CHAPTER 4

Discussion and conclusion

Spirogyra neglecta is freshwater green algae grown in north Thailand. Our group has reported their biological activities such as antioxidant activity (Thumvijit *et al.*, 2013a), antimutagenicity (Thumvijit *et al.*, 2013b) and antihepatocarcinogenicity (Thumvijit *et al.*, 2014). The first part of this study, we focused on comparative study of *S. neglecta* extract and dried *S. neglecta* mixed diet on both the initiation and post-initiation stages of 1, 2-dimethylhydrazine (DMH)-induced colorectal carcinogenesis in rats. The treatment of *S. neglecta* extract suppressed colonic aberrant crypt foci (ACF) formation in DMH-treated rats, while administration of dried *S. neglecta* mixed diet did not inhibited colonic preneoplastic lesion development. It was in line with our previous results concerning diethylnitrosamine-induced hepatocarcinogenesis in rats (Thumvijit *et al.*, 2014). The amounts of total phenolic compounds, total carbohydrates, and sulfate of the *S. neglecta* extract were higher than raw materials (Thumvijit *et al.*, 2013a). Several studies has reported that macroalgae are a source of polysaccharides with anticancer property (Jiao *et al.*, 2009, Kaeffer *et al.*, 1999). The polysaccharide contained in *S. neglecta* extract might be one of active compounds that why the hot water extract of *S. neglecta* had greater anticarcinogenicity than dried algae.

Cancer chemoprevention was defined as prevention, delay, inhibition and reversion of multistage carcinogenesis by administrating phytochemicals derived from natural products (Mehta *et al.*, 2010). The *S. neglecta* extract, which was given 1 weeks before DMH initiation and continued for 5 weeks, significantly reduced the formation of ACF in the proximal and distal parts of the colon. Although hepatic CYP2E1 expression was induced in DMH-treated rats, *S. neglecta* extract did not modulate the metabolic activation of DMH in the liver. However, *S. neglecta* extract significantly decreased the activity of UDP-glucuronyltransferase when compared to the DMH alone group.

This result suggested that *S. neglecta* extract might reduce the conjugation and transportation of conjugated MAM to colon. Glutathione *S*-transferase (GST) is considered to be a detoxifying enzyme for protection against some reactive metabolites of procarcinogens (Dasgupta *et al.*, 2003). The decrease of GST activity was found in DMH-treated rats possibly due to the excessive utilization during detoxifying process. Notably, *S. neglecta* extract increased activities of GST and glutathione peroxidase in the DMH-treated rats. As described by previous report, astaxanthin and carotenoids which produced by marine algae enhanced the activities of some antioxidant enzymes that were correlated with the reduction ACF formation in DMH-induced rats (Prabhu *et al.*, 2009). It might be suggested that *S. neglecta* extract blocked DMH-induced the initiation stage of colorectal carcinogenesis in rats by the modulation of detoxifying and antioxidant enzymes. Cell proliferation and apoptosis are common processes concerned to examine the ability of potential chemopreventive agents (Caderni *et al.*, 2000, Sengottuvelan *et al.*, 2006). This study found the reduction of ACF formation was concomitant with the decrease of PCNA-positive cells and increase of apoptotic cells in colonic mucosa of rats treated by *S. neglecta* extract after DMH initiation. Therefore, the anti-proliferating and apoptosis inducing properties of *S. neglecta* extract might suppress the ongoing formation of aberrant crypt foci to precancerous lesions in DMH-initiated rats. From this part, we could summarized that *S. neglecta* extract might act as a blocking agent in initiation stage and a suppressing agent in post-initiation stage of colorectal carcinogenesis.

According to the inhibitory effect of *S. neglecta* extract on both initiation and post-initiation stage of colorectal carcinogenesis leading to investigate the chemopreventive compounds which presented in *S. neglecta* extract. Several researches reported about the beneficial properties of sulfated polysaccharide isolated from marine algae including anticancer activity (Fedorov *et al.*, 2013, Raposo *et al.*, 2015). The polysaccharide rich extract and chloroform fraction, the remaining part obtained during defatted and decolorized process, were evaluated their chemical composition, antimutagenicity and anticarcinogenicity. The contents of total carbohydrates, sulfate and uronic acid in polysaccharide rich extract were higher than *S. neglecta* extract and these constituents were not found in chloroform fraction. It was suggested that polysaccharide might be a major component in *S. neglecta* extract. Several researches reported that polysaccharides could be found in algae as cell wall constituents such as cellulose in brown and green

