CHAPTER 4
DISCUSSION

At present, more than 346 million people worldwide diagnosed with type 2 diabetes or T2DM. It is a heterogeneous disorder that is characterized by hyperglycemia and insulin resistance ((WHO), 2006). There are several risk factors to develop T2DM, including age, family history, genetic and dietary life styles (Olafsdottir et al., 2009). Moreover, obesity is known to be the most frequent related with T2DM (Boney, 2012). The experimental models for studying T2DM have been extensively established. The 60% high fat-diet (HFD) was demonstrated insulin resistance condition in rats and rabbits (Flanagan et al., 2008; Tanaka et al., 2007; Zhao et al., 2008). In addition, the combination of HFD and low single-dose of streptozotocin (STZ) injection in non-genetic, out-bred rats had shown the initial beta cell dysfunction, hyperglycemia and insulin resistance, which is similar to that of T2DM in human (Srinivasan et al., 2005). Therefore, the combination of HFD and single dose of STZ was utilized as an experimental model in this study. Consistently, the significant increase in the levels of fasting plasma glucose, triglyceride, and HOMA index was exhibited in DM rats, suggesting that hyperglycemia, hypertriglyceridemia and insulin resistance had been developed in our experimental rat model which is similar to that T2DM as seen in human.

The present study has shown that a significant increase in hyperglycemia was reduced by the oral administration of 1000 mg/kg BW of SN extract. Similarly, hypertriglyceridemia and HOMA index also reduced by administration of SN extract. Previous study showed that SN had a high concentration of polyphenolic compound which is antioxidant (Peerapornpisal et al., 2009). In addition, tannin or tannic acids, one of the components in polyphenol, have shown multiple biological effects *in vitro*, including anticancer, antioxidant, and antimicrobial activities (Hagerman et al., 1998; Okuda et al., 1995). Moreover, it has been shown that tannic acid stimulated glucose transport by phosphorylation of the insulin receptor (IR) and Akt (Liu et al., 2005). Therefore, the component in SN, probably tannin, might be an important molecule to

improve hyperglycemia, hypertriglyceridemia and insulin resistance in T2DM rats. In our study, T2DM rats supplemented with vitamin C, an antioxidant control, also showed a significantly decreased in plasma triglyceride (Table 4). These data were very similar to previous study in diabetes patients, showing a substantial decreased in total cholesterol and triglyceride levels after taking vitamin C supplementation at the dose of 1000 mg for 8 months (Sokoloff et al., 1967). Subsequent studies also showed that vitamin C deficiency by food deprivation had reduced in the catabolic rate of LDL, HDL and bile acid syntheses, which caused a higher total and LDL cholesterol concentrations in guinea pigs rats (Ginter, 1989; Hemilä, 1992). However, the precise mechanism of its action has not yet investigated. Similar to vitamin C and SN extract, the beneficial effects of antioxidant supplements, such as, green tea, aloe vera and grape seed extract have been also shown in metabolic disorders which were coronary heart disease and diabetes mellitus (Cabrera et al., 2006; Okyar et al., 2001; Suwannaphet et al., 2010).

Elevated levels of circulating blood glucose caused by T2DM induced increase in glucose autoxidation and protein glycation have been recently illustrated (Wright et al., 2006). These consequences lead to enhanced production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide and hydroxyl radicals (Batkova et al., 2008). Moreover, ROS increased lipid peroxidation in leucocytes, plasma, liver, pancreas, skeletal muscles, brain, and kidney tissues in STZ induced diabetic rats was also revealed (Matsunami et al., 2010). In our study, we have demonstrated that an increase in renal MDA, a lipid peroxidation end-product, in T2DM rats was significantly reduced by SN extract (Figure 1). In agreement with our data, lipid peroxidation was decreased in mice treated with the dietary astaxanthin-rich algal or vitamin C compared to that of either *Helicobacter pylori* infectious animals or mice fed with normal diet (Wang et al., 2000). Besides DM, a high ROS production was also linked to other pathological status, including Alzheimer's disease, chronic obstructive pulmonary disease, and ischemia/reperfusion injury (Pratico et al., 1998; Roberts and Morrow, 2000). However, a low to moderate levels of ROS have shown the opposite effect to play important physiological roles in cellular functions, such as, immune response, apoptosis, vascular tone, hormonal

regulation, signal transduction, activation of transcription factors, and antioxidant gene expressions and adaptive responses to several enzymes (Singh, 2009).

