CHAPTER 1

Introduction

1.1 Statement of the problem

Colorectal cancer is the third most common cancer in the world (Society, 2014). In Thailand, colorectal cancer is the third rank in male and the fourth rank in female (Imsamran *et al.*, 2015). It has annually increased from 12.9 in male and 9.2 in female during 2004-2006 (Khuhaprema *et al.*, 2012) to 14.4 in male and 11.2 in female during 2010-2012 (Imsamran *et al.*, 2015). Risk factors of colorectal cancer are associated with either genetic alteration or environment. Inflammation acts as a key regulator of promotion and progression stages of carcinogenesis by several mechanisms including acceleration of cell cycle progression and cell proliferation, evasion from apoptotic cell death, and stimulation of angiogenesis (Philip *et al.*, 2004). Chronic inflammatory diseases particularly ulcerative colitis and Crohn's disease increased risk of colorectal cancer (Rhodes *et al.*, 2002).

The animal models of carcinogen induced colorectal carcinogenesis have become the prominent model for studying pathogenesis of colorectal carcinogenesis and evaluating chemopreventive agents for colorectal cancer (De Robertis *et al.*, 2011). Chemical carcinogens such as 1, 2-dimethylhydrazine (DMH) and azoxymethane (AOM) are widely used to induce colon tumors in rodents with pathological features similar to human sporadic colorectal cancer (Johnson *et al.*, 2013). Aberrant crypt foci (ACF) are putative preneoplastic lesions of colorectal cancer (Suzui *et al.*, 2013). They can be easily induced by DMH or AOM in colon of rodents. The ACF system is also a rapid and reliable assay for screening of chemopreventive agents (Krishnan *et al.*, 2000). Several models of sporadic and inflammation-related colorectal carcinogenesis in rodents have been developed in the last decade, including chemically induced colorectal carcinogenesis models and genetically engineered mouse models (De Robertis *et al.*, 2011). Among of

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chemically induced colorectal carcinogenesis models, the model of the combination of DMH or AOM with the inflammatory agent, dextran sodium sulfate, in rodents has been established to shortly induce colorectal cancer and to resemble the ACF-adenoma-carcinoma sequence that occurs in human colorectal cancer (Tanaka *et al.*, 2003).

Nowadays, numerous dietary constituents from natural products have been used for preventing several diseases, including cancer (Mehta *et al.*, 2010). Algae especially marine algae, have served as important sources of bioactive natural substances (Pal *et al.*, 2014). Moreover, numerous compounds isolated from algae have been shown to possess several biological activities and potential health benefits (Sharifuddin *et al.*, 2015). Therefore, a new trend to isolate and identify bioactive compound(s) from algae has been risen. Algae contain large amounts of polysaccharides. The cell walls of algae are rich in sulfated polysaccharides such as fucoidans in brown algae, carrageenans in red algae and ulvans in green algae. These sulfated polysaccharides exhibited many beneficial biological activities such as anticoagulant, antiviral, antioxidative, anticancer and immunomodulating activities (Costa *et al.*, 2010). Therefore, sulfated polysaccharides derived from algae have great potential for further development as products in pharmaceutical and medical applications.

Spirogyra neglecta (Hassall) Kützing is freshwater green macroalgae. This alga is consumed as a tradition food in the Northern Province of Thailand. *S. neglecta* contains high amounts of nutrients and dietary fiber (Thumvijit *et al.*, 2013b). Our group reported *S. neglecta* showed antioxidant (Thumvijit *et al.*, 2013a), antimutagenic (Thumvijit *et al.*, 2013a) and cancer chemopreventive (Thumvijit *et al.*, 2014) activities. Therefore, the main objective of this study is to determine chemopreventive effect of *S. neglecta* on either chemical induced or inflammation associated colorectal carcinogenesis.

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1.2 Literature reviews

1.2.1 Colorectal carcinogenesis

1) Epidemiology of colorectal cancer

Colon cancer is a major cause of cancer mortality in many industrialized countries (Haggar *et al.*, 2009). In Thailand, colon cancer is the third and fourth leading cancer in male and female, respectively (Imsamran *et al.*, 2015). It has annually increased from 12.9 in male and 9.2 in female during 2004-2006 (Khuhaprema *et al.*, 2012) to 14.4 in male and 11.2 in female during 2010-2012 (Imsamran *et al.*, 2015). The incidence of colorectal cancer associated several risk factors including age, hereditary factors environment and lifestyle. Thus, physical activity, obesity, cigarette smoking and heavy alcohol consumption play an important role in the development of colorectal cancer (Haggar *et al.*, 2009).

2) Molecular mechanisms of colorectal carcinogenesis

Colorectal carcinogenesis generally occurs in a slow and stepwise process of accumulating mutations under the influence of genetic and environmental factors. This process is called adenoma-carcinoma sequence. The adenomatous polyp is the precursor lesion that can become colorectal adenocarcinoma (Levine *et al.*, 2006). During various stages of colorectal carcinogenesis, several genetic mutations may be found. However, malignant transformation in the colon do not need all of these sequences (Figure 1.1).

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Activation of the Wnt/β-catenin signaling pathway has been recognized as one of the main events in the development of colonic polyps (Fredericks *et al.*, 2015). Most sporadic colorectal cancer is associated with aberration of the Wnt/β-catenin signaling pathway and adenomatous polyposis coli (APC) gene dysfunction (Figure 1.2) (Fredericks *et al.*, 2015). APC is a tumor suppressor gene that plays an important role in the Wnt/β-catenin signaling pathway. APC mutation is found in adenomatous polyps before other genetic alterations have occurred (Takahashi *et al.*, 2004). In the presence of Wnt/β-catenin signaling or mutated APC, phosphorylation of GSK-3β is inactivated and leads to reduced β-catenin phosphorylation. The cytoplasmic β-catenin translocated to the nucleus and binds with Tcf/Lef transcription factors, leading to transcription of canonical

Wnt target genes such as c-myc, cyclin D1 and matrix metalloproteinase matrilysin. These genes, which are regulated by the β-catenin/Tcf/Lef complex, play critical role in various steps of neoplastic transformation. *C-myc* controls neoplastic growth, while *cyclin D1* controls cell cycle progression and the *matrix metalloproteinase matrilysin* regulates tumor invasion (Pandurangan, 2013). Another event regularly that observed at the early stages of adenoma development is DNA hypermethylation of CpG-islands in the promoter regions of genes involved in cell cycle control and DNA repair, such as p16INK4a and hMLH1 (Fredericks *et al.*, 2015).

On the progression to intermediate adenoma, the event observed is K-ras oncogene mutation. The K-ras oncogene mutation is found in approximately 50% of sporadic colorectal adenomas and carcinomas (Rajagopalan *et al.*, 2003). The mutation in K-ras leads to activation of the Ras/Raf/MAPK signaling pathway, resulting in the abnormal activation of cell proliferation and growth and contribute to malignant progression (Karreth *et al.*, 2009). Microsatellite instability (MSI) and activation of cyclooxygenase-2 (COX-2) were also observed in this stage (Ullman *et al.*, 2011).

Loss of tumor suppressor genes such as DCC and DPC4 and LOH of the second intact alleles often observed during development of intermediate adenoma to malignant adenocarcinoma. The mutation of tumor suppressor p53 was observed in the carcinomas (Fredericks *et al.*, 2015).

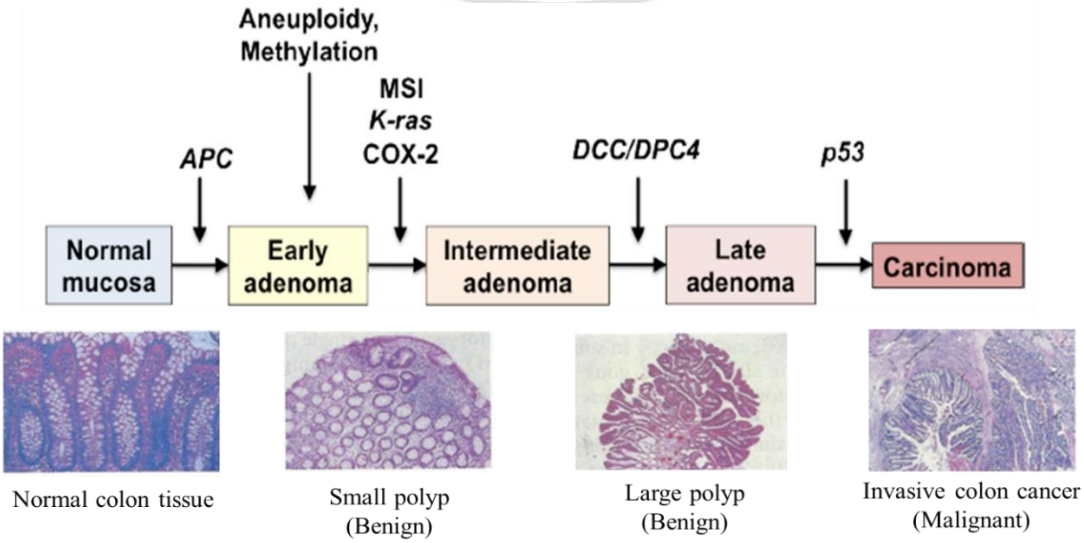

Figure 1.1 Gene alteration during colorectal carcinogenesis (Ullman *et al.*, 2011).

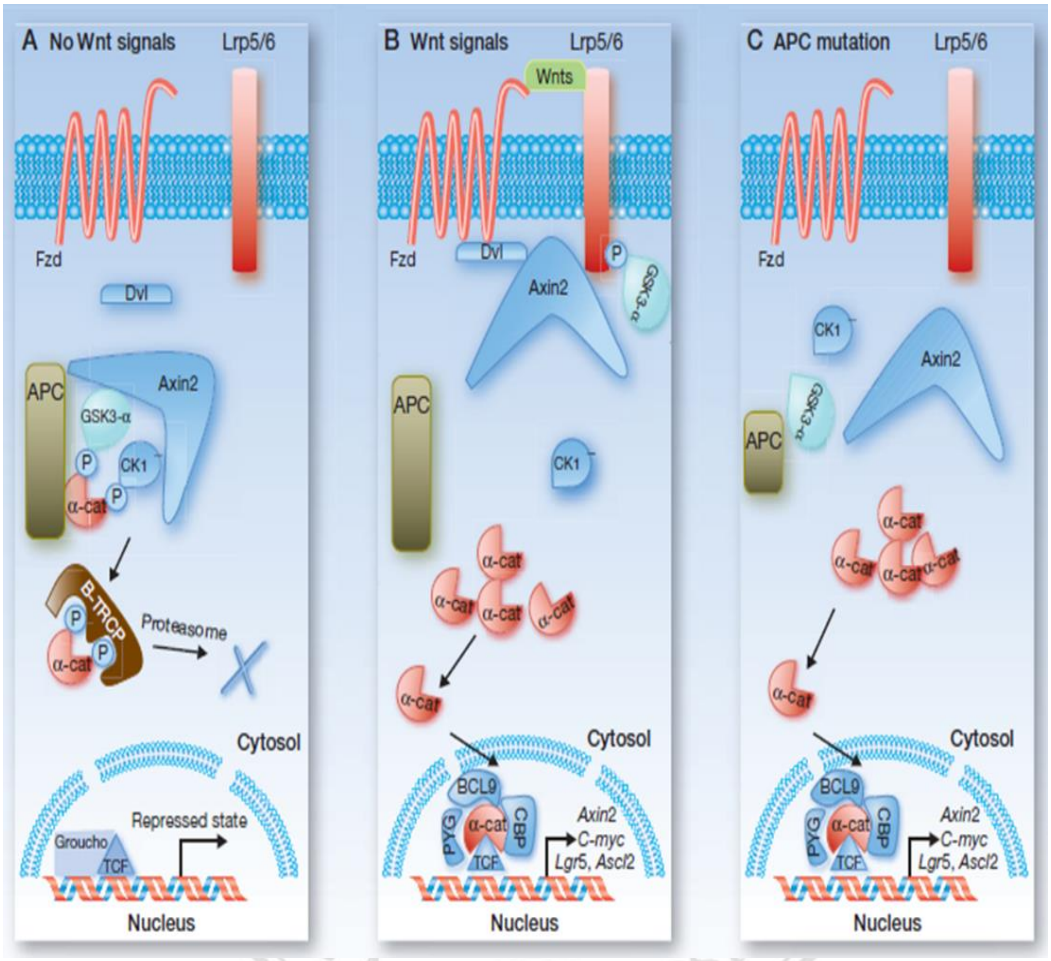

Figure 1.2 The Wnt canonical pathway. A; the absence of Wnt ligands, B; the presence of Wnt ligands, C; truncating mutation in APC (de Sousa *et al.*, 2011).