algae (Kraan, 2012). Moreover, major monosaccharides in polysaccharide rich extract were glucose, fucose, galactose, rhamnose and arabinose. The monosaccharide profile of polysaccharide rich extract was in line with polysaccharide extract from *S. neglecta* of the other group (Surayot *et al.*, 2015). In addition, identification of sulfate moiety in the polysaccharide rich extract is one of the key information for confirmation of sulfated polysaccharides. FT-IR spectroscopy is a useful tool for the preliminary identification of sulfated polysaccharide produced by algae (Gomez-Ordonez *et al.*, 2011). Characterization of polysaccharide rich extract by FT-IR showed typical absorption bands of sulfated polysaccharides especially a band at 1248 cm^{-1} attributed to S=O stretching vibration and it could suggest that the presence of sulfate esters in crude polysaccharide. Moreover, the band at 1642 and 1412 cm^{-1} is allocated to asymmetric and symmetric stretching vibration of -COO- of uronic acid (Ananthi *et al.*, 2010). The absorption bands at 1078 and 1045 cm^{-1} in the range of 1200-1000 cm^{-1} in FT-IR spectrum suggested that the monosaccharides in polysacchararide rich extract has a pyranose ring (Ding *et al.*, 2010a). Moreover, the absorption band at 914 cm^{-1} are typical for D-Glucose in the pyranose form (Liu *et al.*, 2013). The band at 874 cm^{-1} indicated the presence of β-glycosidic linkages (Ding *et al.*, 2010a). Based on the FT-IR spectrum it could be expected that polysaccharide rich extract might be a sulfated polysaccharide which displayed pyran type sugar rings, and the polysaccharide might be connected to β-glycosidic bond. Overall, polysaccharide rich extract derived from *S. neglecta* might be a sulfated heteropolysaccharides. However, the structure characteristics of polysaccharide rich extract, especially the chain linkage, conformation of sugar units and position of sulfate, might be further completed and confirmed by NMR.

DNA mutation is recognized as the initial stage of chemical induced carcinogenesis. The blocking of DNA damage would be the first way of defense system against cancer progression. Next, we identified the antimutagenicity of *S. neglecta* extracts using *Salmonella* mutation assay. CF showed effective antimutagenicity against heterocyclic amine induced mutagenesis. Moreover, we found gallic acid was a major phenolic compound in *S. neglecta* extract and chloroform fraction, whereas gallic acid content in chloroform extract was lower than *S. neglecta* extract. We suggested that gallic acid was not antimutagenic compound in chloroform fraction. From chemical analysis, chlorophyll and carotenoid contents were found in only chloroform fraction. Likewise, several studies

reported antimutagenicity of macroalgae derived pigments including carotenoids, chlorophyll and its derivatives against various dietary and environmental mutagens (Ferruzzi et al., 2007). Therefore, we could suggested that antimutagenic compounds which occurred in chloroform fraction might be some beneficial pigments including carotenoids and chlorophyll. However, *S. neglecta* extract and polysaccharide rich extract were lacked of antimutagenicity in this assay. The molecular weights of sulfated polysaccharides isolated from *S. neglecta* were range from 164 to 1460 kDa (Surayot *et al.*, 2015). Some low molecular weight (30 kDa) glucomannans exhibited antimutagenicity against mutagen-induced mutagenesis in the *S. typhimurium* strain TA97 and TA100 (Vlckova *et al.,* 2004). It was assumed that active polysaccharides in *S. neglecta* were high molecular weight. To confirm cancer chemopreventive properties of *S. neglecta,* animal model was further performed.

In contrast to antimutagenic result in *Salmonella* mutation assay, polysaccharide rich extract exhibited the strongest antitumor promoting activity when compared to the other extracts. It could particularly reduce a large size of ACF and decreased proliferating index in colonic mucosa of DMH initiated rats. The previous study reported that the water soluble sulfated polysaccharides, comprised of two main glucose and galactose, derived from green alga had potent antitumor activity in both *in vitro* and *in vivo* (Ji *et al.*, 2008). Moreover, sulfated glucorhamnans or sulfated rhamnan isolated from green seaweed also exhibited anticancer in both *in vitro* and *in vivo* (Jiao *et al.*, 2009, Karnjanapratum *et al.*, 2011). In our study, polysaccharide extract mainly contained several monosaccharides including galactose, arabinose, fucose and rhamnose. The monosaccharide constituents of sulfated polysaccharides might be one of main factors that associated with their anticancer activity. It was suggested that chemopreventive effect of polysaccharide rich extract derived from *S. neglecta*, might be partly due to suppression of cell proliferation in colonic mucosa of carcinogen-induced preneoplastic lesion in rats. However, the structure of anticarcinogenic sulfated polysaccharide remains unclear though at the very least the results suggest that a sulfated polysaccharide might be one of the active compounds in *S. neglecta* extract.