The alterations of membrane transport proteins in several pathological conditions have been extensive revealed. In T1DM rats, a decrease of $Na^{+}K^{+}$-ATPase and glucose transporter in liver, brain and heart tissues have demonstrated whereas the expressions of these two transporters were increased in the kidneys (Fedorak et al., 1987). Moreover, hyperuricemia in rats had shown a decrease in renal basolateral PAH uptake, corresponding to the reduction of rOat1 and rOat3 mRNA and protein expressions (Habu et al., 2003). Furthermore, previous study had found that chronic renal failure reduced Oat1 expression, and decreased renal secretion of organic anions in rats (Monica Torres et al., 2005). Despite previous studies have demonstrated that rOCTs and mOat3 had reduced expressions and functions in STZ induced T1DM (Lungkaphin et al., 2012; Thomas et al., 2003), the differences of physiological PAH and ES transport mediated by rOat1 and 3, which occurred in parallel with rOat3 protein expressions in our study were not observed. These differences could be due to the severity of pathogenesis, probably glycemic status, leading to develop different degrees of oxidative stress conditions and gene expressions between T1DM and T2DM. Likewise, long-term exposure of several high glucose concentrations enhanced ROS production from protein glycation and glucose autoxidation in a dose dependent manner, which in turns catalyzed lipid peroxidation in both endothelial cells and isolated rat glomeruli (Brownlee, 2004; Ha et al., 1994).

The characteristics of T2DM by excessive hepatic glucose production, decrease insulin secretion, and insulin resistance have been known for decades (DeFronzo, 1997; Kahn, 1994; Porte, 2001; Reaven, 2000). Furthermore, it has been shown that hyperglycemia induced T2DM leads to impaired insulin signaling cascade in several tissues, including skeletal muscles, adipocytes and kidneys (Srinivasan et al., 2005; Thirone et al., 2006). Moreover, Oat1 and 3 were up-regulated by either EGF or insulin through cAMP-PKA pathway (Barros et al., 2009; Soodvilai et al., 2004). In the present study, we have shown that the insulin stimulated PAH and ES uptake mediated by rOat1 and 3 were present in normal rats whereas the insulin effect was completely blunted in T2DM. Interestingly, the oral administration of SN extract

significantly restored insulin stimulated Oat1 and 3-mediated PAH and ES transport in DM rats. Surprisingly, insulin pre-incubation did not stimulate PAH and ES transport in SN supplemented in normal rat. The explanation of this matter is still unknown. However, recent study had shown that tannic acid inhibited cAMP activity resulted in a decrease of lipolysis in rat adipocytes (Wrisez and Lambert, 2001). Hence, tannic acid in SN extract might inhibit cAMP-dependent protein kinase, leading to attenuate up-regulation of Oat1 and 3 functions by insulin in normal rat supplemented by SN.

This finding is given the possibility that SN extract might primarily counteract against ROS through oxidative stress genes. As known that the imbalance between the generation of ROS and the ability to dispose by antioxidant system is the major factor to generate oxidative stress (Maneesh and Jayalekshmi, 2006). In addition, the pre-dominant scavenger enzymes involved in detoxifying ROS in mammalian systems are catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Mates et al., 1999). Previous study demonstrated that catalase and Cu-Zn SOD were increased their mRNA expressions in STZ-induced diabetic rat kidneys (Sechi et al., 1997). Furthermore, STZ diabetic transgenic mice which overexpressed Cu-Zn SOD gene improved glomerular injury and bovine aortic endothelial cells overexpressed SOD inhibited LDL oxidation (Craven et al., 2001; Fang et al., 1998). Moreover, Cu-Zn SOD overexpression also improved lipid peroxidation and glyco-oxidation formation in isolated rat glomeruli (Curcio et al., 1995; Dileepan et al., 1993; Tesfamariam and Cohen, 1992; Williamson et al., 1993). Nonetheless, the differences in Cu-Zn SOD, GPx or catalase gene expressions did not observe between NC, DM and DM+SN1000 in our study. These data were similar to previous studies that indicated no significantly change in total SOD, GPx and catalase enzyme activities in T1DM rat kidneys (Godin et al., 1988; Kakkar et al., 1995; Limaye et al., 2003). Therefore, the changes of enzyme expressions could depend on several variations, such as, experimental models, animal age, pathological severity, specific organ, and the measurement techniques (Limaye et al., 2003; Maritim et al., 2003).

