H_9), 261 (M^+-H_2O-C_9H_{13}), 255 and 213 (Cong, 1987).

β-Sitosterol: Its EIMS spectrum exhibited a molecular ion ($M^{+\cdot}$) peak at m/z 414 and fragment ions at 399 (M^+-CH_3), 396 (M^+-H_2O), 381 (M^+-CH_3-H_2O), 329 (M^+-H_2O-C_2H_5), 303 (M^+-H_2O-C_7H_9), 273, 255 and 213 (Cong, 1987).

Stigmasterol: Its EIMS spectrum exhibited a molecular ion ($M^{+\cdot}$) peak at m/z 412 and fragment ions at 397 (M^+-CH_3), 394 (M-H_2O), 379 (M^+-CH_3-H_2O), 369 (M^+-C_3H_7), 273 and 255 (Cong, 1987).

4.3.2 *Examination of antiulcer effect of Hr fractions against ethanol-induced gastric lesions*

The hexane, chloroform and ethanol extracts of Hr and the fractions 0-3 of the hexane extract were examined for their antiulcer effect using the ethanol-induced gastric lesion model. The detailed protocol for this pharmacological test was described in Chapter 2.

4.3.2.1 Effects of the different extracts of Hr seed on ethanol-induced gastric lesions

The hexane-extract, chloroform-extract and ethanol-extract of Hr were tested for their antiulcer effect against ethanol-induced gastric lesion. The results are given in Table 4.6.

Table 4.6 Effect of different extracts of Hr seed on gastric lesions induced by Ethanol

Treatment	Dose (g/kg)	Lesion index (mm^2) M ± SEM (n=10)	Percentage of protection (%)
Control		72.7±5.8	
Hexane-extract	0.3	40.7±5.2*	44.0
Hexane-extract	0.6	30.67±4.9**	57.8
$CHCl_3$-extract	0.6	38.5±11.4*	47.0
EtOH-extract	0.6	mucosa exfoliation	

Rats were pretreated (4ml/kg, i.g.) with the different extract solutions or vehicle 30min before administration of ethanol (60%, 6ml/kg, i.g.), the gastric lesions in rats were measured 30 min after the ethanol treatment. *p<0.05, **p<0.01, ***p<0.001 compared with the control group. The statistical significance was analysed by Tukey's test followed analysis of variance (ANOVA).

The experimental results showed that intragastrically administrated ethanol (60%, 6ml/kg) produced linear hemorrhagic lesions along the long axis of the stomach. Pretreatment with the hexane-extract (0.3-0.6 g/kg) and chloroform-extract (0.6 g/kg) of Hr exhibited a significant gastric protective effect ($p<0.01$ and $p<0.05$, respectively) in this lesion model. However, the ethanol-extract of Hr at dose of 0.6 g/kg did not show any protective effect on gastric mucosa, but aggravated the damage. Comparing the potency of chloroform extract with that of hexane extract, the later showed higher gastric protective activity in this test model.

4.3.2.2 Effects of the Hr fractions isolated from the hexane-extract on ethanol induced gastric lesions

The fractions 0-3 were isolated from the hexane extract by column chromatograph. Each fraction was tested pharmacologically for its antiulcer effect against ethanol-induced mucosal damage. The results are shown in Table 4.7.

Table 4.7 Gastric protective effects of different fractions of h-extract

Treatment	Dose (g/kg)	Lesion index (mm^2) M ± SEM (n=10)	Percentage of protection (%)
Control		58.93±4.28	
Hexane-extract	0.16	33.90 ±5.71*	42.5
fr-o	0.16	29.50±5.23**	49.9
fr-1	0.16	20.56±6.62***	65.1
fr-2	0.16	26.14±6.23**	55.6
fr-3	0.16	18.00±7.36***	69.5

Rats were pretreated (i.p.) with the different fractions of Hr extract or vehicle independently 30 min before the administration of ethanol (60%, 6ml/kg, i.g.). Gastric lesions were measured 30 min after the ethanol treatment. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the control group. The statistical significance was analysed by Tukey's test followed analysis of variance (ANOVA).