Inflammation acts as a key regulator of promotion and progression stages of carcinogenesis. In colorectal carcinogenesis, patients with inflammatory bowel diseases

(IBD) such as ulcerative colitis (UC) as well as Crohn's disease (CD) are at the risk for the development of colorectal carcinogenesis (Mattar *et al.*, 2011). This study was to further investigate chemopreventive effect of *S. neglecta* extracts on colitis and inflammation-associated colorectal carcinogenesis in animal models. The effect of *S. neglecta* extracts on DSS-induced colitis in mice was determined. The histological structures of DSS-induced colitis characterized by edema, ulcer, erosion, inflammatory cell infiltration and crypt loss (Cooper *et al.*, 1993), were observed in our study. The *S. neglecta* extract, polysaccharide rich extract and chloroform fraction tended to improve histological appearance to the normal structure. DSS induced apoptosis and cell cycle arrests might lead to destroy epithelium barrier and led intestinal bacterial into mucosal layer and inflammation occur (Hans *et al.*, 2000). Administration of *S. neglecta* extract and polysaccharide rich extract markedly recovered cell proliferation and apoptosis to the normal level. These findings suggested *S. neglecta* extract and polysaccharide rich extract may have a protective effect on DSS-induced colitis by decreasing colonic mucosal damage through induction of cell cycle and decreased of cell apoptosis.

Furthermore, the pathogenesis of UC also remains unclear. We also investigated alterations of protein expression which could explain the mechanisms of DSS-induced colitis and further to explain the preventive effect of *S. neglecta* extract and polysaccharide rich extract on DSS-induced colitis in mice. It is evident that mitochondrial dysfunction might play one role of these pathogenesis (Hsieh *et al.*, 2006, Sifroni *et al.*, 2010). Mitochondrial dysfunction leads to lacking of energy supplier, increased ROS generation, and causing apoptotic cell death (Hsieh *et al.*, 2006). From proteomics analysis, DSS administration induced down-regulation of COX5A that is one subunit of cytochrome c oxidase or complex IV. Sifroni *et al.* have been reported that activities of mitochondrial respiratory chain enzymes in UC patients including complex II-IV were decreased (Sifroni *et al.*, 2010). Likewise, there are several studies reported about ROS production in UC patients (Nishikawa *et al.*, 2005, Rana *et al.*, 2014) and chemical-induced colitis in animal model (Damiani *et al.*, 2007). When ROS overproduction occurred in the cell, cell need to accomplish antioxidant system such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and glutathione (GSH) for removal of ROS (Medhi *et al.*, 2008). According to the proteomic analysis, GR was down-regulated in DSS-treated mice

leading to increased ROS production during DSS administration. Not only the mitochondrial dysfunction but the abnormality of mucosal structure or epithelial barrier also has been reported that associated with the UC pathogenesis. Keratin8 (KRT8) and keratin18 (KRT18) are the major cytoskeletal intermediate filament in the intestinal epithelia (Ding *et al.*, 2010b). KRT8 is the major type II keratin in small and large intestine. It forms with type I keratins KRT18, 19, or 20 depend on the type of cell and tissue. The major role of KRT8 is to maintain normal epithelial architecture (Coulombe *et al.*, 2002). Our proteomic analysis showed KRT8 and KRT18 were down-regulated in DSS-induced colitis. Several studies reported KRT8-null mice developed chronic inflammation (Baribault *et al.*, 1994, Habtezion *et al.*, 2005). Moreover, the results of genome-wide microarray analysis found the changes in protein translation of KRTs that altered colonic epithelial barrier function in UC patients (Treton *et al.*, 2011). In addition, microfilament-associated proteins tropomyosin 1 and 2 (Tpm1 and Tpm2) presented in eukaryotic cells having different functions, were detected with low level in colon mucosa of mice treated with DSS (Lin *et al.*, 1997). Nevertheless, *S. neglecta* extract and polysaccharide rich extract administrations ameliorated DSS-induced down-regulation of Tpm1 and Tpm2. The previous study has been reported that Tpm1 gene was down-regulated in trinitrobenzenesulphonic acid (TNBS)-induced rat colitis (Martinez-Augustin *et al.*, 2008). Hence, down-regulation of cytoskeleton proteins including KRTs and Tpms leaded to injury of colonic epithelial structure, allowed gut bacteria enter into the lamina propria and trigger colonic inflammation. Therefore, maintenance of colonic epithelial structure by *S. neglecta* extract and polysaccharide rich extract might play a role in protection against DSS-induced inflammation.

