CHAPTER 1

INTRODUCTION

Seaweeds or marine algae contain different vitamins, minerals, trace elements, protein, iodine, dietary fiber, essential fatty acids and bioactive substances (1-4). Marine algae are consumed in diet and as medicine in Asian countries such as Japan, China and Korea. Traditional medicines in Asia have used the marine algae for the treatment of cancer and to provide many health benefits (5, 6). Cell walls of marine algae characteristically contain sulfated polysaccharides (7), which are not toxic to humans, and are known to exhibit different biological properties, such as anticoagulant, anti-inflammatory, antiviral and antitumor activities (8, 9). The phenolic compounds presence in the seaweeds: *Sargussum ringgoldianum*, *Padina natillarum* and *Kappaphy alvarezzi* have been found to display antioxidant activity (10, 11).

Peptic ulcers (PU) are lesions in the stomach "gastric ulcer" or duodenum "duodenal ulcer" that occur as a result of the activity of acid and pepsin (12). Peptic ulcers are thought to be due to an imbalance of aggressive factors, such as acid production or pepsin, and defensive factors, such as mucus production, bicarbonate, and blood flow (13). Important mucosal defense mechanisms, which are complex process of "cytoprotection", include prostaglandins (particularly PGE_2), nitric oxide, calcitonin gene-related peptide, and a variety of gastrointestinal hormones.

Aggressive factors

1. Acid and pepsin

Gastric secretion contains mainly acid and pepsin. The secretion of gastric acid is controlled by multiple central (neuronal) and peripheral (endocrine and paracrine) factors (14). Each factor attributes to a common final physiological event

that secretion of H^+ by parietal cells, which are located in the body and fundus of the stomach.

Gastrin

Gastrin is present in the antral G cell and its principle biological activity is considered to be its role as a stimulant of hydrochloric acid secretion by gastric parietal cells (15). Gastrin stimulates acid secretion predominantly in an indirect manner by causing the release of histamine from ECL cells.

Acetylcholine (ACh)

Vagus nerve or parasympathetic nerve innervated gastrointestinal tract releases ACh, which acts directly on the parietal cells to increase acid and pepsin secretion (15). Moreover, stimulation of vagus nerve increases gastrin secretion from G cells in the gastric antrum. Vagal stimulation and the effect of gastrin stimulate release of histamine from the enterochromaffin-like (ECL) cells in the fundus (16).

Histamine

Histamine is released from ECL cells through multifactorial pathways and is a critical regulator or acid production through the H_2 subtype of receptor that link to the stimulation of adenylcyclase (AC), causing activation of the cyclic AMP (cAMP) (17).

Pepsinogens

Pepsinogen released from the chief cells. Pepsinogen occurs in response to two types of signals: 1) stimulation of the peptic cells by acetylcholine released from the vagus or other enteric nerves 2) stimulation of secretion in response to acid in the stomach (18).

2. Non-steroidal anti-inflammatory drugs (NSAIDs) (12)

NSAIDs are the most common cause of peptic ulcer disease in patients without *H.pylori* infection. Topical effects of NSAIDs cause submucosal erosions. In addition, by inhibiting cyclooxygenase, NSAIDs inhibit the formation of prostaglandins and their protective cyclooxygenase-2 mediated effects i.e., enhancing gastric mucosal protection by stimulating mucus and bicarbonate secretion and epithelial cell proliferation and increasing mucosal blood flow.

3. *Helicobacter pylori*

A Gram-negative spiral bacteria, *Helicobacter pylori* (*H. pylori*), has been associated with gastritis and subsequent of gastric and duodenal ulcer, that developed gastric adenocarcinoma (12). *H. pylori* bacteria adhere to the gastric mucosa; the presence of an outer inflammatory protein and a functional cytotoxin-associated gene island in the bacterial chromosome increases virulence and probably ulcerogenic potential (19).