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3) Colitis-associated colorectal cancer

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are related to development of colorectal cancer. There are prominent differences in the histomorphology during sporadic and colitis-associated colorectal carcinogenesis development. The colitis-associated colorectal carcinogenesis has believed to occur by a progression from normal, indefinite dysplasia, low-grade dysplasia, high-grade dysplasia and carcinoma, respectively (Figure 1.3) (Ullman *et al.*, 2011). Genetic analysis showed a similar genetic alteration in colitis-associated colorectal carcinogenesis compared to sporadic colorectal carcinogenesis, suggesting that colitis-associated colorectal carcinogenesis also is a multistep process (Itzkowitz *et al.*, 2004). However, genomic alteration in colitis-associated colorectal carcinogenesis is different from sporadic colorectal carcinogenesis (Figure 1.3). The mutation of p53 is commonly found earlier in indefinite dysplasia, than other genes including DCC, K-ras and APC (Itzkowitz *et al.*, 2004).

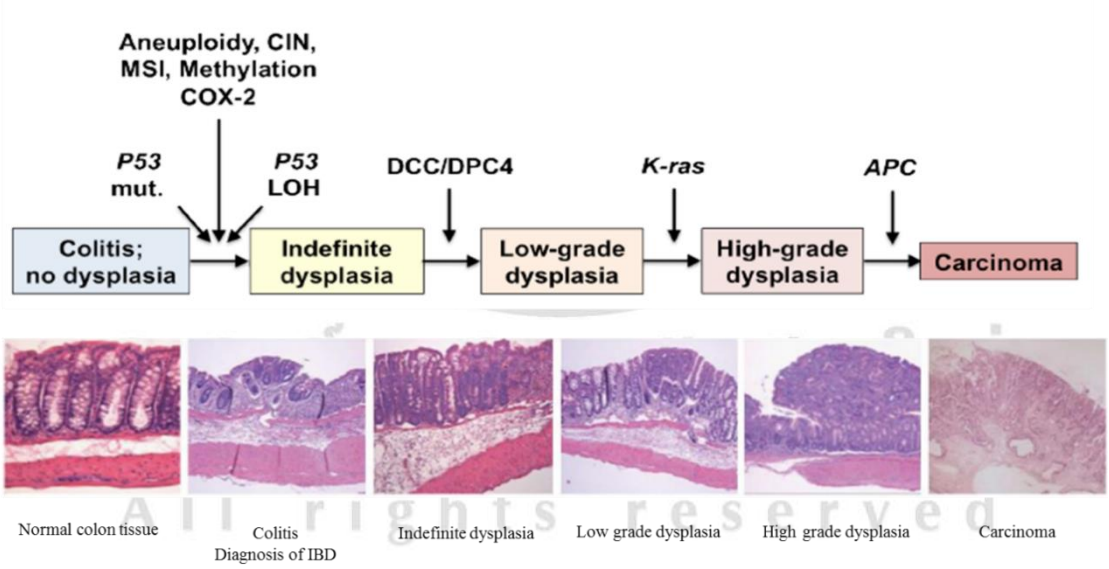

Figure 1.3 Gene alteration during colitis-associated colon cancer (Ullman *et al.*, 2011).

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4) Experimental colorectal carcinogenesis

The animal models of colorectal cancer have provided data on the initiation, promotion and progression of carcinogenesis, including particular information on cellular transformation and the subsequent events leading to the formation of neoplastic lesions (Rosenberg *et al.*, 2009). These animal models are induced by carcinogens (Rosenberg *et al.*, 2009) and modified by genetic alteration (Taketo *et al.*, 2009). The established models can be used for cancer chemopreventive studies as well.

4.1) Chemical induced models of colorectal cancer

The chemical induced animal models have been useful for understanding of molecular mechanisms in the sporadic colorectal cancer. Dimethylhydrazine (DMH) and its metabolite, azoxymethane (AOM), are commonly used as colon specific carcinogens for understanding of the molecular mechanisms underlying sporadic colorectal carcinogenesis. Metabolism of DMH and AOM involves several xenobiotic-metabolizing enzymes (Figure 1.4) (Rosenberg *et al.*, 2009). DMH is metabolized to AOM and methylazoxymethanol (MAM) in liver and transfered to colon via bile or blood system. Before transferring to colon, MAM is conjugated in the liver with glucuronic acid or sulfate or glutathione. This conjugate is transported to colon and converted to a reactive methyl cation by bacterial enzymes. A reactive methyl cation alkylates macromolecules and deoxyguanosines at the O^6 and N^7 positions (O^6-methyl-deoxyguanosine and N^7-methyl-deoxyguanosine) (Rosenberg *et al.*, 2009).

DMH and AOM administration induces mutation of β-catenin, which includes the GSK-3β phosphorylation site. The mutation constitutively activates the Wnt-signaling pathway. There are many similar events between human colon cancer and AOM- or DMH-induced colon cancer in rats (Taketo *et al.*, 2009).

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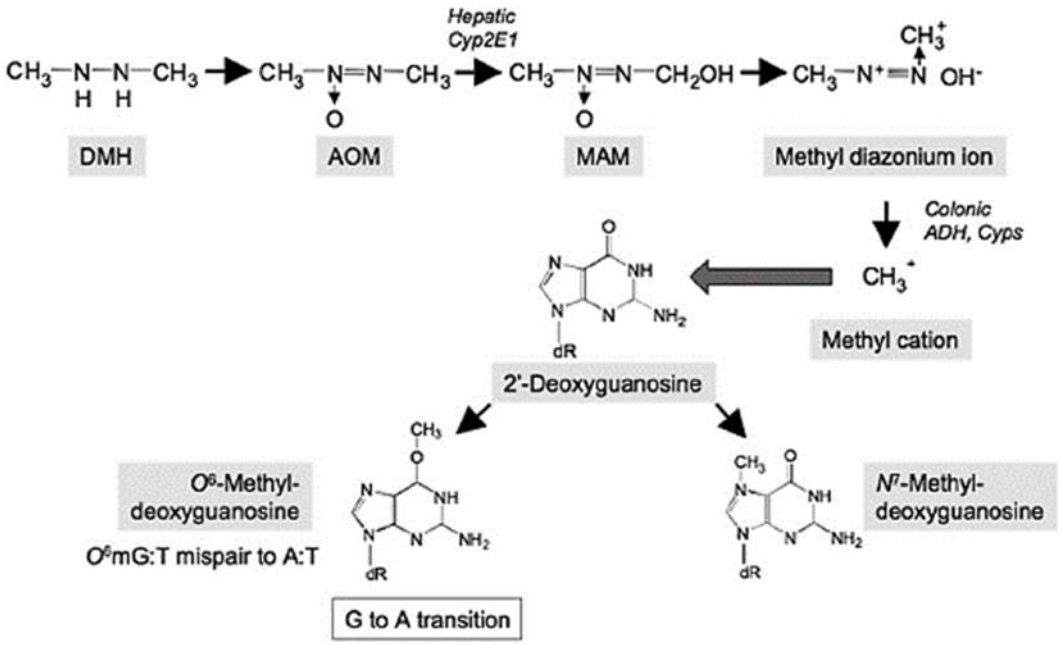

Figure 1.4 Metabolism of 1, 2-dimethylhydrazine and azoxymethane (Rosenberg *et al.*, 2009).

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4.1.1)The AOM/DSS model of colitis-associated cancer

Many studies have suggested that chronic or constant mucosal inflammation may result in tumorigenesis through several mechanisms including induction of genetic mutation, increase of cryptal cell proliferation, change of crypt cell metabolism and alteration of the bacterial flora profile (Rosenberg *et al.*, 2009). The inflammation associated colorectal cancer model supports the hypothesis that chronic inflammation in IBD plays a critical role in epithelial malignant neoplasia in a large intestine (Seril *et al.*, 2003).

A novel colitis induced mouse colorectal cancer model initiated with AOM and promoted by dextran sodium sulfate (DSS) has been developed to obtain the understanding of the pathogenesis of IBD-associated colorectal cancer (De Robertis *et al.*, 2011). In colitis-associated colorectal cancer model, mice were injected with a low dose of a colon specific carcinogen, AOM (10 mg/kg bw), followed by one or more consequent cycles of the nongenotoxic irritant DSS to develop tumors. The subsequent dysplasia and neoplasm show positive staining for β-catenin, COX2 and iNOS, but did not show evidence of p53 immunoreactivity (Tanaka, 2012). This novel mouse model combining AOM with DSS might be used for investigating colitis-associated colorectal cancer development. Moreover, mice received a single low dose of various colon carcinogens such as DMH and PhIP followed by exposure of 2% DSS for 1 week, have shown a high incidence of tumor formation within 20 weeks (Rosenberg *et al.*, 2009). In summary, the AOM or DMH/DSS model with mice and rats were established to be a useful tool for investigating the pathogenesis and chemoprevention of colitis-associated colorectal carcinogenesis.

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4.2) Genetic animal models of spontaneous colorectal cancer

4.2.1)The *Apc*^{Min} mice

The *Apc*^{Min} mouse is one of germ-line mutant mouse of intestinal neoplasia, which carries an APC gene mutation, thereby mimicking human familial adenomatous polyposis. The mutation within APC shares the common feature to destroy APC function and to destabilize the cytoplasmic β-catenin complex, which results in constitutive active Wnt-signaling pathway (Fodde *et al.*, 2001).

4.3) Genetic animal models of spontaneous colitis and colitis-associated colorectal cancer

4.3.1) STAT3-immune knockout mice

STAT3 is an oncogenic transcription factor, which regulates the transcription of several survival and anti-apoptotic genes such as *c-Myc*, *Bcl-XL* and *cyclin-D1* (Bowman *et al.*, 2000). Additionally, it regulates the expression of intestinal defensive proteins in intestinal epithelial cells such as RegIIIβ/PAP and several mucins (Bollrath *et al.*, 2009). Activation of STAT3 was observed in several human cancers including colorectal adenocarcinoma and adenoma (Bowman *et al.*, 2000). The STAT3-immune knockout (STAT3-IKO) mice develop spontaneous chronic colitis and colonic polyps (Deng *et al.*, 2010). Approximately 16% of STAT3-IKO mice, invasive adenocarcinoma was developed from polyps, which is a frequency comparable to human IBD patients (Deng *et al.*, 2010). The colitis and colitis-associated colorectal cancer are probably promoted by IL-10 signaling deficiency in STAT3-IKO mice. The overproduction of cytokines such as IL-6, IL-23 and IL-22 was observed in STAT3-IKO mice might lead to STAT3 activation in intestinal epithelial cells (Deng *et al.*, 2010). These findings suggest that loss of STAT3 signaling is required for the spontaneous generation of colitis-associated colorectal cancer in mice.