The molecular mechanisms of the excess glucose induced cell injury in T2DM have been widely investigated. High glucose concentration was shown to activate

PKC in kidney tissues by various mechanisms, including de novo synthesis of DAG, and activation of PLC (King et al., 1996; Xia et al., 1996). Furthermore, PKC activity was shown to be lasted long within minutes and persistently increased in several days in both *in vitro* (Haller et al., 1995; Williams et al., 1992) and in STZ induced hyperglycemic rats (Inoguchi et al., 1992; Kunisaki et al., 1996). In addition, the activation of specific PKCα, ε and βII isoforms were found in diabetic nephropathy rats whereas activated PKCζ was likely to show protective effect in hyperglycemia rats (Kang et al., 1999b; Meier et al., 2007; Noh and King, 2007). Consistently, our data showed that PKCα was activated and its phosphorylated form translocated from cytosol to plasma membrane in T2DM rat (Figure 17B). We also found that the administration of SN at the dose of 1000 mg/kg BW significantly decreased phosphorylated PKCα in both whole cell and cytosolic fractions, suggesting that SN could de-activate PKCα in T2DM rat kidneys. Besides PKCα, The activation of NFκB by high glucose also decreased the expression of Na^+/glucose transporter (SGLT) and then led to renal dysfunction which was indicated to be the initial consequence of diabetic complications (Chen et al., 2003; Lee and Han, 2007). Moreover, activated PKCα associated with the nuclear translocation of p65NFκB in the presence of high glucose in rat glomerular mesangial cultured cells (Kumar et al., 2001). In response to cellular disturbances, such as, inflammation or oxidative stress, NFκB in cytosolic compartment of isolated mitochondria and rat hepatocytes were also demonstrated to be activated by dissociation from IκB (King and Loeken, 2004). Similarly, our present study demonstrated that p65NFκB was significantly increased in nuclei fraction of T2DM, suggested that p65NFκB was activated and phosphorylated NFκB translocated into the nucleus under diabetic condition. In contrast, nuclei fraction of DM treated with SN at the dose of 1000 mg/kg BW was markedly decreased compared to that of its expression in nuclei fraction from DM rat kidneys (Figure 16), suggesting that SN extract de-activated and inhibited nuclear translocation of p65NFκB. Hence, this transcription factor might be an important target to improve regulatory function of renal Oat1 and 3 in T2DM.

The involvement of PKCζ expression and activity have illustrated in several diabetic conditions (Cross et al., 1995; Zhang et al., 2010). For instance, PKCζ

translocated from cytosol to plasma membrane after incubation with high glucose in skeletal muscle tissues (Zhang et al., 2010). Recent studies had also shown that hyperglycemia and insulin could stimulate PKCζ activity in diabetic rat renal tissues (Kang et al., 1999a; Sheu et al., 2004; Zdychova et al., 2008). Regarding to the regulation of transporters, insulin stimulated mouse and rat Oat1 and Oat3 functions were mediated by trafficking process of these transporters though PKCζ activation (Barros et al., 2009). In contrast, the activation of PKCα by angiotensin II exhibited an inhibitory effect of both hOAT1 and hOAT3 functions (Duan et al., 2010; Li et al., 2010), Consistently, our study found that despite PKCζ was activated in T2DM rat similar to that of previously seen in other studies (Kang et al., 1999b; Sheu et al., 2004; Zdychova et al., 2008; Zhang et al., 2010), there was a significantly increase in total PKCζ protein expressions in both whole cell and cytosolic fractions after SN extract supplementation (Figure 18). Thus, this data suggested that SN extract activated new PKCζ protein synthesis in T2DM rat kidneys. Moreover, the data also demonstrated that p-PKCζ was highly expressed in both whole cell and membrane fractions while reduced its expression and phosphorylation in cytosol after supplemented with SN extract. Therefore, it can be concluded that SN extract activated p-PKCζ as a result of its translocation from cytosol to the plasma membrane.

Taken together, it is likely that the molecular mechanisms of SN extract acted directly to transcription factor NFκB and specific PKC isoforms, which were PKCα and ζ, leading to retrieve rOat1 and 3 functions at the post-translational regulation in T2DM rats (Figure 19). Nevertheless, the precisely potential molecules in SN extract require further investigation.

According to the daily intake of SN in human, the dose of SN extract in the current study must be taken into account. Previous study suggested that dose translation from rat to human should be considered using body surface area normalization method (Reagan-Shaw et al., 2008). Hence, daily intake 1000 mg/kg BW could correlate to only 162 mg/kg BW for human daily intake. Based on biological activity of SN extract, it was shown that 1 g of SN extract contains polyphenolic, antioxidant compounds, equivalent to that extracted from 77.7±3.6 mg

GAE (Boonchum et al., 2011; Ngozi et al., 2010). In addition, recent study has shown that a single oral administration of SN extract at a very high dose (5000 mg/kg BW) had no acute toxic effect (Peerapornpisal et al., 2010). In the current study, the data also demonstrated that SN extract at the dose of 1000 mg/kg BW did not change lipid peroxidation, oxidative stress makers, and renal transport function in normal rats. Therefore, the dose used in this study is safe and sufficient for daily intake in human. However, the therapeutic effects of SN extract in clinical trials are needed further investigations.