The results showed that all fractions possess gastric preventive effects against ethanol-induced mucosal damage. Comparing the activity of different fractions at the same dose, the potency of these fractions in a descending order is: fraction3 > fraction 1 > fraction 2 > fraction 0 > Hr extract.

4.3.2.3 Effects of Hr components on gastric lesions in different lesion models

4.3.2.3.1 Effects of Hr components on ethanol-induced lesions

β-sitosterol (0.1g/kg) and linolenic acid (0.24g/kg), the components of the Hr extract, were tested using the ethanol-induced lesion model. The gastric protective effect of these compounds was compared with that of the Hr extract. The results are given in Table 4.8.

Table 4.8 Effect of Hr ingredients on ethanol-induced gastric lesions

Treatment	Dose (g/kg)	Lesion index (mm^2) M ± SEM (n=10)	Percentage of protection (%)
Control(60% EtOH)	-	72.7±5.8	
Hr extract	0.6	30.7±4.9**	57.8
Cimetidine	0.1	50.0±4.8	31.2
β-sitosterol	0.1	47.6±6.5	34.5
Linolenic acid	0.24	11.0±3.0***	84.9

Rats were pretreated (4ml/kg, i.g.) with the test samples or vehicle 30min before the administration of ethanol (60%, 6ml/kg, i.g.). The gastric lesions were measured 30 min after the ethanol treatment. Cimetidine was used as a control compound. *$p<0.05$, ***$p<0.001$ when compared with the control group. The statistical significance was analysed by Tukey's test followed analysis of variance (ANOVA).

The linolenic acid showed strong preventive action ($p<0.001$) on gastric mucosa lesions induced by ethanol, but β-sitosterol at dose of 100 mg/kg did not show any gastric protective effect in this pathological model. There is no significant difference in the gastric protective effect between the Hr extract (0.3 and 0.6 g/kg) and linolenic acid (0.24g/kg) suggesting linolenic acid is one of the active components of the Hr extract. Cimetidine (0.1g/kg), a control compound used in this experiment, did not show any gastric protective effect against the ethanol-induced gastric lesions. The result agreed with the previous report (Kuwayama et al., 1987). As cimetidine is an inhibitor of acid secretion, this finding suggested that the stimulation of gastric acid secretion might not be the major pathogenic factor in the ethanol-induced ulceration.

4.3.2.3.2 Effects of Hr components against stress-induced gastric lesions

β-Sitosterol, the component of the Hr extract, with the positive control compound cometidine, was tested in the stress-induced lesion model. The result was compared with that of Hr extract (Table 4.9).

Administration of the β-sitosterol (0.1g/kg) decreased the stress-induced gastric mucosal lesions approximate 39% indicating that β-sitosterol possessed certain degree of gastric protection although it did not achieve a significant level compared with the control. Administration of cimeditine (0.1g/kg) markedly inhibited the gastric lesions in rats, suggesting the increase of gastric acid secretion was involved in the pathogenesis of the stress-induced gastric lesions.

Table 4.9 Effects of Hr ingredient on gastric lesions induced by stress

Treatment	Dose (g/kg)	Lesion index (mm^2) M ± SEM (n=8)	Percentage of protection (%)
Control		9.38±1.53	
Hr extract	0.6	3.57±1.11*	61.9
Cimetidine	0.1	3.13±1.08**	66.6
β-sitosterol	0.1	5.71±0.92	39.1

Thirty minutes after the test samples or vehicle were administered (i.g.), the rats were subjected by restrains plus water immersion at 24±1°C for 6h. The gastric lesions in rats were then measured and expressed as lesion index (LI). *$p<0.05$, **$p<0.01$ when compared with the control group. The statistical significance was analysed by Tukey's test followed analysis of variance (ANOVA).