Cytokines promote colitis-associated tumor development such as TNF, IL-6 and IL-1 (Rubin *et al.*, 2012). In IPA upstream regulator analysis, activated TNF-α, a mainly cytokine involving in promotion and progression of cancer was found in DSS-treated mice. However, *S. neglecta* extract and polysaccharide rich extract administration ameliorated TNF-α activation which could be one of their anti-inflammatory mechanisms. Moreover, IPA showed that c-MYC and n-MYC oncoproteins were activated but p53 was suppressed in DSS-treated mice. The overexpression of c-MYC has been expressed in approximately 70% of colorectal cancers associated with dysplasia of colonocytes (Chu *et al.*, 2007). The c-MYC regulated several biological activities

including cell proliferation, cell growth and cell transformation. Additionally, the important molecular checkpoint, p53 pathway, was suppressed in response to the severe damage of colonic epithelium induced by DSS administration. However, the mechanism of DSS-induced apoptosis in this model seemed to be independent of p53 activation. The previous studies found that an absence or low existence of p53 mutations were found during chemical induced colon tumorigenesis in rodents (De Robertis *et al.*, 2011). Moreover, administration of *S. neglecta* extract or polysaccharide rich extract impacted on numerous regulators of cell proliferation and suppressed activity of MAP kinases such as p38 MAPK, ERK1/2 and MAP2K1, which could explain their effects on cell proliferation in colonic mucosa. Altogether, the results from proteome and IPA analyses indicated that DSS-induced colitis in mice might be associated with colorectal carcinogenesis by controlling inflammatory cytokines, proliferation and apoptosis. Amelioration of DSS-induced colitis by *S. neglecta* extract and polysaccharide rich extract administration might cause prevention of colorectal carcinogenesis. In DSS-induced colitis model, *S. neglecta* extract and polysaccharide rich extract presented protective effects. Their modifying effects seem to be related to suppression of colonic epithelium from DSS-induced cell cycle arrest and cell death, improvement of colonic epithelial structure and mitochondria function as well as removal ROS in colonic mucosa.

The last experiment was focused on the effect of *S. neglecta* extracts on inflammation promoted preneoplastic lesion in rat colon. The ACF marker was detected 15 weeks after double injection of DMH and 7-day administration of DSS. We found DSS did not promote ACF formation initiated by DMH. Lower number of ACF but more nodules in colonic mucosa was observed in DMH/DSS-treated group when compared to DMH alone group. It might be suggested that colonic aberrant crypts were gradually developed to benign tumor during promotion stage. Therefore, dysplastic crypts, adenomas and adenocarcinomas in colon might be a reliable marker for chronic inflammation associated colon carcinogenesis (Onose *et al.*, 2003). From this reason, anticarcinogenicity of *S. neglecta* extracts in this model was not detected although they exhibited cancer chemopreventive effect in our former experiment. In this protocol, we also found the other imperfection on experimental design of DSS-induced chronic inflammation in rat colon. Not all proinflammatory cytokine markers, only the IL-1β and iNOS, were statistically increased. It might be due to the amount of DSS was not reach

to an inflammatory dose. The previous study reported that colonic inflammation was accomplished by the cyclic administration of a low dose of DSS (Clapper *et al.*, 2007, Marin *et al.*, 2013). It might be a reason why the anti-inflammatory activity of polysaccharide extract was not observed in low dose of DSS-induced chronic colitis in rats although this beneficial effect of the extract was found in high dose of DSS-induced acute colitis in mouse model.

In conclusion, *S. neglecta* could prevent the early stages of DMH-induced colon carcinogenesis in rats. The possible inhibitory effects were due to the modulation of xenobiotic metabolizing and antioxidant enzymes and inhibition of cell proliferation as well as induction of apoptosis. Furthermore, *S. neglecta* extract and polysaccharide rich extract also diminished chemical-induced colitis in mice by reducing ROS, maintaining normal mitochondrial function, attenuating pathological apoptosis leading to protecting colonic epithelium damage. Sulfated heteropolysaccharide might be one of cancer chemopreventive ingredients in *S. neglecta*.