CHAPTER 3

RESULTS

Effect of *Spirogyra neglecta* on general characteristics and plasma parameters in type 2 diabetic rats

As shown in Table 4, the body weight (BW), kidney weight per body weight (KW/BW) ratio, and insulin levels were not significantly different among experimental groups. However, DM rats had significant increased in the levels of fasting plasma glucose (298.4±46.2 mg/dl), triglyceride (266.5±59.3 mmol/l), and HOMA index (29.0±10.2) compared to that of NC (130.9±23.1 mg/dl, 141.0±37.8 mmol/l, 17.2±5.4, respectively, $p<0.05$). Similarly, DM+vitC had also shown a significant increase in both plasma glucose and HOMA index (277.5±62.5 mg/dl, 38.4±20.1, respectively) compared to that of NC. On the other hand, fasting plasma glucose, triglyceride and HOMA index were significantly reduced in DM+SN1000 (164.8±90.3 mg/dl, 156.9±34.5 mmol/l, 15.1±7.9, respectively. $p<0.05$) relatively to that of DMC. This result indicated that SN was able to reduce blood glucose, triglyceride, and insulin resistance in diabetic condition.

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Table 4 Effect of *Spirogyra neglecta* extract on body weight, kidney weight per body weight ratio and biochemical plasma parameters in experimental rats

	NC	NC+SN1000	DMC	DM+SN1000	DM+vit.C
Body weight (g)	516.0±57.3	493.3±40.3	500.0±48.6	520.0±49.3	445.0±22.9
Kidney weight/Body weight ratio	4.7±0.5	4.9±0.3	5.0±1.1	4.3±0.9	6.3±1.9†
Glucose (mg/dl)	130.9±23.1	127.7±19.4#	298.4±46.2*	164.8±90.3#	277.5±62.5*
Triglyceride (mmol/l)	141.0±37.8	101.6±23.5#	266.5±59.3*	156.9±34.5#	118.2±8.7#
Insulin (ng/ml)	2.2±0.5	1.6±0.5	1.0±0.6	1.1±0.2	2.2±0.5
HOMA-IR	17.2±5.4	16.0±8.2#	29.0±10.2*	15.1±7.9#	38.4±20.1*

HOMA index; homeostasis model assessment of insulin resistance , NC; normal control, NC+SN1000; Normal and SN supplement at at the dose of 1000 mg/kg BW, DMC; Diabetic mellitus control, DM+SN1000; Diabetes and SN supplement at the dose of 1000 mg/kg BW, DM+vit.C; Diabetes and ascorbic acid (vit.C) treatment at the dose of 200 mg/kg BW. Each value is expressed as mean ± SEM. *p<0.05 indicates the significant differences from normal (NC), #p<0.05 indicates the significant differences from T2DM (DMC) and † p<0.05 indicates the significant differences from Diabetes and SN supplement at the dose of 1000 mg/kg BW (DM+SN1000).

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Effect of *Spirogyra neglecta* on lipid peroxidation in renal cortical tissues

To determine oxidative stress condition in renal cortical tissues, we conducted thiobarbituric acid reactive substances (TBARS) assay. As shown in Figure 8, total malondialdehyde (MDA) level, end-product of lipid peroxidation, was significantly increased in DMC compared to that of NC (19.8±2.4 and 11.9±0.8 nmol/ml, respectively), showing a high oxidative stress condition in T2DM rat kidneys. In contrast, MDA levels were markedly reduced in DM+SN1000 and DM+vit.C relatively to that of DMC (8.8±1.1 and 13.3±1.4 nmol/ml, respectively), suggesting that SN at the dose of 1000 mg/kg BW had an antioxidant effect in rat kidney tissues similar to that of vitamin C in this T2DM rat model. Moreover, normal rat supplemented with SN extract at the same concentration showed the similar extent of the total MDA level as seen in NC, implying that SN extract at the dose of 1000 mg/kg BW did not increase oxidative stress in normal condition.