4.3.2.3.3 Effect of β-sitosterol against gastric lesions induced by pylorus ligation

The gastric protective effects of the Hr extract, β-sitosterol and the positive control compound cimetidine against pylorus ligation-induced gastric damage were examined in this study. The results are given in Table 4.10.

Cimetidine at dose of 0.1 g/kg completely inhibited the formation of the gastric lesions. β-sitosterol, a component of the Hr extract, inhibited the gastric mucosal lesions by approximate 46%, however the inhibition did not achieve to a significant level. This finding suggests that β-sitosterol can only partially contribute to the protective activity of Hr, the activity of other Hr components cannot be ruled out.

Table 4.10 Effect of the Hr extract and its component against pylorus ligation-induced lesion

Treatment	Dose (g/kg)	Lesion index M ± SEM (n=6)	Percentage of protection (%)
Control	-	2.17 ±0.70	
Hr extract	0.6	0.33 ± 0.33*	84.8
Cimetidine	0.1	0 *	100
β-sitosterol	0.1	1.17 ±0.6	46.1

After the rat's pylorus was ligated, test samples or vehicle were injected into duodenum immediately. Gastric lesions were measured 6h after the pylorus ligation. * $p<0.05$ when compared with the control group. The statistical significance was analysed by Tukey's test followed analysis of variance (ANOVA).

4.4 Discussion

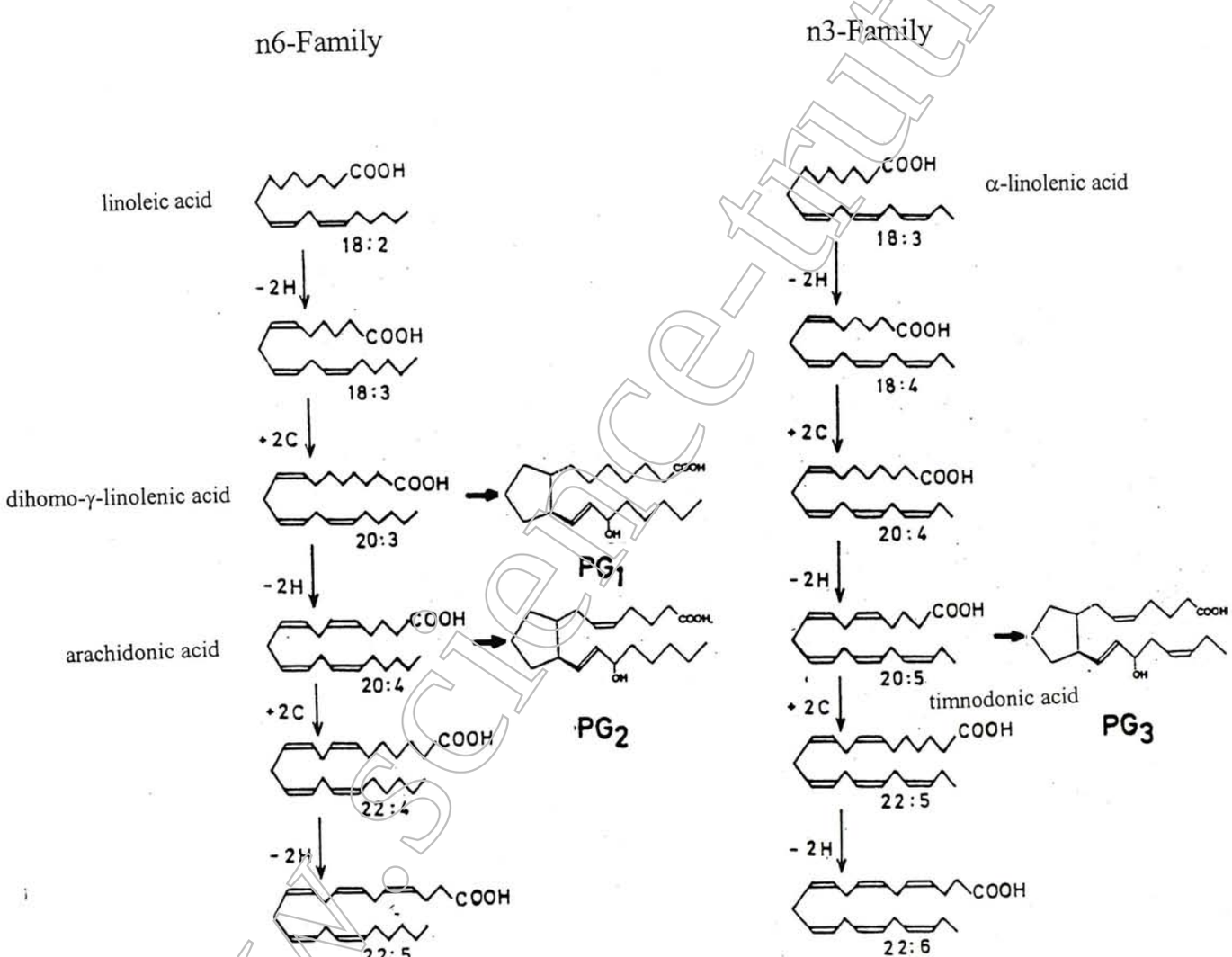
It was found in the present study that the Hr extract contained vitamins (A, C, E), terpenoids (e.g. campesterol, β-sitosterol, stigmasterol) and a number of fatty acids, such as oleic acid (octadec-9-enoic acid, 17.75%), linoleic acid (Octadeca-9,12-dienoic acid, 37.24%) and linolenic acid (Octadeca-9,12,15-trienoic acid, 29.66%). Thus, it is reasonable to assume that the multiple components of Hr extract may contribute to the antiulcer effects.