4. Physiological and psychological stress (20)

Both physiological and psychological stresses play a role in the development of peptic ulcer in some patients. The most important structures for CNS stimulation of gastric acid secretion are the dorsal motor nucleus of the vagus nerve, and the hypothalamus. Efferent fibers originating in the dorsal motor nuclei descend to the stomach via the vagus nerve.

Defensive factors

1. Gastric mucus

The mucous neck cells located in the gastric gland and the surface epithelial cells of the stomach secrete mucus which is viscous and sticky and contains glycoproteins called mucins. Mucus is an insoluble viscous gel which acts as a barrier, protecting the gastric mucosa from acid, pepsin, bile salts, alcohol and other injurious agents (21). In addition, it is secreted in response to some stimuli that enhance acid and pepsinogen secretion, especially by acetycholine released from parasympathetic nerve ending near gastric glands. Moreover, prostaglandins also stimulate the synthesis, secretion of mucus and cause mucous gel layer thickness (17).

2. Gastric mucosal blood flow

The gastric mucosal blood flow is an important part of the defense, as the circulating blood dilutes, neutralizes and carries away noxious substances. The blood stream also has an important function in transporting oxygen, nutrients and gastric hormones to the different mucosal cell types. The gastric mucosal blood flow is regulated at the level of the submucosal arterioles and is under the intricate control of

the central and enteric nervous systems, autocrine and paracrine regulation of hormones and growth factors, and mucosal production of eicosanoids (22-24).

3. Protaglandins

Prostaglandin E2 (PGE_2) and prostacyclin (PGI_2) are believed a key mediator in gastric mucosal defense to have potent anti-ulcer and cytoprotective properties. They inhibit acid production by binding to the EP3 receptor on parietal cells to prevent gastric ulcer (25). Prostaglandins prevent stasis of gastric mucosal blood flow; induce superoxide dismutase, bicarbonate and mucus secretion and decrease HCl secretion (26-30).

4. Gastric mucosal barrier (31)

The protective mucus gel that form on the luminal surface of the stomach, and alkaline secretions entrapped within it, constitute a gastric mucosal barrier that prevents damage to the mucosa by gastric contents. The mucus allows the pH of the epithelial cells to be maintained at nearly neutral pH, despite a luminal pH of about 2. Mucus also slows the diffusion of acid and pepsin to the epithelial surface.

5. Bicarbonate

The surface epithelial cells secrete bicarbonate into the mucus gel. The bicarbonate neutralizes back-diffused acid and creates a pH gradient in the mucus layer, with a neutral pH at the cell surface when the luminal pH is low. Bicarbonate can be produced from carbon dioxide and water in the gastric mucosal surface epithelial cells by the enzyme carbonic anhydrase (22).

6. Nitric oxide (NO)

Nitric oxide has been reported to influence different components of gastric mucosal defense, such as mucosal blood flow, mucus secretion and mucosal permeability (22, 32-34).

Physiological and pharmacological regulation of gastric secretions: the basis for therapy of peptic ulcer disease by Hoogerwerf and Pasricha are illustrated in Figure 1. Gastric acid secretion is a complex, continuous process in which multiple central and peripheral factors contribute to a common endpoint: the secretion of H+ by parietal cells. Neuronal (acetylcholine, ACh), paracrine (histamine), and endocrine (gastrin) factors all regulate acid secretion. Their specific receptors (M_3, H_2, and CCK_2 receptors, respectively) are on the basolateral membrane of parietal cells in the body and fundus of the stomach. The H_2 receptor is a G-protein coupled receptor (GPCR) that activates the Gs-adenylylcyclase-cyclic AMP-PKA pathway. ACh and gastrin signal through GPCRs that couple to the Gq-PLC-IP_3-Ca^{2+} pathway in parietal cells. In parietal cells, the cyclic AMP and the Ca^{2+}-dependent pathways activate H^+,K^+-ATPase (the proton pump), which exchanges hydrogen and potassium ions across the parietal cell membrane. This pump generates the largest known ion gradient in vertebrates, with an intracellular pH of about 7.3 and an intracanalicular pH of about 0.8. (16)