In conclusion, this study was demonstrated that high-fat diet with low-single dose of STZ induced hyperglycemia, hyperlipidemia, and renal oxidative stress condition in T2DM rat model. This experimental induction in rats did not change basal level of rOat1 and 3 functions. However, the impairments of up-regulation of rOat1 and 3 by insulin were demonstrated in T2DM rats. This defect was shown to associate with oxidative stress pathway through the activation of intracellular signaling molecules, such as, NFκB, PKCα and PKCζ. Interestingly, dietary supplement by SN extract could restore hyperglycemia, hypertriglyceridemia, and reduced oxidative stress by deactivation of NFκB and PKCα in kidney tissues. In addition, SN extract also improved rOat1 and 3 regulations by alteration of the intracellular signaling mechanisms, including stimulated PKCζ protein synthesis, membrane expression and phosphorylation**.** Thus, these findings indicate that SN extract could be a potential candidate used as a natural supplement to improve hyperglycemia-induced oxidative stress in T2DM.

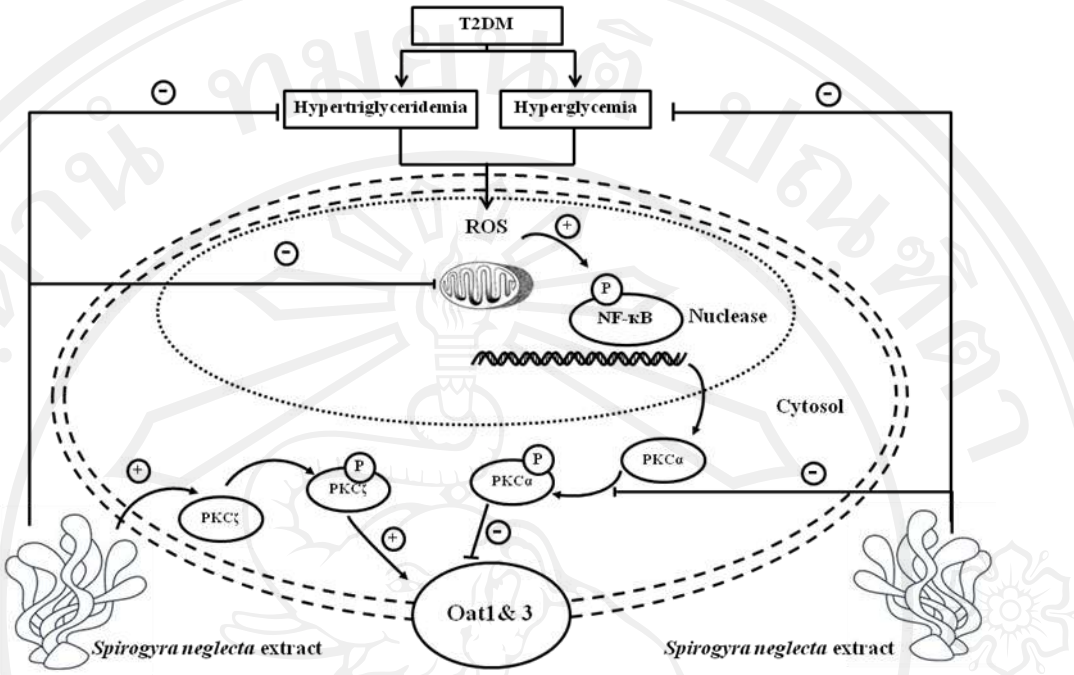

Figure 19 The possible mechanisms of *Spirogyra neglecta* extract restores rOat1 and rOat3 functions and regulations in T2DM rat model.

The general characteristics of T2DM rats were improved by *Spirogyra neglecta* extract, including hyperglycemia, hypertriglyceridemia and insulin resistance. Moreover, the pre-dominant antioxidant capacity of *Spirogyra neglecta* extract in T2DM on intracellular signaling cascade was shown as seen by the reduction of lipid peroxidation, activation of NFκB and PKCα, and increased phosphorylated PKCζ and its protein expression**.** Although the consequences of T2DM rat model did not affect physiological rOat1 and 3 functions, the defect on up-regulation of rOat1 and 3 by insulin stimulation was completely restored by *Spirogyra neglecta* extract.