4.4.1 Role of fatty acids in the stomach protection

Dietary fatty acids are commonly present in the plant seeds, and important essential fatty acids in the diet are linoleic acid (18:2 n6) and α-linolenic (18:3 n3) acids, which both occur in plants (Bruneton, 1995). In the mammalian organism, these

fatty acids can be desaturated and elongated to form the "derived" essential fatty acid, dihomo-γ-linolenic acid (20:3 n6), arachidonic acid (20:4 n6) and timnodonic acid (20:5 n3), the three precursor acids of PGs (Paceasciak et al, 1983, cited in Figure 4.6).

Figure 4.6 The biosynthesis pathways of prostaglandins



Homo-γ-linolenic and arachidonic acids convert into PG_1 series (containing one double bond) and PG_2 series (two double bonds), respevtively. These polyunsaturated fatty acids and their precursor, linoleic acid, are members of the "n6-family" of fatty acids. Timnodonic acid, a member of " n3-family" derived from α-linolenic acid, is the precursor of PG_3 series (three double bonds). As the mammalian organism cannot induce double bonds at the n-3 and n-6 position of long-chain fatty acids, this partly explains why these fatty acids must be provided in the diet (Vergroesen, 1977). Polyunsaturated fatty acids are normal constituents of phospholipids in cell membranes (Bakhle, 1983) and seem to be importance for maintaining the fluidity and integrity of the membranes (Berlin et al., 1980). In gastrointestinal mucosa, the biosynthesis of PGs most likely due to a physiological need for maintaining mucosal cellular integrity (Whittle, 1977; Konturek et al., 1986).