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5) Aberrant Crypt Foci

Aberrant crypt foci (ACF) are an important marker of colorectal cancer (Fenoglio-Preiser *et al.*, 1999). ACF in methylene blue stained rodent colon treated with colon specific carcinogens were firstly described by Bird (Figure 1.5). ACF were defined as crypts that have the following: altered luminal openings, thickened epithelia, and larger than neighboring normal crypts (Bird *et al.*, 1989). The chemicals induced colorectal cancer models, rats treated with colon specific carcinogens such as DMH and AOM develop aberrant crypts that represent microadenomas. Moreover, many studies have shown a dose-response relationship between carcinogens and the number of induced ACF (McLellan *et al.*, 1991). Characteristics of ACF, such as size and crypt multiplicity, also have been shown to be predictors of the incidence of colorectal cancer in carcinogen-treated animals (Pretlow *et al.*, 1992). The ACF system is a rapid and reliable assay system for the quick and reliable screening of potential chemopreventives agents (Gupta *et al.*, 2007).

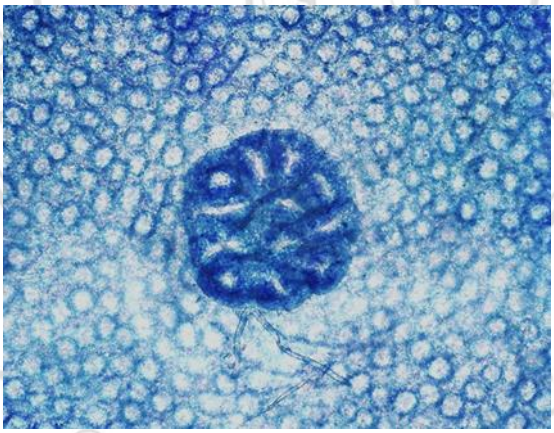

Figure 1.5 Aberrant Crypt Foci (ACF) in rat colon.

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1.2.2 Cancer chemoprevention

Chemical carcinogenesis is a multistep process, including initiation, promotion and progression (Figure 1.6). Initiation involves ultimate carcinogen that bind directly to DNA and lead to DNA damage. This step is rapid and irreversible. Promotion involves epigenetic factors that influence the proliferation of the initiated cell and lead to be a benign lesion. This process is generally reversible. Progression, the final stage of neoplastic transformation, involves the growth of a tumor with invasive and metastatic potential. This step is also generally irreversible (Barrett *et al.*, 1987).

Cancer chemoprevention is involved in the prevention, delay and reversal of cancer by consumption of dietary agents capable of modulating the process of carcinogenesis (De Flora *et al.*, 2005).

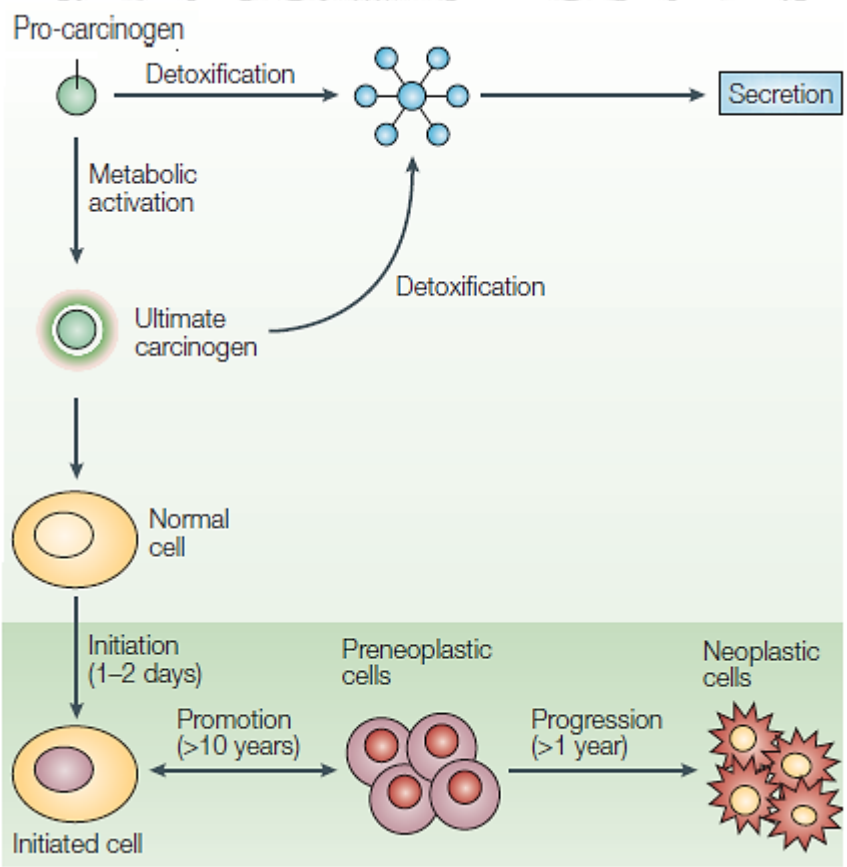

Figure 1.6 The process of chemical carcinogenesis stages (Surh, 2003).

1) Chemoprevention strategies

The goal of chemoprevention is to either delay or reverse at all stages of the development of carcinogenesis. Chemoprevention can be classified into 3 strategies, including primary prevention, secondary prevention and tertiary prevention (Figure 1.7). The primary prevention has the goal of preventing carcinogenesis in healthy and high risk person such as smokers. This strategy includes inhibition of mutation and cancer initiation, either in the extracellular environment or inside cells, followed by inhibition of tumor promotion. The secondary prevention acts to inhibit progression of a diagnosed benign tumor towards malignancy. The strategies of tertiary prevention are to inhibit invasion and metastasis and to prevent development of tumor or recurrence (De Flora *et al.*, 2005).

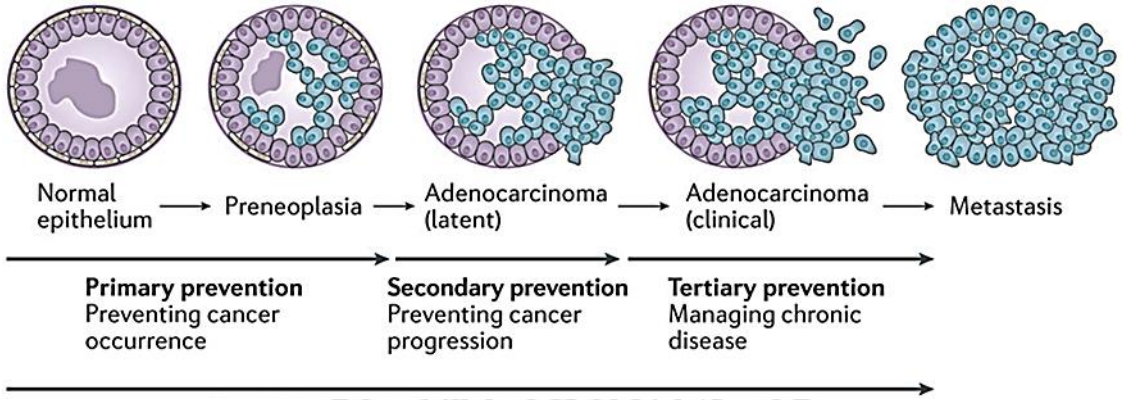

Figure 1.7 The strategies of cancer prevention (Le Magnen *et al.*, 2016).

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2) Classification of chemopreventive agents

The classification of chemopreventive agents is difficult to categorize because the exact modes of action are not clarify for several compounds. Moreover, many chemopreventive agents act through more than one mechanism. The classification pattern developed by Wattenberg (Wattenberg, 1985) is based on the time period during which agents administrate to display their activities in animal models of carcinogenesis. On this pattern, chemopreventive agents are classified into 2 main categories; blocking agents and suppressing agents (Figure 1.8).

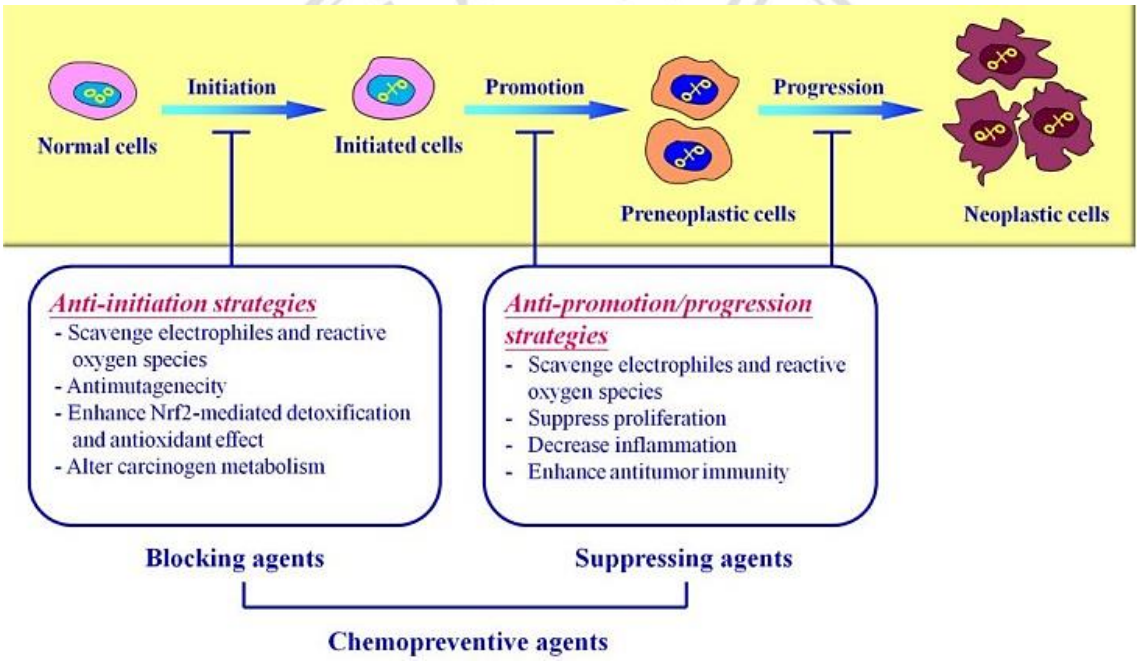

Figure 1.8 The categories of chemopreventive agents (Kuo *et al.*, 2012).

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1.1) Blocking agents

Blocking agents commonly influence on the initiation stage. They prevent activation of carcinogens undergoing metabolic activation or enhance detoxification systems or trap reactive carcinogenic species before reaching critical target sites (Shukla *et al.*, 2004). The molecular blocking mechanisms of phytochemical compounds are described below.

1.1.1) Alteration of carcinogen metabolism via phase I enzymes

Cytochrome P450 (CYP) enzymes are phase I xenobiotic metabolizing enzymes, commonly catalyze the oxidative metabolism of various xenobiotic chemicals, including drug and chemical carcinogens (Rendic, 2002). The objective of the reaction by phase I xenobiotic metabolizing enzymes is to produce more hydrophilic compounds and less toxic metabolites. However, some CYP enzymes such as CYP1A1, CYP3A, and CYP2E1, have been shown to involve in the metabolic activation of several carcinogens such as benzo[a]pyrene, N-nitrosodimethylamine and aflatoxin B1 (Gonzalez *et al.*, 1994).