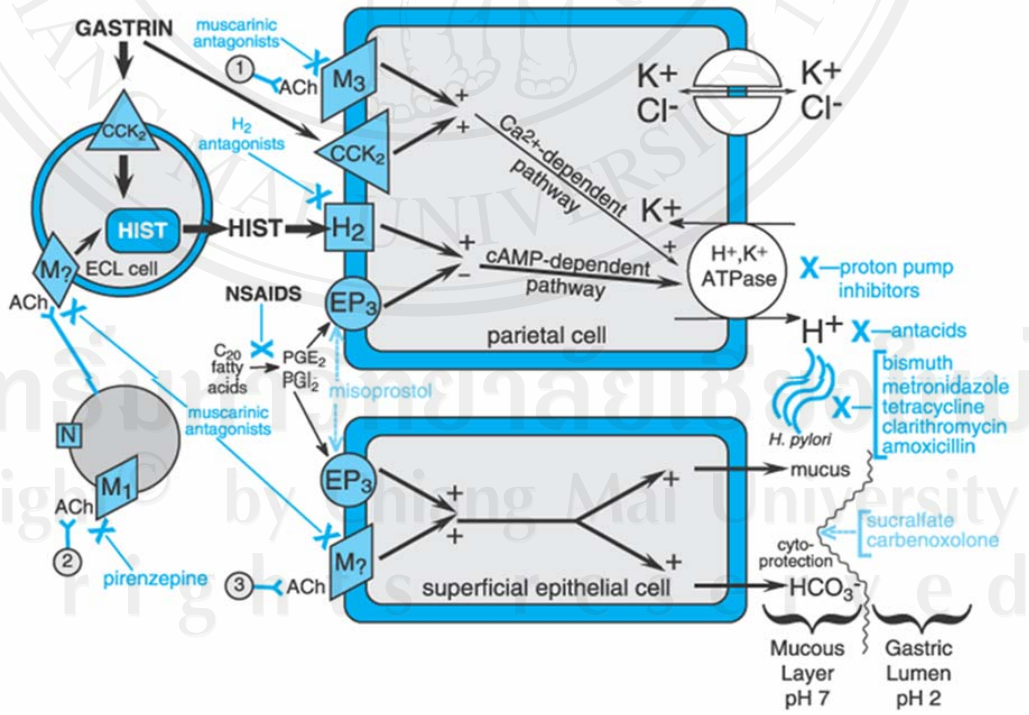

Figure 1 Physiological and pharmacological regulation of gastric secretions: the basis for therapy of peptic ulcer disease (16)

Drugs treatment of peptic ulcer

The goals of Therapy for peptic ulcer are relief from pain, promotion of healing and prevention of gastric recurrence. Drugs used in the treatment of acid-peptic disorders may be divided into two classes: agents that reduce intragastric acidity and agents that promote mucosal defense.

1. Neutralize gastric acid: Antacids (e.g. $Al(OH)_3$, $Mg(OH)_2$ and $CaCO_3$)

Antacids are weak bases that react with gastric hydrochloric acid to form a salt and water. Although their principle mechanism of action is reduction of intragastric acidity, they may also promote mucosal defense mechanisms through stimulation of mucosal prostaglandin production. Their usefulness in peptic ulcer disease appears to lie in their ability to reduce gastric acidity and, since pepsin is inactive in solutions above pH4.0, to reduce peptic activity (25).

2. Gastric anti-secretory drugs

2.1 H_2 receptor antagonists

H_2-receptor antagonists inhibit the secretion of gastric acid. Histamine, released primarily from mast cells, binds to H_2-receptors and activates adenylate cyclase, and thereby increases intracellular cyclic adenosine monophosphate (cAMP). The increased levels of cAMP activate the proton pump of the parietal cell to secrete hydrogen ions against a concentration gradient in exchange for potassium ions. H_2-receptor antagonists competitively and selectively inhibit the action of histamine on the H_2-receptor of the parietal cells, thus reducing basal and stimulated gastric acid secretion (12).