In the present study, the major components of Hr seed extract are also found to be fatty acids including linoleic acid and linolenic acid (Table 4.5). It was also demonstrated that linolenic acid, one of the major components of the Hr extract significantly inhibited the formation of gastric lesions induced by ethanol (Table 4.8). These plant components have been found to be able to stimulate release of PGs over 400-folds above basal values with 5-10 minutes in rat gastric lumen (Doyle et al., 1989). Therefore, these fatty acids of Hr may help in the generation of PGs and subsequently help in the stomach protection. Moreover, the presence of these fatty acids seems to be related to the cytoprotective effect of Hr. For instance, hexane- and chloroform-extracts of Hr contained similar amount of fatty acids and they all showed significant antiulcer effect against ethanol-induced mucosal damage. Whilst the ethanol-extract of Hr did not contain fatty acids and failed to show any activity (Table 4.6).

It was suggested that the direct absorption of the soluble fatty acids by the gastric mucosa may play an important role in preventing gastric mucosa from damage (Chow et al., 1978; 1979; Tarnawski et al., 1983). However, the correlation between gastric protective effect of fatty acid and the content of prostaglandins in gastric mucosa is not conclusive (Mandel et al., 1994). It is known that endogenous PGs have a short biological half-life (minutes) after they have formed in the gastric mucosa and entered subsequently the portal circulation. They can be removed during the first pass through the liver and lungs and inactivated by 15-hydroxy PG-dehydroganase (15HD) (Konturek et al., 1986). Thus, a continuous supply of fatty acids is required for PG synthesis and subsequently the treatment of gastric mucosal damage. Experimental evidence suggested that the chronic treatment with dietary linoleic acid could increase the gastric mucosal defence and decrease gastric acid secretion (Tarnawski et al., 1986a). In addition, fatty acids have been proved to have beneficial influence on acceleration of repairing process of microvessels against mucosal lesions (Tarnawski et al., 1986b). Thus, the fatty acids present in the Hr extract may contribute to the cytoprotective activity of Hr.

4.4.2 Role of vitamins in the gastric protection

The present study indicates the presence of vitamins A, C and E in the Hr extract. These components may also show a positive contribution to the gastric protective effect.

Vitamin E is an well known free radical scavenger presented in various organisms and prevents the propagation of the peroxidative process (Infante, 1986). Vitamin E has several isomeric forms, namely α-, β-, γ-, and δ-tocopherol. The antioxidative activities of these isomers vary and α-tocopherol is proved to have highest

biologically activity (Infante, 1986). Thus the quantitative analysis of α-tocopherol was performed in this study.

It was suggested that gastric lesions may involve the degradation of the polyunsaturated fatty acids in the cellular membranes, and lipid-soluble vitamins, such as V_E, and V_A may produce a beneficial effects on gastric mucosa by their free radical scavenging actions (Javor et al., 1986). Previous study demonstrated that vitamin E showed gastric protective effect against various necrotizing agents and stress. It inhibited gastric acid secretion and maintained the basal levels of gastric nonprotein sulfhydryl compounds (Moutairy et al., 1996). However, the present study indicates that the content of vitamin E in the Hr extract is only 0.0026%, therefore, the cytoprotective effect of Hr cannot be solely accounted by the presence of vitamin E.

It was reported that vitamin C and carotenes had synergetic effect on vitamin E to enhance its antioxidative activity (Strohschein et al., 1998). Vitamin A and beta-carotene were also found to have cytoprotective effects on the inhibition of necrotizing agent-induced gastric lesions through scavenging free radicals (Javor et al., 1984). Therefore, the contribution of vitamins to the protective effect of Hr might be mainly associated to their antioxidative effects.

4.4.3 Role of plant terpenoids in the stomach

Previous studies suggested that β-sitosterol-β-D-glucoside and its aglycone, two components of *Hippophae rhamnoides*, inhibited stress-induced gastric lesions in rats and prevented acetic acid-induced stomach injury in mice (Zhang, 1989). These findings suggest that plant sterols and terpenoids may have a positive contribution to the antiulcer activity of Hr.