The initiation stage of carcinogenesis might delay or reverse by numerous chemopreventive agents via alteration of carcinogen metabolism via phase I enzymes. Fucoxanthin, a carotenoid that found in brown algae, is known to have anticarcinogenic and anti-tumor activities. Carotenoids modulated the expression and activity of CYPs. Some reports presented fucoxanthin induced *cyp1a1* mRNA expression, however, it inhibited its enzyme activity (Satomi *et al.*, 2013).

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1.1.2) Enhancement of Nrf2-mediated detoxification and antioxidant activity

The detoxification by phase II enzymes is important in cellular response to electrophilic and oxidative toxicants. The phytochemical antioxidants exert preventive effects not only by scavenging free radicals, but also by inducing the expression of detoxifying/antioxidative genes. These genes encode enzymes such as glutathione peroxidase, gamma-glutamylcysteine synthetase (γ-GCS), GST, NQO and heme

oxygenase-1 (HO-1). Many basic leucine zipper (bZIP) transcription factors, including NRF, JUN, FOS, FRA, MAF and AH receptor bind to these ARE sequences and modulate expression of some of detoxifying/antioxidative genes (Figure 1.9).

Several chemopreventive agents are able to decrease the risk of initiation of cancer by increasing detoxification of activated metabolites via phase II xenobiotic metabolizing enzymes and antioxidant enzymes to scavenge free radicals such as fucoxanthin, phlorotannin and indole-3-carbinol. Phlorotannin found in brown algae enhanced cellular antioxidant defense system through induction of HO-1 via ERK-Nrf2-ARE signaling pathway (Kang *et al.*, 2007). Fucoxanthin induced phase II enzymes through activation of the ARE transcription system (Ben-Dor *et al.*, 2005). Indole-3-carbinol (I3C), found in cruciferous vegetables, is a powerful inducer of both phase I and II xenobiotic metabolizing enzymes (Manson *et al.*, 2000). I3C affected on AFB1 metabolism by inducing various CYP isozymes including, 1A1, 1A2, 3A and 2B1/2 resulting less toxic metabolites of AFB1, AFQ1 and AFM1. Moreover, I3C also induced phase II enzymes such as GST A5, which conjugated AFB1-epoxide to glutathione and eliminated from the body (Manson *et al.*, 2000).

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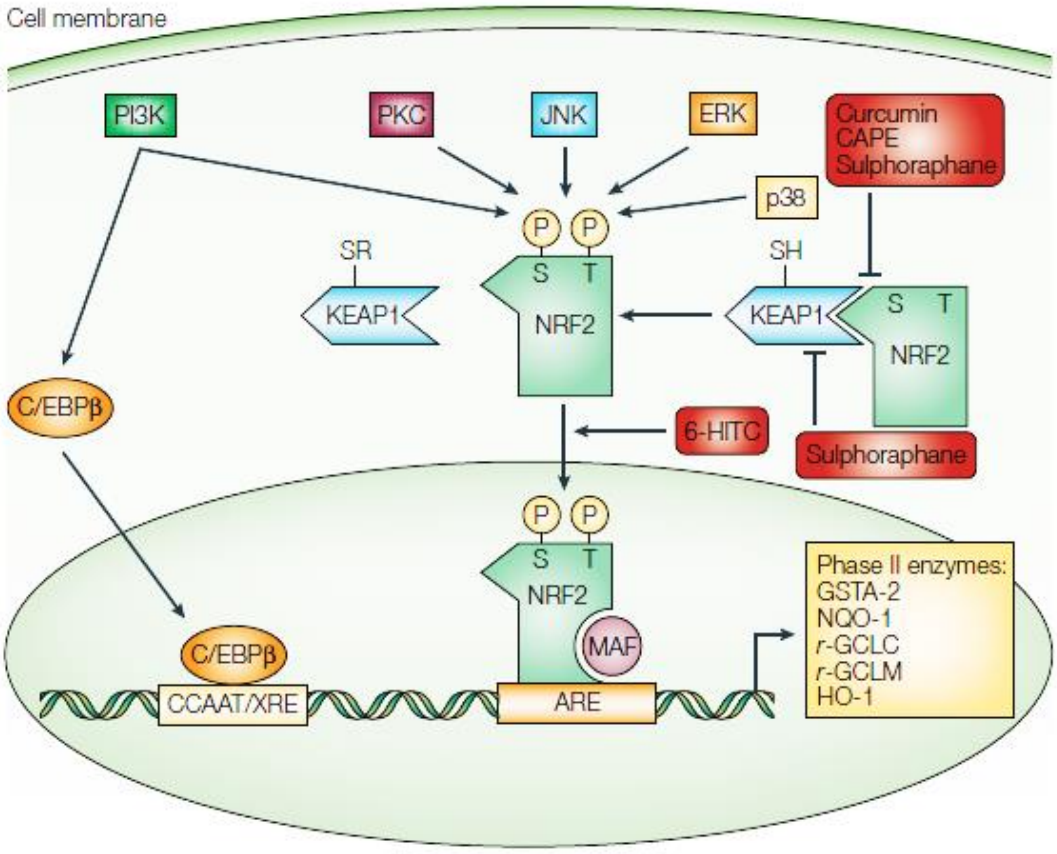

Figure 1.9 Transcriptional activation of detoxify and antioxidant enzymes by NRF2 (Surh, 2003).

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1.2) Suppressing agents

Suppressing agents inhibit the malignant transformation of initiated cells, in either promotion or progression stage. They modulate signal transduction, promote intercellular communication, restore immune response, induce apoptosis, correct DNA methylation imbalance, inhibit basement membrane degradation and inhibit arachidonic acid metabolism (Manson *et al.*, 2000). The molecular mechanisms of suppressing agents are described below.

1.2.1) Inhibition of NF-κB activation

NF-κB is a transcription factor for expression of several genes involved in inflammation, immunity and adhesion (Surh, 2003). The activation of signaling pathway by cytokines or oxidative stress leads to phosphorylation and degradation of IkB, allowing NF-κB translocate to nucleus and activates several target genes (Figure 1.10). Several phytochemical agents have been shown to suppress NF-κB activation in malignant cells or NF-κB activation induced by cytokines or oxidative stress (Bremner *et al.*, 2002). Curcumin inhibited NF-κB activation by inhibiting the ability of the IKKα/β complex to phosphorylate IkB, which in turn may contribute to increase apoptosis (Plummer *et al.*, 1999). Fucoxanthin was a potent inhibitor of cell migration, MMP activation and inhibition of NF-κB translocation (Nguyen *et al.*, 2014).

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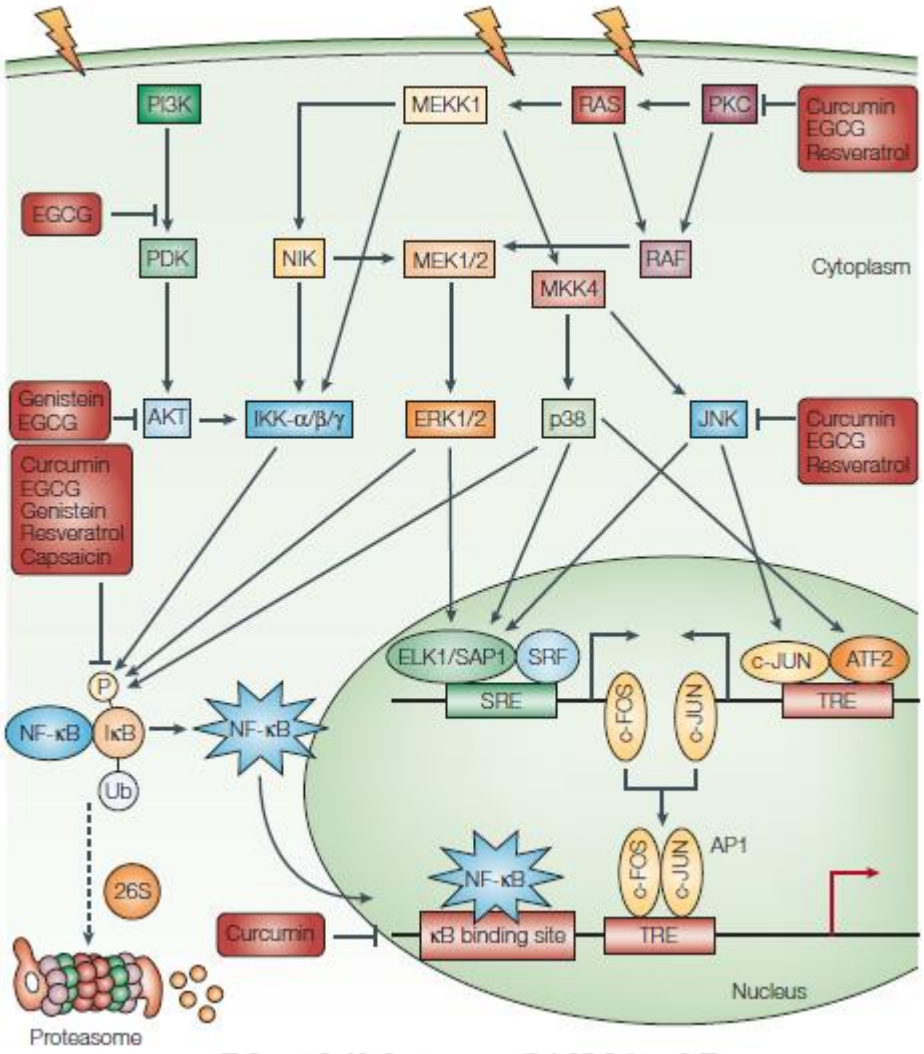

Figure 1.10 The activation of NF-κB (Surh, 2003).

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1.2.2) Cell cycle arrest

Uncontrolled proliferation is a well-known character of carcinogenesis (Hanahan *et al.*, 2000). The cell cycle consists of four phases, including G1, S, G2 and M phase. Several proteins are known to regulate cell cycle. The C/EBP family of transcription factors plays an important role in controlling cell proliferation and differentiation (Johnson, 2005). The eukaryotic cell cycle is regulated through the consequent activation and inactivation of cyclin-dependent kinases (Cdks) that drive cell cycle progression through phosphorylation and dephosphorylation of several regulatory proteins (Johnson, 2005). In normal cells, Cdks exist mainly in complexes of Cdk, cyclin, proliferating cell nuclear antigen (PCNA), and 21 kDa protein (p21). From G1 to S phase is regulated by the accumulation of cyclins D, E, and A, which bind to different Cdk catalytic subunits. The transition from early to mid G1 phase is regulated by activation of Cdk4-cyclin D and/or Cdk6-cyclin D complex. The activation of Cdk2-cyclin E complex is necessary for the transition of mid G1 to S phase. Progression from the late G1 to the S phase also requires the presence of the Cdk2-cyclin A complex (Figure 1.11).

The induction of cell cycle arrest is considered to be a promising chemopreventive strategy. Resveratrol, an active compound in grape, caused cell cycle arrest via up-regulation of p21, p27, p16, and down-regulation of cyclin D1, cyclin E, Cdk2, Cdk4 and Cdk7 in human colon carcinoma cells (Tyagi *et al.*, 2005). Moreover, some flavonoids in fruits and vegetables induced cell cycle arrest in various cancer cells (Dani *et al.*, 2008) (Zhang *et al.*, 2008) (Pan *et al.*, 2002). Several studies reported about chemopreventive properties of phytochemicals from algae on cell cycle arrest including sulfate polysaccharides, fucoxanthin and astaxanthin. The algal sulfated polysaccharide extract from red marine alga induced G1 phase arrest and apoptosis in human breast cancer cell line (Murad *et al.*, 2016). Fucoxanthin showed cancer preventive mechanism via the cell cycle arrest during the G_0/G_1 phase mediated through the up-regulation of $p21^{WAF1/Cip1}$ in human colon adenocarcinoma cell lines (Das *et al.*, 2005). Astaxanthin, red ketocarotenoid, also stopped cell cycle progression (Palozza *et al.*, 2009).