2.2 Proton pump inhibitors (eg. omeprazole, lansoprazole, rabeprazole pantoprazole and esomeprazole)

The proton pump inhibitors (PPIs) are highly specific inhibitors of gastric acid secretion. They act by irreversibly binding to K^+-H^+ATPase (an enzyme that transports acid across the parietal cell), these drugs inhibit basal and stimulated gastric acid secretion in a dose-dependent and sustained fashion. PPIs cause almost

total elimination of acid release because they inhibit the terminal step in the acid production cycle (12).

2.3 Muscarinic antagonists (eg. pirenzepine and telenzepine)

The anticholinergic compounds can reduce basal acid production by 40-50 %. Cholinoceptor antagonists are now rarely used and only as adjuncts to H_2-receptor antagonists, especially in patients' refractory to treatment with the latter or those with nocturnal pain.

2.3 Octreotide

Octreotide is a long-acting synthetic somatostatin analog. It has been found to significantly inhibit the secretion of several circulating peptide hormones and to inhibit gastric and pancreatic secretion.

3. Mucosal protective agents

3.1 Sucraflate

Sucralfate consists of the octasulfate of sucrose to which Al $(OH)_3$ has been added. In an acid environment (pH <4), sucralfate undergoes extensive cross-linking to produce a viscous, sticky polymer that adheres to epithelial cells and ulcer craters for up to 6 hours after a single dose. In addition to inhibiting hydrolysis of mucosal proteins by pepsin, sucralfate may have additional cytoprotective effects, including stimulation of local production of prostaglandins and epidermal growth factor (25).

3.2 Prostaglandin Analogs (eg. Misoprostol)

Misoprostol is a synthetic analog of prostaglandin E, is approved for the prevention of ulcers induced by the administration of NSAIDs. Its cytoprotective effect is mediated through enhancement of the synthesis and secretion of mucus and bicarbonate and inhibition of gastric secretion (25).

3.3 Collodial bismuth compounds

Bismuth subsalicylate is probably coat ulcers and erosions, creating a protective layer against acid. Bismuth compounds have direct antimicrobial activity against *H. pylori* and pepsin. It may also stimulate prostaglandin, mucus, and bicarbonate secretion (25).

3.4 Carbenoxolone

Carbenoxolone is a synthetic derivative of glycyrrhizic and has been shown to be effective in healing both gastric and duodenal ulcers. The mechanism of action of carbenoxolone is not clear but is thought to involve an increase in the production, secretion and viscosity of intestinal mucus.

4. Eradication of *H.pyroli*

Regimens for *H. pylori* eradication have been proposed. The combination therapies consist of proton pump inhibitor or H2-receptor antagonist or bismuth compounds with two or three antibiotics (plus acid-suppressive therapy) (25).

Free radicals

Free radicals are defined as any species capable of independent existence that contain one or more unpaired electrons in an orbital (35). They are generated in living organisms during metabolism. The free radicals include reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chloride species (RCS) (36, 37).

Reactive oxygen species (ROS) are small, highly reactive and the most important ones. They are produced in the forms of superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\bullet}$) (36, 37). Excessive amounts of ROS lead to oxidative stress which can initiate biomolecular oxidations resulting in cell injury, DNA damage, lipid peroxidation and cell death. The oxidative stress has been suggested to play roles in numerous diseases and disorders such as atherosclerosis, cancer, aging, peptic ulcer, stroke, etc. (37-41).

Oxidative stress

The term oxidative stress refers to a serious imbalance between production of reactive species and antioxidant defence (42) Sies *et al.* (1999) defined it as a disturbance in the prooxidant antioxidant balance in favor of the former, leading to potential damage. Possible causes of oxidative stress are: (42)

1. Diminished levels of antioxidants by

- mutation of antioxidants defense enzymes: superoxide dismutase, glutathione peroxidase
- toxins that deplete antioxidant defences.
- deficiencies in dietary minerals (e.g. Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Se) and/or antioxidants can also cause oxidative stress.