It has also been demonstrated in previous studies that terpenoids or essential oils can act as penetration enhancers. When these oils are applied transdermally as an adjunct in topical formulations, diffusion and partition of drugs into the dermal tissues are enhanced (Williams et al., 1991; Hori et al., 1991, Cornwell et al., 1996). Although interactions among the Hr ingredients were not tested in the present study, the synergetic effect among the plant components (e.g. fatty acids, vitamins and triterpenoids) cannot be ruled out as one of mechanisms related to the gastric protective action of Hr.

4.4.4 Summary

In the present study, multiple plant ingredients were found in the active fractions of Hr extract. As Hr possessed both anti-secretory effect on gastric acid and cytoprotective effect on chemical- and drug-induced stomach damage, it is hardly to attribute such broad actions to activities of one or two Hr components. Therefore, it is reasonable to assume that multiple constituents of Hr contribute to the gastric protective effects. In addition, the ratio of each type of ingredients in an extract may also be necessary for the protection actions. Disruption of such ratio may also lead to alterations in the activities. Further studies should be undertaken to clarify the interactive nature among these plant components.

Chapter 5

General Discussion

The present investigation confirmed that the pathogenesis of gastric ulcer is complex. As summarised in Table 2.1, a number of pathogenic factors can induce gastric lesions, such as high-concentration alcohol, necrotizing agents, smoke, stress, drugs (NASIDs, reserpine, etc), ischaema, excessive acid secretion, disorders in the central nervous system and abnormal physiological conditions (imbalance of hormone, 5HT, histamine, etc). These pathogenic factors can disturb the normal functions of the stomach, e.g. decrease of secretion of mucus, bicarbonate and non-protein sulfhydryl compounds; increase in H^+ back-diffusion, gastric acid secretion and gastric acidity; reduction of Na^+ transport; alteration of gastric mucosal blood flow, micro-circulation and gastric motility; increase in local ischaemia, vascular contraction and release of vasoactive products, histamine and ACTH-sterol hormones; decrease in transmucosal potential difference; disturbances of PGs synthesis; enhancement of leukotriene production, cell membrane permeability and the generation of free radicals; stimulation of local nerves, and induction of CNS disorders. These pathological alterations in the stomach may all either directly or indirectly damage mucosal integrity and finally induce ulcer. Six lesion models were established in the present study including ethanol-, necrotizing agents-, NSAIDs-, stress- and pylorus ligation-induced acute gastric lesions and an acetic acid-induced chronic ulcer. The pathogenesis of the gastric damage induced by these models relates to the most

pathogenic factors mentioned above, therefore, the anti-ulcer effect of *Hippophae rhamnoides* can be extensively evaluated in these models.

Because of the complexity of ulcer formation, the use of single drug may be not sufficient to treat peptic ulcers. In addition, peptic ulcers usually last a considerable period, long-term using a single remedy may lead to disturbance of the normal physiological conditions of the body and induce side effects. To overcome the disadvantages of using a single drug, combination therapy is widely accepted in the management of peptic ulcers. To further develop this idea, selection of potent plant medicines that consist of a number of ingredients and usually have less side-effects than a single entity, may be considered for development of antiulcer agents.

Natural products, including herbs and extracts of plants, have been used for the treatment of human diseases for thousands of years. It has been reported that 45% of the world population rely on natural remedies for their health care (Farnaworth et al., 1985). In China, great credence is given to traditional medicines, and herbal remedies play an important role in the health care system that represent 30 to 50% of the total consumption of medicines (Xiao et al., 1987). The importance of plants as resources of drug discovery has been recognized worldwide especially for searching for lead compounds for new drug development. In addition, the use of alternative medicines for prevention of diseases is on a rise worldwide in this decade.

Traditional Chinese medicines have been used for the treatment of various diseases including peptic ulcers. These natural remedies provide a rich resource for search for drug candidates. In the present study, *Hippophae rhamnoides* L. (Hr) was selected for evaluation of its antiulcer activity.