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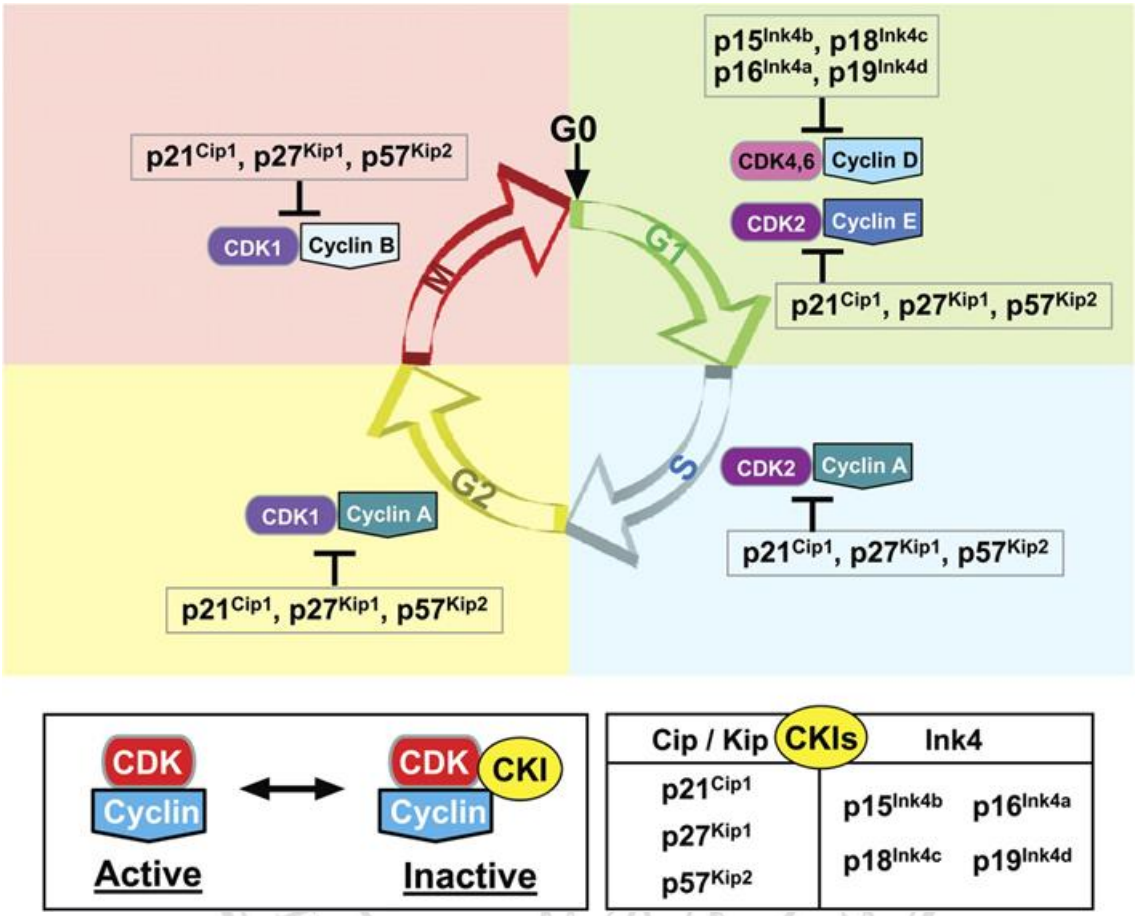

Figure 1.11 Mammalian cell cycle regulation by Cdk-cyclin complex and CKIs (Fuster *et al.*, 2010).

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1.2.3) Apoptosis

Apoptosis, a programmed cell death, plays an important role in development and tissue homeostasis. There are several intracellular signaling pathways involving cellular apoptosis, including members of the caspase family. The morphological and biochemical features of apoptosis are characterized by cell shrinkage, membrane blebbing, chromatin condensation and formation of DNA ladder with multiple fragments caused by DNA cleavage (Fink *et al.*, 2005). Apoptosis is divided into two pathways, including the death receptor (extrinsic) and mitochondrial (intrinsic) pathway, which are activated by caspase 8 and caspase 9, respectively (Figure 1.12).

Apoptosis induction may be considered one of the important targets in a cancer chemopreventive strategy by reversion of the conversion of normal cells to malignant cells. Several studies reported that many phytochemicals from natural products could inhibit the tumor growth by targeting one or more signaling intermediates leading to induction of apoptosis. Epicatechin, found in tea, induced cell cycle arrest and apoptosis in various cancer cells (Granado-Serrano *et al.*, 2007). Genistein, an isoflavonoid found in soybean, induced apoptosis by activation of calpain-caspase and ASK-1 signaling (Shim *et al.*, 2007). Resveratrol induced apoptosis through mitochondrial pathways in DMBA/TPA induced mouse skin tumorigenesis (Kalra *et al.*, 2008). Limonene, a terpenoid found in citrus fruits, cherries, apricots and grapes, induced apoptosis through caspase dependent mitochondrial death pathways (Jia *et al.*, 2013). Astaxanthin promoted apoptosis through down-regulation of phosphorylated AKT, changes in apoptosis-related proteins, including Bax, Bcl-2 and Bcl-XL and in MAP kinases signaling (Palozza *et al.*, 2009). Fucoidan, a sulfated polysaccharide found in brown algae, induced apoptosis in human colon cancer cells via both the death receptor-mediated and mitochondria-mediated apoptotic pathways (Kim *et al.*, 2010). Porphyrans, the sulfated polysaccharides produce by red algae, promoted apoptosis via inducing caspase-3 activation (Kwon *et al.*, 2006). Siphonaxanthin, a carotenoid from marine green algae, induced apoptosis in human leukemia cells (Ganesan *et al.*, 2011). Phloroglucinol induced apoptosis in human breast cancer cells (Kong *et al.*, 2009).

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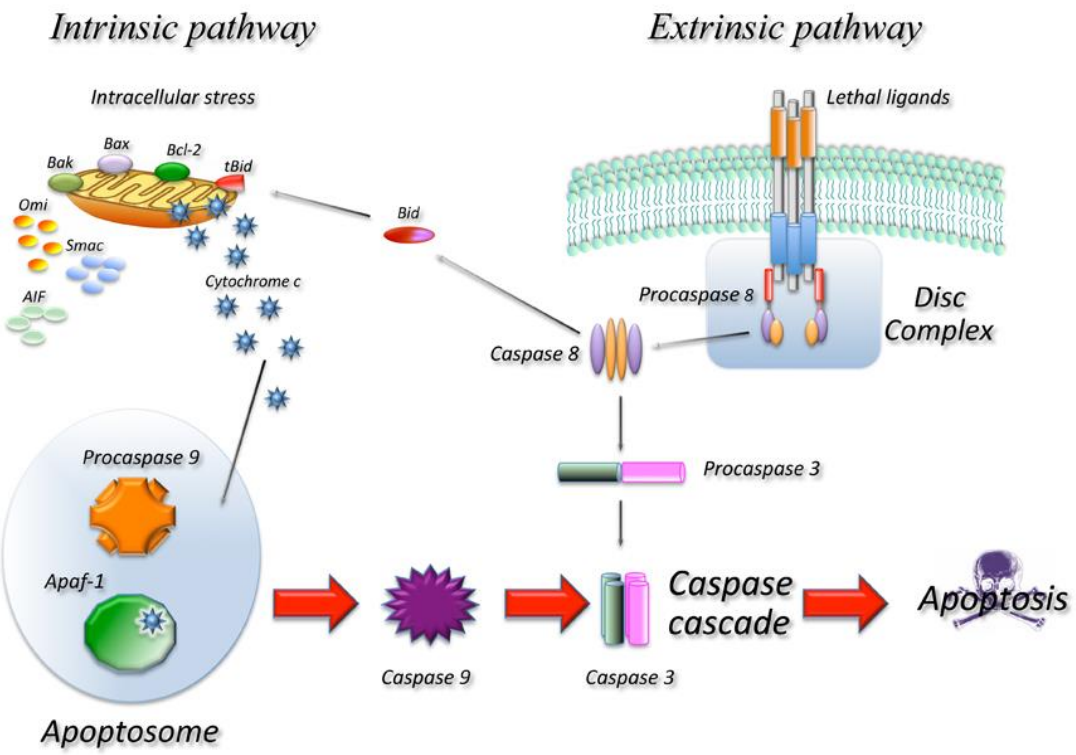

Figure 1.12 The intrinsic and extrinsic apoptosis pathways (Favaloro *et al.*, 2012).

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1.2.3 Algae

Algae are photosynthetic organisms that can convert sun light into chemical energy compounds in the process of photosynthesis. The algae synthesize various compounds with diverse structures and functions protect them from physiological stressors (Kim *et al.*, 2015). Algae can be divided into microalgae and macroalgae. Microalgae are very small plant-like organisms and also called phytoplankton. They are commonly in both sea and fresh water. The classes of microalgae are classified following 4 main classes, including diatoms (*Bacillariophyceae*), green algae (*Chlorophyceae*), golden algae (*Chrysophyceae*) and blue-green algae (*Cyanophyceae*) (Rajkumar *et al.*, 2014). Macroalgae are multicellular plants growing in both sea and fresh water. They can be classified according to the presence of specific pigments into 3 classes. The first class is *Chlorophyceae* which its green color resulting from the presence of chlorophyll a and b. There are few reports of novel secondary metabolites among the chlorophyta. The second class is *Phaeophyceae*, which brown color resulting from the presence of the xanthophyll and fucoxanthin, chlorophyll a and c, β-carotenes and other xanthophylls. The storage materials of brown algae are generally complex polysaccharides and higher alcohols. The major carbohydrate storage is laminarin. The cell walls are made of cellulose and alginic acid. The last class is *Rhodophyceae,* which its red color resulting from the presence of phycobilins such as phycoerythrin and phycocyanin, chlorophyll a, β-carotene and a number of unique xanthophyll. The walls are made of cellulose, agars and carrageenan. The red algae are the most important sources of carrageenan (Bold *et al.*, 1985).

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1.2.4 Bioactive compounds in algae

Macroalgae provide an abundant variety of metabolites and natural bioactive compounds with antimicrobial, antioxidant and anticancer activities, such as polysaccharides, polyunsaturated fatty acids, phlorotannins and other phenolic compounds, and carotenoids (Pal *et al.*, 2014, Perez *et al.*, 2016, Zong *et al.*, 2012).

1) Sulfated polysaccharides

Polysaccharides are polymers comprised of monosaccharides linked together through glycosidic bonds. These structures can be linear or contain branched side chains (Zong *et al.*, 2012). Polysaccharides can be classified into two groups based on their source. Natural polysaccharides are obtained from various organisms such as algae, plants, microorganisms, and animals. On the other hand, semi-synthetic polysaccharides are produced by the chemical or enzymatic modification of the parent natural polysaccharides (Zong *et al.*, 2012).