2. Increased production of reactive species by

- exposure of cells or organisms to elevated levels of O_2
- toxins that are themselves reactive species or are metabolized to generate reactive species
- excessive activation of "natural" systems producing such reactive species (e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases)

Consequences of oxidative stress can include (42)

1. Adaptation of the cell or organism by upregulation of defense systems, which may (a) completely protect against damage; (b) protect against damage to some extent but not completely; or (c) "overprotect" (e.g. the cells is then resistant to higher levels of oxidative stress imposed subsequently).

2. Cell injury: This involves damage (oxidative damage) to any or all molecular targets: lipids, DNA, protein, carbohydrate, etc.

3. Cell death: The cell may (a) recover from the oxidative damage by repairing it or replacing the damaged molecules, or (b) it may survive with persistent oxidative damage or (c) oxidative damage, especially to DNA, may trigger cell death, by apoptosis or necrosis.

Lipid peroxidation

In living animal cells, peroxidized membranes lose their permeability, becoming rigid, reactive and nonfunctional. Lipid peroxidation can produce singlet oxygen, hydroperoxides and lipid epoxides (43). The peroxidation of lipids is commonly described as an oxidative, oxygen-dependent deterioration of fats, notably the unsaturated fatty acids (44). Many damaging aldehydes are formed during lipid peroxidation, particularly Malondialdehyde (MDA, propanedial) and 4−Hydroxy

nonenal (4−HNE). MDA is a major metabolite of arachidonic acid (20:4) [fatty acid with 20−carbons & 4 double-bonds]. MDA assays (notably TBARS -- ThioBarbituric Acid-Reacting Substances) have been widely used as a measure of cell membrane lipid peroxidation (43, 45). Figure 2 depicts the basic reaction sequence of lipid peroxidation and final products.