Evidence collected in this study suggest that the seed extract of *Hippophae rhamnoides* possesses gastric protective effect against ethanol-, necrotizing agents-,

NSAID-, stress- and pylorus ligation-induced acute gastric lesions. It also has ulcer-healing effect against acetic acid-induced chronic ulcers. It is widely accepted that the occurrence of peptic ulcer is a result of an imbalance between aggressive factors and defensive factors in the gastroduodenal mucosa. Taking this into account, the preparation of *Hippophae rhamnoides* can be considered as a good candidate for further developing anti-ulcer agent because its activities are associated with both protection of gastric mucosal integrity and inhibition of aggressive factors (e.g. gastric acid).

The cytoprotective effect of the Hr extract has been proved in the present study with ethanol- and necrotizing agent-induced lesion models. These stomach irritants induced gastric lesions via disruption of the defensive system in gastric mucosa. This included the decrease in GMBF and gastric motility; increase of the permeability of mucosa and ischemia in the tissue, enhancement of the release of free radicals and endogenous inflammatory mediators (e.g. LTs, histamine); induction of vascular injury that lead to back-diffusion of hydrogen ion; and inhibition of secretion of mucus and bicarbonate which disrupt the gastric mucosal barrier directly (Szabo et al., 1985; Szabo, 1987). The Hr extract at dose of 0.3-0.6 g/kg (i.g.) prevented the ulcer formation and protected mucosal integrity. Direct evidences have been collected to indicate the mechanisms of this protective action. It was demonstrated that the Hr extract can improve mucosal blood flow, modify the pathological changes of gastric motility, stimulate mucus secretion and GSH generation, increase free radical scavenging, neutralize gastric acid by increase of thickness of mucus layer and accelerate tissue regeneration.

In the present study, activity of Hr on inhibition of gastric acid secretion was demonstrated with pylorus ligation- and stress-induced lesion models. Gastric

secretion, no doubt, has a close relationship with gastric ulcer. The pylorus ligation-induced lesion model involved an accumulation of gastric acid and pepsin, the major causative factors for induction of mucosal damage. The present time-course study on pylorus ligation-induced lesion model exhibited that the occurrence and development of the gastric lesions are associated with accumulation of acid in the gastric lumen and increase of acid and pepsin output. Pretreatment with the Hr extract prevented pylorus ligation-induced lesion formation mainly via the inhibition of gastric acid and pepsin secretion.

Stress-induced gastric lesions involve complex mechanisms including the disturbance of gastric mucosal microcirculation, increase in gastric secretion, hypermotility of the stomach and disorders of the central nervous system. The Hr extract significantly inhibited the development of gastric lesions induced by stress and the mechanism of actions were also related to inhibition of gastric acid and pepsin secretion.

Drug-induced gastric lesions were investigated via NSAID-induced lesion model. The pathogenesis of this type of ulcer includes inhibition of prostaglandin production in the gastric mucosa and damage of the mucosal cells. The Hr extract reduced the NSAID-induced stomach damage and the mechanism of action may involve normalising PG synthesis and inhibiting gastric acid secretion.

The ulcer healing effect of Hr was evaluated with acetic acid-induced chronic gastric ulcer. This type of ulcer is similar to human peptic ulcers and pretreatment with Hr extract for 12 days was found to be able to increase mucus secretion, accelerate ulcer recovery and promote tissue regeneration. The mechanism of action may also relate to the inhibitory effect on gastric acid secretion that can help in the healing process.

In conclusion, Hr possesses multiple beneficial effects on the gastric mocosa. It can strength the mucosal defensive system, protect the integrity of gastric mucosa and

inhibit the aggressive factors in the ulcer formation. As cytoprotective and anti-ulcer effects of plant unsaturated fatty acids, phenolic compounds and terpenoids have be demonstrated in a number of studies, the presence of these plant components in the Hr extract should give a positive contribution to its anti-ulcer activities. Therefore, Hr can be considered as a potential plant anti-ulcer agent for the prevention and treatment of peptic ulcers.

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