Marine macroalgae contain large amounts of polysaccharides mainly in cell wall structure, mucopolysaccharides and storage polysaccharides (Kumar *et al.*, 2008). From the nutritional proportion, macroalgae are low in lipid content but high in carbohydrate content. The major carbohydrates in macroalgae are dietary fibers. The total polysaccharide contents in the macroalgae species are ranged from 4-76 % of the dry weight (Pal *et al.*, 2014). The polysaccharide cell wall mainly consists of cellulose, hemicelluloses, and neutral polysaccharides. Green algae contain sulfated galactans, sulfated rhamnose and aldobiouronic acid whereas brown algae contains alginic acid, fucoidan or sulfated fucose, laminarian or β-1, 3 glucan. While red algae contain carrageenan, amylopectin like sugar also known as floridean starch, water soluble sulfated galactan, as well as porphyran as mucopolysaccharide that are presented in the intracellular spaces (Murata *et al.*, 2001).

The classification of sulfated polysaccharides extracted from macroalgae has been illustrated in Figure 1.13.

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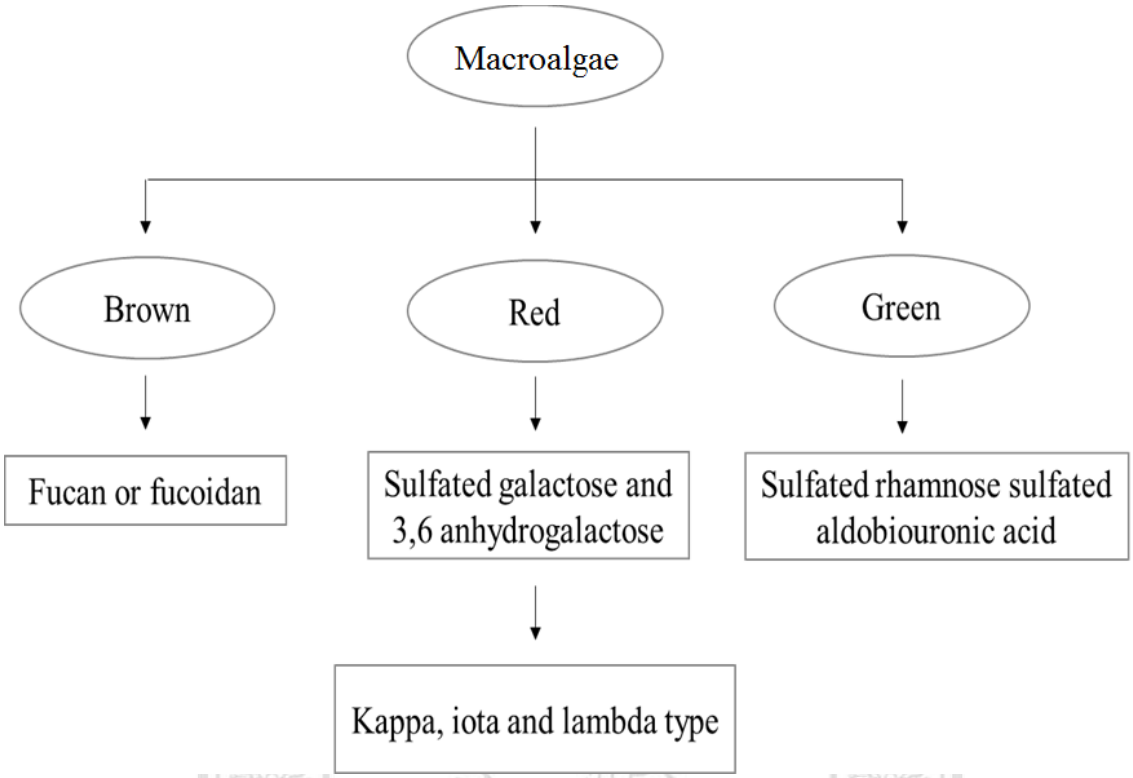

Figure 1.13 Classification of the sulfated polysaccharides in algae (Patel, 2012).

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1.1) Fucoidan

Fucoidan is a common term of sulfated polysaccharides derived from brown algae and brown seaweed. Fucoidan is mainly composed of L-fucose and sulfate group with small amounts of other monosaccharides including mannose, galactose, glucose and xylose, uronic acids and acetyl groups (Figure 1.14). Some fucoidan has branched structures. Fucoidan from different algal species has diverse structures not only in position, sulfation degree and molecular weight, but also in the main carbohydrate chains (Li *et al.*, 2008).

Fucoidan has various important biological activities including anticoagulant, antithrombotic, antivirus, immunomodulatory, anti-inflammatory, antioxidant and anticancer activities (Li *et al.*, 2008). The anticancer properties of fucoidan have been reported. Fucoidan inhibited cell proliferation of various cancer cell lines including prostate cancer PC-3, cervical cancer HeLa, alveolar carcinoma A549 and hepatocellular carcinoma HepG2 cells (Synytsya *et al.*, 2010). Moreover, fucoidan also induced apoptosis via caspase-independent and dependent apoptotic pathway (Foley *et al.*, 2011, Zhang *et al.*, 2011). The differences of apoptotic mechanisms probably depend on the structure of fucoidan and cell types. The pro-apoptotic effect of fucoidan was involved in ERKs and p38 activation and blocking of PI3K/Akt signaling pathway in colon carcinoma cells (Hyun *et al.*, 2009). Furthermore, fucoidan also suppressed tumor growth by inhibiting angiogenesis (Narazaki *et al.*, 2008). The sulfate content in fucoidan was related to proliferation of human stomach cancer cell line (Cho *et al.*, 2011). The antiangiogenic and antitumor activities of fucoidan can be highly potentiated by increasing the sulfate groups in fucoidan (Koyanagi *et al.*, 2003). Likewise, the inhibitory effect of various sizes of fucoidan on cancer cell growth has been reported. The anticancer activity of fucoidan could be increased by lowering their molecular weight (Yang *et al.*, 2008). Thus, the molecular mechanisms of anticancer and cancer preventive actions of fucoidan are very complicated and may include inhibitory effects against cancer cell proliferation and induction of tumor cells apoptosis. The cancer preventive action of fucoidan comprises useful properties as anti-inflammatory, anti-adhesive (Cumashi *et al.*, 2007) and antioxidant (Wang *et al.*, 2010).

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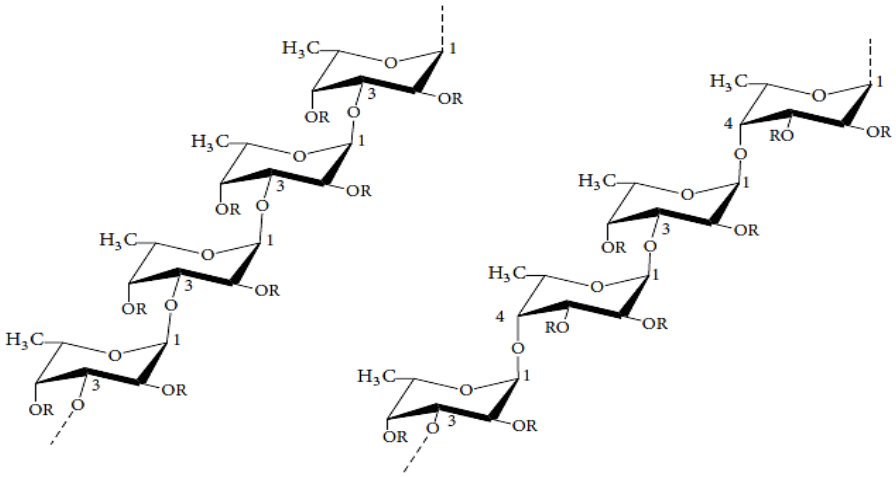

Figure 1.14 Two types of homofucose backbone chain in fucoidan isolated from brown macroalgae. R groups illustrate the position for attachment of noncarbohydrate (sulfate and acetyl groups) and carbohydrate (α-L-fucopyranose and α-D-glucuronic acid) substituents (Zorofchian Moghadamtousi *et al.*, 2014).

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1.2) Carrageenan

Carrageenan is one type of well-known polysaccharides contained in red algae and usually contained repeating disaccharides of β-(1→3)-linked and α-(1→4)-linked galactopyranosyl (Galp) residues. They consist of galactose or galactose and 3, 6-anhydrogalactose monosaccharide units and are differ from other polysaccharides in monosaccharide composition, degree and position of sulfation, and molecular weights (Usov, 2011). Three groups of carrageenan, kappa- (κ), iota- (ι) and lambda- (λ) carrageenan are categorized (Figure 1.15).

Carrageenan exhibited cancer preventive activity associated with their antiviral, antioxidant properties and stimulation of antitumor immunity. The low molecular weight κ-carrageenan presented antioxidant properties and may be promising for cancer prevention (Sun *et al.*, 2010). Moreover, κ-carrageenan oligosaccharides exerted antitumor effects by stimulating the immune system (Hu *et al.*, 2006). However, the harmful gastrointestinal effects of carrageenan were found in animal experiments. In animal models, both degraded and undegraded carrageenan have been related to development of ulcerative colitis (Benard *et al.*, 2010, Tobacman, 2001). Moreover, degraded and undegraded carrageenan were a known carcinogen and co-carcinogen in animal models (Tobacman, 2001). However, some groups reported that administrated undegraded carrageenan has not found to be carcinogenic in rat colorectal cancer (Hagiwara *et al.*, 2001). Therefore, further researches are needed to study the harmful effects of degraded and undegraded carrageenan.

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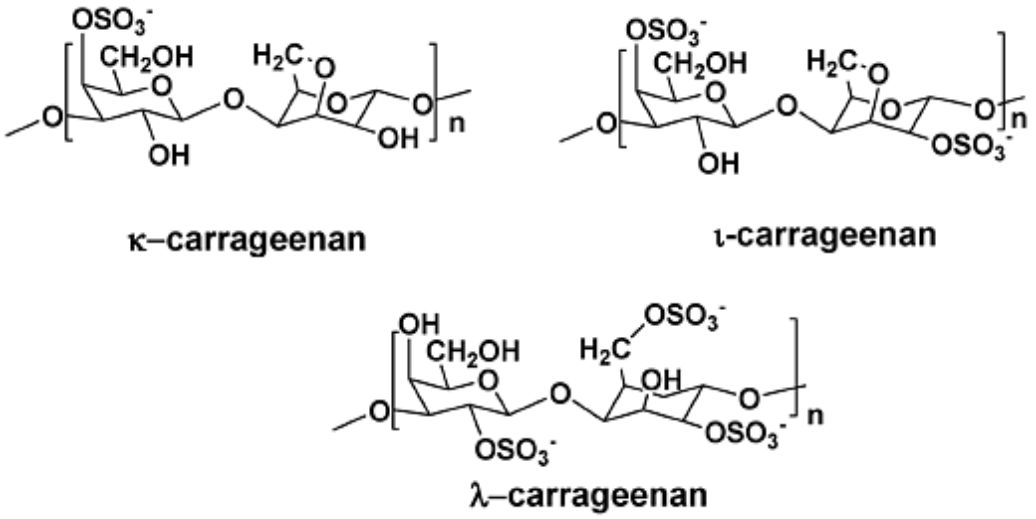

Figure 1.15 Repeating units of some carrageenan (de Jesus Raposo *et al.*, 2015).

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1.3) Ulvan

Marine green algae have been less studied on sources of poly-saccharides with anticancer and cancer preventive properties in comparison to brown and red algae. Ulvan is water soluble sulfated polysaccharides from the cell wall of green algae (Figure 1.16). It is composed of repeating disaccharide moieties, containing sulfated rhamnose and uronic acid (glucuronic or iduronic). Some ulvan also includes xylose residues (Costa *et al.*, 2010). The sulfated polysaccharides isolated from several green algae presented antioxidant (Sathivel *et al.*, 2008), antiproliferative (Pal *et al.*, 2014), anti-inflammatory (Kim *et al.*, 2011) and antitumor (Ji *et al.*, 2008) activities. The strong immunomodulatory and antioxidant activities of polysaccharides from green algae suggest their potential cancer preventive activity.