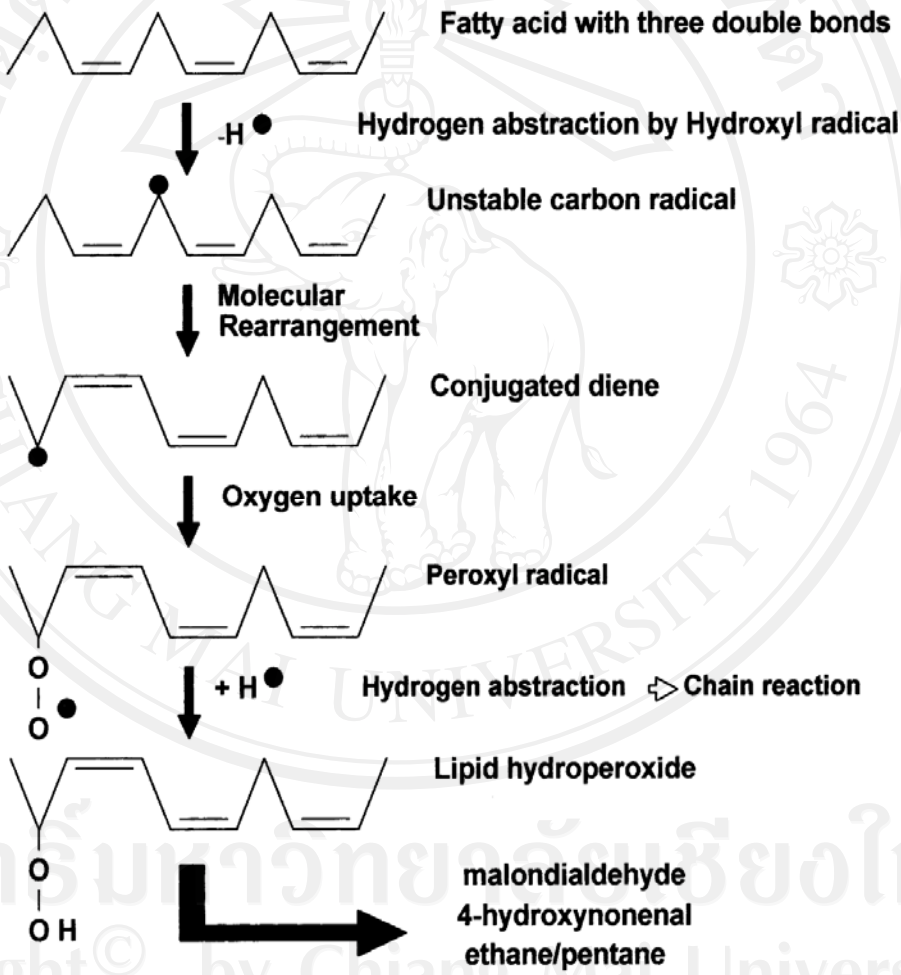

Figure 2 Basic reaction sequence of lipid peroxidation and final products (42-45)

Roles of free radicals in the gastric ulcer

The importance of reactive oxygen species in the mechanism of acute gastric superficial erosions has been demonstrated by the fact that several scavengers reduced these erosions induced by indomethcin (46, 47) ethanol (46, 48, 49) and stress (50). Although more direct evidence for the tissue-damaging actions of reactive oxygen species in gastric tissue has come from studies where local generation of superoxide or hydrogen peroxide caused gastric mucosal bleeding and mucosal necrosis (51, 52). The role of these species in the pathogenes is of human gastric ulcer remains to be elucidated (53).

Roles of free radical in the hepatic damage

Oxidative stress plays roles in liver pathologies and progression, thus the use of antioxidants has been proposed as therapeutic agents, as well as drug coadjuvants, to counteract liver damage (54). Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages (55-58).

Carbon tetrachloride (CCl_4) is a classical hepatotoxicant that causes rapid liver damage progressing from steatosis to centrilobular necrosis (59, 60). CCl_4 is known to induce reactive oxygen formation, deplete GSH of phase II enzyme, and reduce antioxidant enzyme and antioxidant substrates to induce oxidative stress that is an important factor in acute and chronic liver injury. The liver injury induced by CCl_4 is resulted from free radicals and lipid peroxidation that cause hepatic cell damage. CCl_4 requires bioactivation by phase I cytochrome P450 system in liver to form reactive metabolic trichloromethyl radical ($CCl_3^{\bullet}$) and proxy trichloromethyl radical ($^{\bullet}OOCCl_3$). These free radicals can bind with polyunsaturated fatty acid (PUFA) to produce alkoxy ($R^{\bullet}$) and peroxy radicals ($ROO^{\bullet}$), that in turn generate lipid peroxide, cause damage in cell membrane, change enzyme activity and finally induce hepatic injury or necrosis (61-64) Figure 3 illustrates biotransformation of carbon tetrachloride