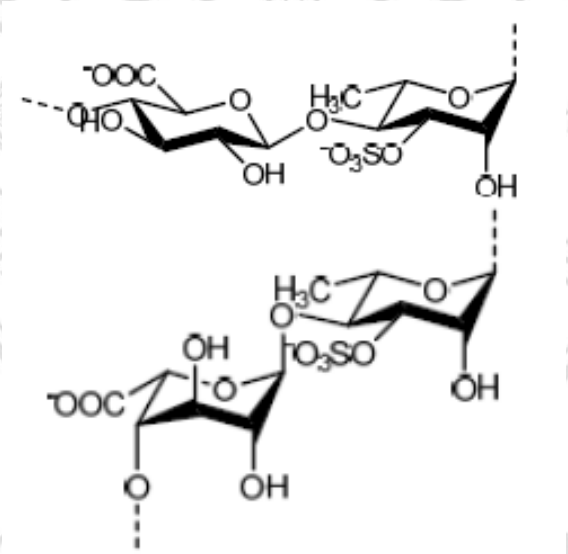

Figure 1.16 Structure of ulvan (Perez *et al.*, 2016).

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2) Polyphenolic compounds

Polyphenol compounds widely produced by plants including algae and have shown various biological activities such as antioxidant (Kim *et al.*, 2005), antimicrobial (Rajauria *et al.*, 2013) and anticancer agents (Namvar *et al.*, 2013). Polyphenol compounds may be classified into different groups including phenolic acids, flavonoids, stilbenes and lignans (Figure 1.17) (Manach *et al.*, 2004). Marine algae also contain high amounts of phenolic acids such as gallic acid, 4-hydroxybenzoic acid, catechin hydrate, epicatechin, catechin gallate, epicatechin gallate, epigallocatechin, epigallocatechin gallate and pyrocatechol (Machu *et al.*, 2015). Macroalgae produce phenolic compounds to protect them against external stress condition (Li *et al.*, 2011). Green and red marine algae have low concentrations of polyphenol compounds as compared to brown marine algae (Holdt *et al.*, 2011). However, Acharaporn and her colleagues reported the phenolic acids contained in *Spirogyra neglecta* extract such as gallic acid, eriodictyol, isoquercetin, kaempferol, quercetin, hydrquinin, rutin, catechin and tannic acid. Especially, isoquercetin and catechin was reported to be the main phenolic acids in extract of *S. neglecta* (Duangjai *et al.*, 2016). Polyphenols are strong antioxidant (Manach *et al.*, 2004) which are close relationship to anticarcinogenic activity (Halliwell, 2007, Pandey *et al.*, 2009).

Phlorotannins are the group of tannin compounds, which belong to the polyphenolic compounds. Phlorotannins, such as eckol or dieckol, (Figure 1.18) have been found only in brown marine algae (Cho *et al.*, 2012). The phlorotannins are polyphenols formed by polymerization of phloroglucinol (Li *et al.*, 2011) and presented various pharmacological activities such as antioxidant, antidiabetic, anti-human immunodeficiency virus, antihypertensive, radioprotective, antiallergic and anticancer activities (Wijesekara *et al.*, 2010). Phlorotannins have very strong antioxidant properties because of their unique structure (Heffernan *et al.*, 2015, Li *et al.*, 2009). Moreover, phlorotannins also have strong antimicrobial activity via attacked microbiological proteins, resulting in inhibition of bacteria (Eom *et al.*, 2012).

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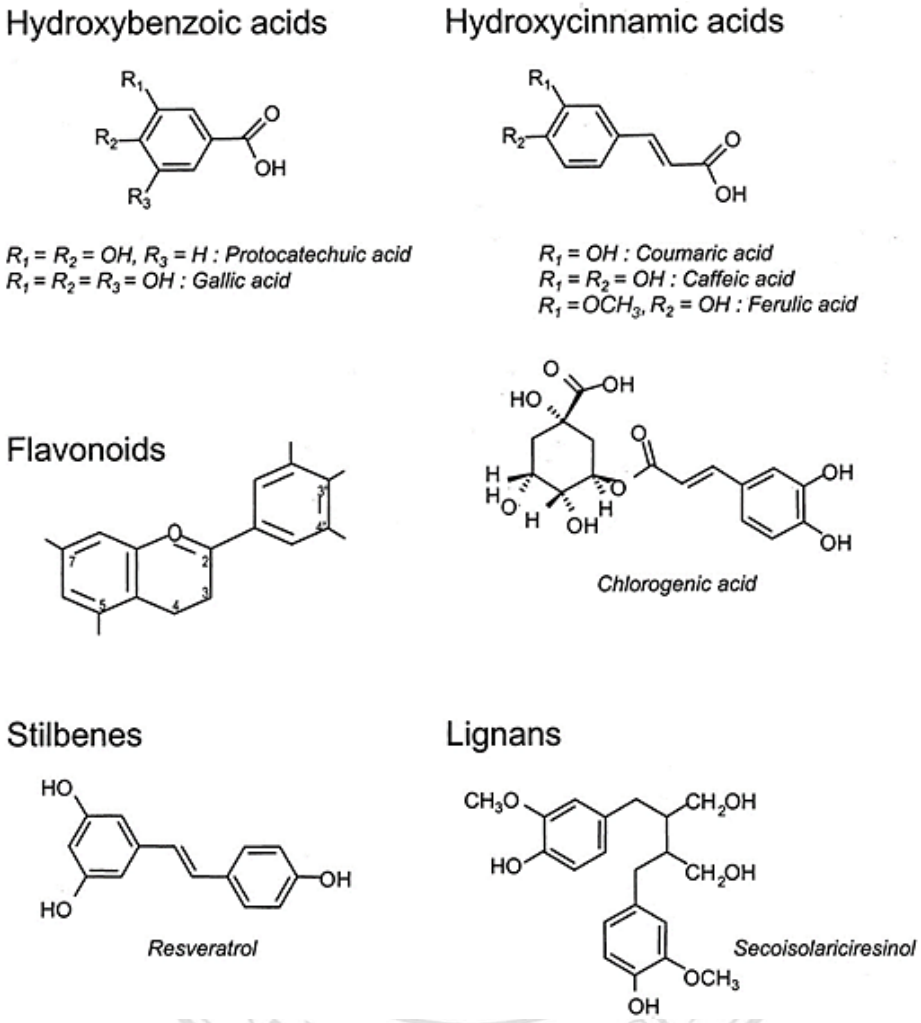

Figure 1.17 Chemical structures of some polyphenols (Manach *et al.*, 2004).

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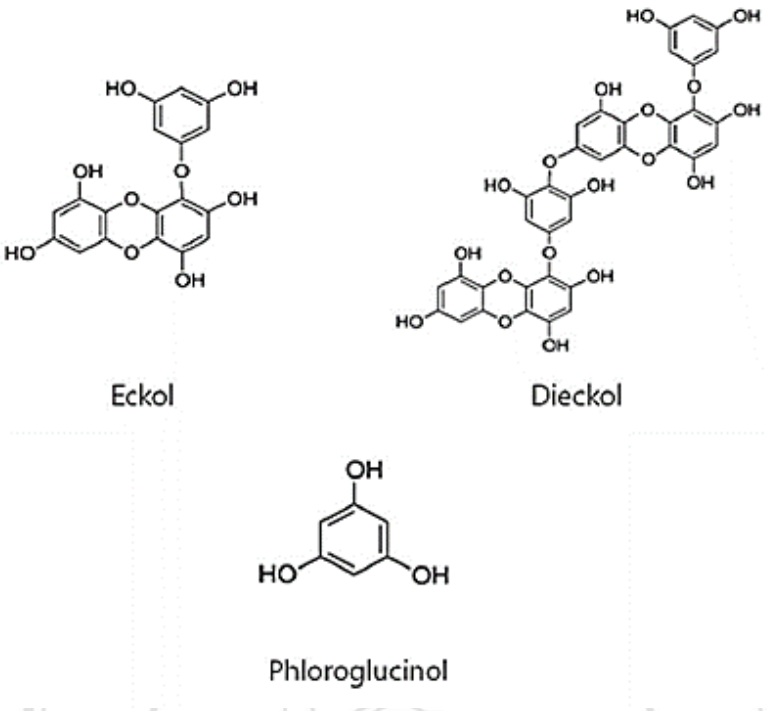

Figure 1.18 Structures of eckol, dieckol and phloroglucinol (Kang *et al.*, 2012).

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3) Proteins

The structure and biological activities of proteins in marine algae are not as widely reported as of polysaccharides. Environmental and seasonal variations have an effect on proteins in marine algae (Harnedy *et al.*, 2011). Usually the content of proteins in marine algae is less than 5% and the content of proteins in brown algae are the lowest (Cerna, 2011). Lectin is an important bioactive proteins extracted from macroalgae (Hori *et al.*, 2000) and involved in numerous biological activities such as host-pathogen interactions, cell-cell communication, induction of apoptosis, cancer metastasis and differentiation, as well as recognizing and binding carbohydrates (Hori *et al.*, 2000). Furthermore, biological activities of marine algal lectins have been reported such as antibiotic, mitogenic, cytotoxic, anti-nociceptive, anti-inflammatory, anti-adhesion and anti-HIV activities (Harnedy *et al.*, 2011, Mori *et al.*, 2005).

Kahalalide F (Figure 1.19), a family of depsipeptides produced by marine green algae, has anticancer and antitumor properties. It is effective in several cancers including lung, colon and prostate cancer (Smit, 2004). Kahalalide F functions by acting on the lysosomal membrane (Stokvis *et al.*, 2002), a mechanism that separates it from all other antitumor agents. It also induces cell necrosis *in vivo* and exhibits cytotoxic activity.

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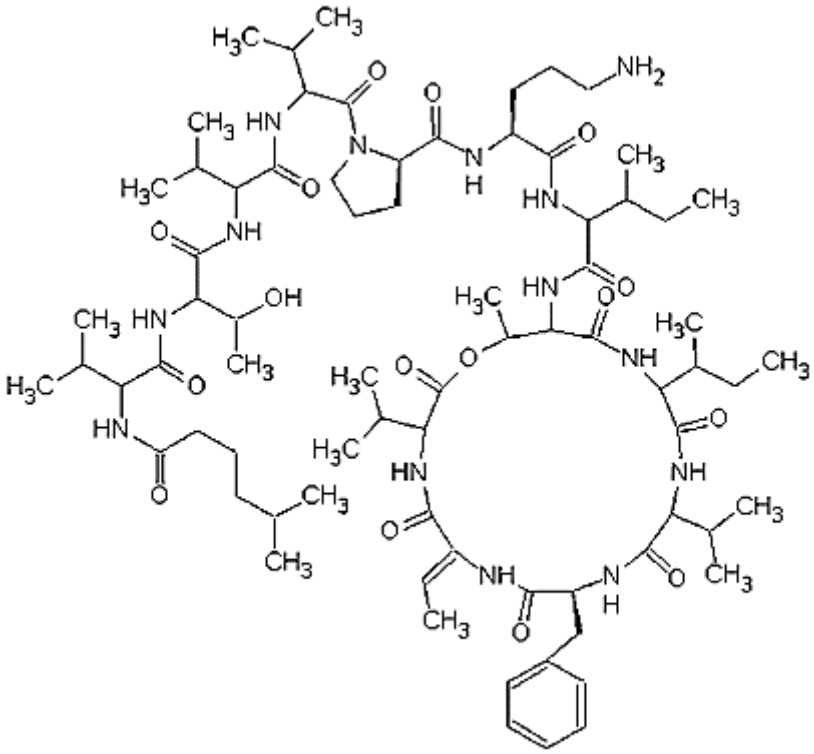

Figure 1.19 Structure of kahalalide F (Smit, 2004).