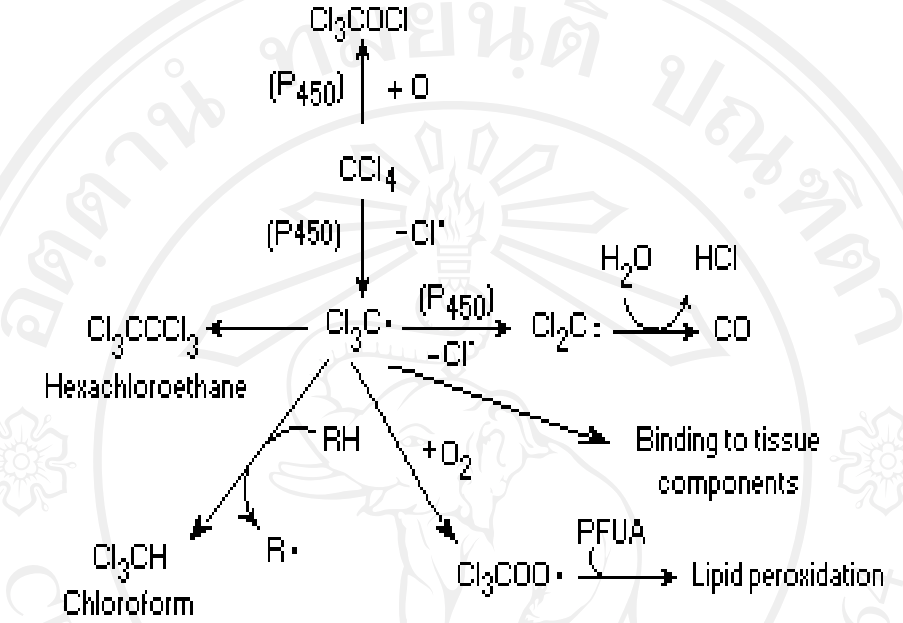

Figure 3 Biotransformation of carbon tetrachloride. (59, 61, 62)

Antioxidant in natural plants

Antioxidants from natural sources are preferred by consumers (65) due to concerns on the toxic and carcinogenic effects of synthetic antioxidants. The plant phenolics can act as ROS scavengers, metal chelators and enzyme modulators and prevent lipid peroxidation (66). Phenolic compounds include a large class of phytochemicals with interesting biological properties (67, 68). In recent years there have been many reports in the literature on the role of these natural compounds in counteracting the negative effects of oxygen and nitrogen reactive species (ROS/RNS), maintaining the redox homeostasis of biological fluids. It is commonly recognized that antioxidants can neutralize potentially harmful reactive free radicals in cells before they induced lipid and proteins oxidation (69). Antioxidants from plants are believed to be useful in preventing aging, atherosclerosis, cancer, peptic ulcer , liver diseases and other degenerative pathologies such as cancer, diabetes, Alzheimer's and Parkinson's diseases (39-41, 70-73)**.**

Some of seaweeds extracts have been demonstrated to have strong antioxidant properties. The phenolic compounds presence in the red seaweed: *Sargussum ringgoldianum* and *Padina natillarum* and in the brown seaweed *Kappaphy alvarezzi* displayed antioxidant activity (10, 11). Furthermore, some seaweed has been found to have complex systems of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase (10).

Seaweeds with anti-gastric ulcer, hepatoprotective and antioxidant activities

Anti-gastric ulcer, hepatoprotective and antioxidant activities of some marine algae have been reported. The aqueous extract of *Gracilaria fisheri* (a red seaweed) showed anti-gastric ulcer when tested in rats of which the gastric ulcers were induced by stress, HCl/ethanol and indomethacin. In addition, an antioxidant activity of *G. fisheri* was observed when tested in (1, 1-diphenyl-2-picrylhydrazyl) DPPH, (2, 2' -azinobis-[3-ethylbenzthiaoline-6-sulphonic acid]) ABTS, lipid peroxidation assays (74). Some of brown algae (*Sargassm* spp.) also exhibited an antioxidant activity. The aqueous extract of *S. polycystum* showed the activity when tested in the 3 models previously stated (75). In addition, it was found that the alcoholic extract of *S.*

polycystum caused an improvement of the hepatic mitochondrial antioxidant defense tsystem against free radicals generated, which might be attributed to the presence of sulfated polysaccharide compounds in the extract (76). Interestingly, quite a number of marine algae have been reported to have hepatoprotective activity. The alcoholic extract of *S. polycystum* exerted a protective effect against acetaminophen induced hepatotoxicity in rats (77). Aqueous extracts of *S. henslowianum, S. siliquastrum* and *Myagropsis myagroides* could protect hepatic damage in rats induced by CCl_4 administration (78). The hepatoprotective activity against galactosamine induced hepatic damage was observed in rats receiving the methanolic extract of *G. edulis* (79). Additionally, the alcoholic and aqueous extracts of *Ulva lactuca* (a marine green alga) showed protective effect against galactosamine induced hepatic damage in rats. The protective effect is suggested to be mediated via the antioxidant defense system of the liver by increasing the activities of antioxidant enzymes and decreasing the levels of lipid peroxides (69).