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4) Pigments

Algae synthesize various pigments for photogenesis. Chlorophyll a and b are major pigments in green algae. The greenish brown color in brown algae is due to the presence of fucoxanthin, chlorophyll a and c. Red algae contain phycobilins such as phycoerythrin and phycocyanin (Pangestuti *et al.*, 2011). Moreover, algae pigments have also been reported several health beneficial properties such as antioxidant, antimutagenic, anti-inflammatory and anticancer activities (Pangestuti *et al.*, 2011).

4.1) Chlorophylls

Chlorophylls are greenish lipid-soluble pigment found in algae and higher plants (Pangestuti *et al.*, 2011). Chlorophylls are substituted tetrapyrrole with a centrally bound magnesium atom. The porphyrin tetrapyrrole is esterified by a diterpene alcohol and phytol to form chlorophyll (Figure 1.20) (Ferruzzi *et al.*, 2007). Four types of chlorophyll in marine algae including chlorophyll a, b, c and d are classified (Ferruzzi *et al.*, 2007, Larkum *et al.*, 2005). The bioavailability of chlorophyll derivatives is limited because of the chlorophyll is unabsorbable in gastrointestinal tract of humans. However, chlorophyll can be converted to pheophytin, pyropheophytin and pheophorbide by normal flora in lower gastrointestinal tract. These derivatives presented antimutagenic effect and may play a vital role in cancer chemoprevention (Pangestuti *et al.*, 2011).

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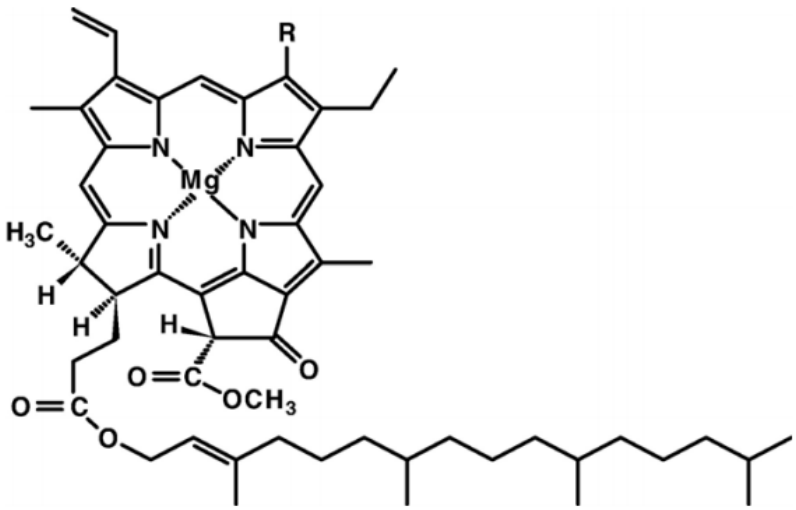

Figure 1.20 Structure of chlorophyll derivatives including chlorophyll a (R =CH_3) and chlorophyll b (R =CHO) (Ferruzzi *et al.*, 2007).

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4.2) Fucoxanthin

Carotenoids contain two main subclasses of nonpolar hydrocarbon carotenes and polar compounds named xanthophylls. Fucoxanthin (Figure 1.21) is one well known example of xanthophylls for anticancer activity (Zorofchian Moghadamtousi *et al.*, 2014). Fucoxanthin showed cancer chemopreventive property on various chemical induced carcinogenesis in animal models including liver, skin and colon (Kim *et al.*, 1998, Nishino *et al.*, 2009). Fucoxanthin exhibited anticancer activity associated with the free radical scavenging activity, induction of apoptosis and the anti-angiogenic effect (Zorofchian Moghadamtousi *et al.*, 2014).

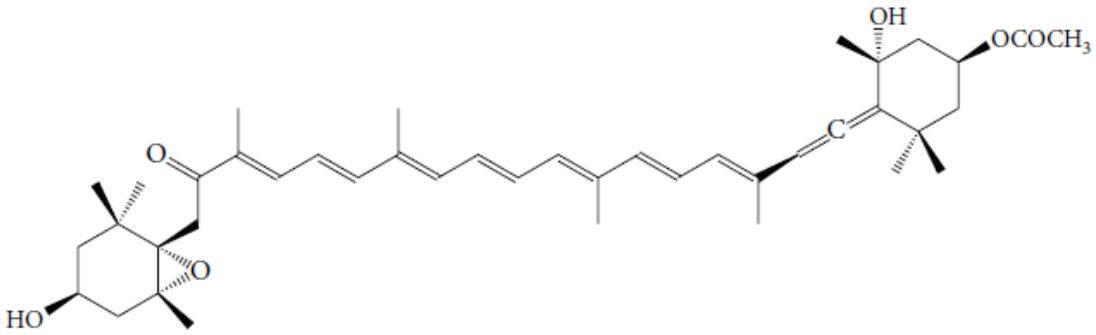

Figure 1.21 Structure of fucoxanthin (Zorofchian Moghadamtousi *et al.*, 2014).

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1.2.5 Beneficial effects of *Spirogyra neglecta*

Spirogyra is a genus of filamentous green algae consisting of more than 300 species in the world. Its chloroplasts display in a spiral shape (Figure 1.22). It produces mucous making slimy layer. *Spirogyra* is found in freshwater source with cool running stream such as shallow pond and ditch.

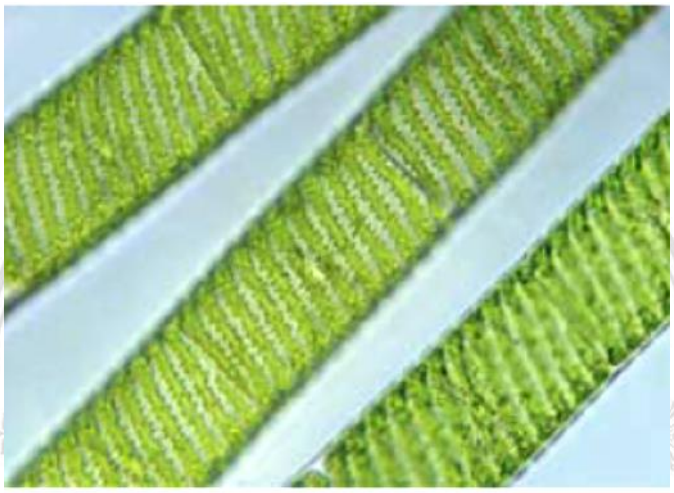

Figure 1.22 *Spirogyra sp.(Kim et al., 2015)*

Spirogyra neglecta (Hassall) Kützing (Figure 1.23) is freshwater green macroalgae. It is consumed as a traditional food in the northern part of Thailand. *S. neglecta* contains high amounts of protein, carbohydrate, fat, sulfate and dietary fiber (Thumvijit *et al.*, 2013a). *S. neglecta* exhibited several biological activities. The hot water extract of *S. neglecta* presented free radical scavenging activity against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Moreover, *S. neglecta* extract showed antioxidant capacity in rat liver by inducing some antioxidant enzymes (Thumvijit *et al.*, 2013a). The *S. neglecta* extract lowered hyperglycemia and hypertriglyceridemia as well as improved insulin resistance and renal oxidative stress in type II diabetic rats (Ontawong *et al.*, 2013). The hot water extract of *S. neglecta* could reduce plasma cholesterol level in *in vitro* system by several modes. It protected cholesterol absorption and inhibited activity of HMG-CoA reductase, a rate-limiting step of cholesterol synthesis. (Duangjai *et al.*, 2016)

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Figure 1.23 *Spirogyra neglecta*

Furthermore, the hot water extract of *S. neglecta* exhibited antimutagenicity against several environmental mutagens and without mutagenicity in *Salmonella* mutation assay. The *S. neglecta* extract strongly reduced the mutagenicity induced 2-aminoanthracene (2-AA), 2-aminofluorene (2-AF), aflatoxin B1 (AFB1) and 2-amino-3, 4-dimethylimidazo [4, 5-f] quinoline (MeIQ) and weakly inhibited mutagenesis induced by 2-Amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP). The possible antimutagenic mechanism of *S. neglecta* extract might be partly due to suppression of some xenobiotic metabolizing enzymes involving in mutagen activation (Thumvijit *et al.*, 2013a). The hot water extract of *S. neglecta* also showed anticarcinogenicity by lessening preneoplastic lesion formation in the liver of DEN-initiated rats. It could inhibit cell proliferation and enhance some detoxifying enzymes (Thumvijit *et al.*, 2014).

Interestingly, Surayot and her colleagues found sulfated polysaccharides obtained from *S. neglecta* were a strong immune stimulator. They strongly stimulated murine macrophages to produce nitric oxide and various pro- and anti-inflammatory cytokines through activation of nuclear factor-kappa B and mitogen-activated protein kinases pathways (Surayot *et al.*, 2015).

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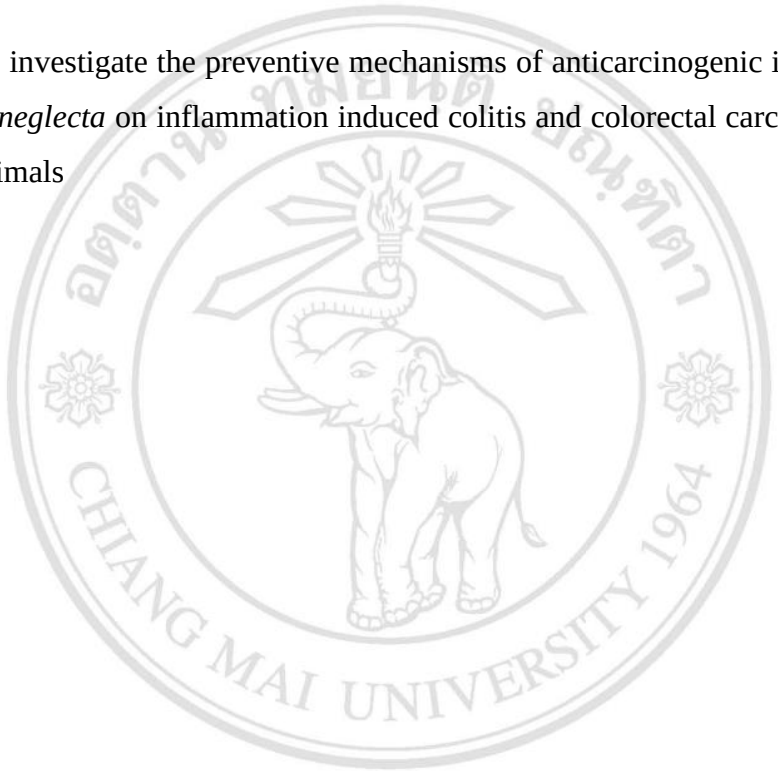
1.3 Objectives

1. To search for anticarcinogenic ingredients in *S. neglecta* on chemically induced early stages of colorectal carcinogenesis in animal and their inhibitory mechanisms

2. To evaluate the effect of anticarcinogenic ingredients in *S. neglecta* on inflammation induced colitis and colorectal carcinogenesis in animals

3. To investigate the preventive mechanisms of anticarcinogenic ingredients in *S. neglecta* on inflammation induced colitis and colorectal carcinogenesis in animals



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