Background of *Caulerpa racemosa* var. *cylindracea*

Caulerpa is a genus of green seaweeds of the Family Caulerpaceae, Division Chlorophyta. As shown in Figure 4, *Caulerpa racemosa* var. *cylindracea* has erect fronds up to 11 cm high bearing un-crowded vesiculate ramuli that are radially or distichously arranged. Fronds are slightly inflated above the attachment to the stolon which is fixed to the substrate by thin short rhizoids (80, 81). Figure 5 shows the ramuli of *Caulerpa recemosa*. Some species of *Caulerpa* (especially *C. lentillifera* and *C. racemosa*) are eaten under the names of sea grape or green caviar (82).

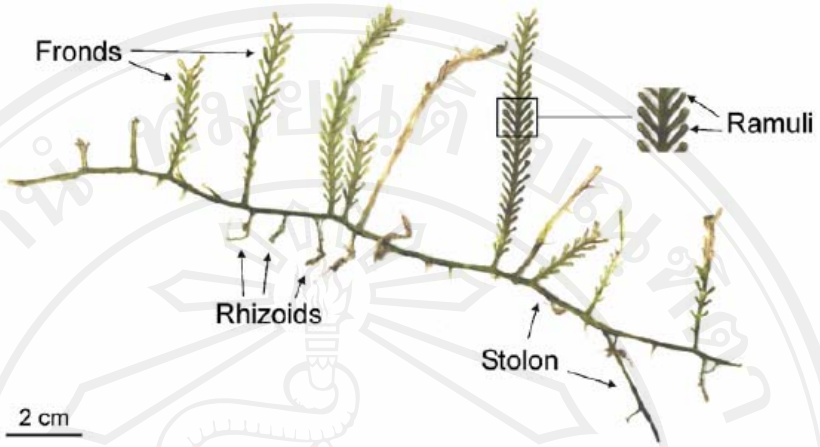

Figure 4 Morphology of the invasive *Caulerpa recemosa* showed stolon, rhizoids, fronds and ramuli (81)

Figure 5 The ramuli of green seaweeds *Caulerpa recemosa* var. *cylindracea*

C. racemosa have been used in traditional medicines to reduce blood pressure and to treat rheumatism (10). Phytochemically, they have been found to contain sulfated polysaccharides, caulerpin, and caulerpenyne. The polysaccharides showed potent inhibitory effects against herpes simplex virus types 1(HSV-1) and types 2 (HSV-2) in Vero cells, and lacking cytotoxic effect (83). The compounds of caulerpin and caulerpenyne displayed anti-tumor activity *in vitro* (84-86) and cytotoxic properties (87-90), respectively. Additionally, caulerpenyne showed anti-proliferative and apoptotic effects (91). Furthermore, *C. racemosa* has been found to contain phenolic substances (10) and antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase (10, 92).

However, no study on the anti-gastric ulcer and hepatoprotective activities of *C. racemosa* (a green algae) has to the best of our knowledge, yet been reported. In our preliminary study, the aqueous extract of *C. racemosa* was found to exhibit antioxidant and anti-gastric ulcer (at the dose 500 mg/kg) activities when it is tested in the ABTS assay and restraint water immersion-induced gastric ulcer in rat model, respectively. Therefore, the present study is carried out to investigate the anti-gastric ulcer and antioxidant activities of *C. racemosa*. The test for hepatoprotective activity of *C. racemosa* is also included in the study since it is likely that its antioxidant activity might play a role in the hepatoprotective activity.

Purposes of the study

The purposes of the present study were:

1. to study the anti-gastric ulcer activity of the aqueous extract of *C. racemosa* in rats
2. to investigate the hepatoprotective activity of the aqueous extract of *C. racemosa* in rats with CCl_4-induced hepatotoxicity
3. to study the antioxidant activity of the aqueous extract of *C. racemosa*
4. to determine total phenolic content of the aqueous extract of *C. racemosa*