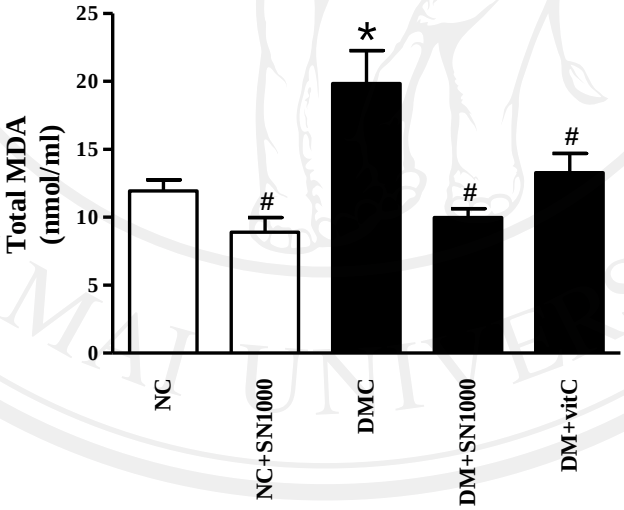

Figure 8 Effect of *Spirogyra neglecta* extract on lipid peroxidation in rat kidney tissues. Renal cortical tissue homogenates were added with enzymatic master mix to produce coloring thiobarbiturate-malondialhyde (MDA-TBARS) substances. The absorbance of MDA-TBAR products were subsequently measured using spectroscopy at the wavelength 595 nm. Each data is expressed as mean ± SEM (n = 6). *$p \leq 0.05$; significantly different from the NC, #$p \leq 0.05$; significantly different from the DMC.

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Measurement of *para*-aminohippurate (PAH) transport mediated by rOat1 and rOat3

We further examined the effects of SN extract on rOat1 and 3 substrate transports in T2DM rats. [^{3}H]PAH is known as typical substrate of rOat1 and 3 while [^{3}H]ES is a specific substrate for rOat3. Renal cortical slices were incubated in the buffer containing 20 µM glutarate and 5 µM [^{3}H]PAH for 30 min. As shown in Figure 9, the uptake of PAH mediated by rOat1 and 3 were not different among experimental groups, indicating that this experimental T2DM did not affect on Oat1 and 3 functions and SN extract itself did not change organic anion substrate transport by either rOat1 or 3 function as indicated by normal rats supplemented with SN extract.

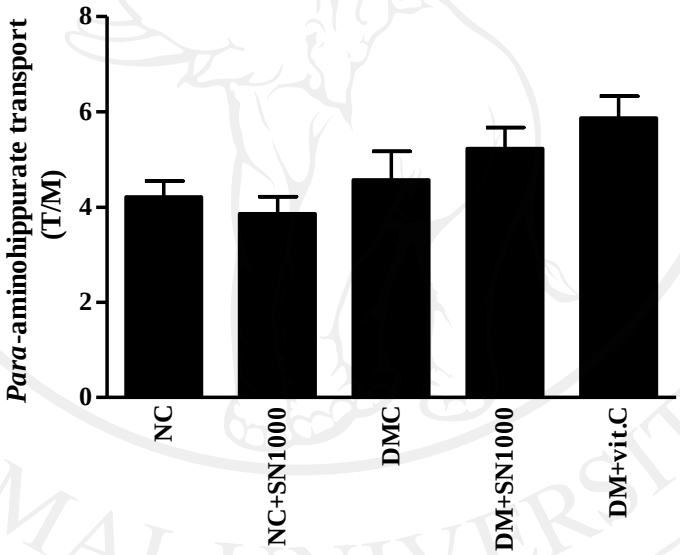

Figure 9 *Para*-aminohippurate transport mediated by rOat1 and rOat3 in rat renal cortical tissues. The renal cortical slices were incubated in the buffer containing 20 µM glutarate and 5 µM [^{3}H] PAH for 30 min at room temperature. Data are expressed as tissue to medium ratios (T/M), i.e. tissue content (DPM/mg) ÷ medium (DPM/µl). Each experiment was performed from separate animals using 5 slices from each animals (n=6).

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Measurement of estrone sulfate (ES) transport mediated by rOat3

Similar to PAH, renal cortical slices were incubated in the buffer containing 20 µM glutarate and 50 nM [^{3}H]ES for 30 min. The slices from each group had no different on ES uptake mediated by rOat3 (Figure 10). This result indicated that T2DM in this experimental rat model and SN extract did not change basal uptake of organic anion by rOat3 function.

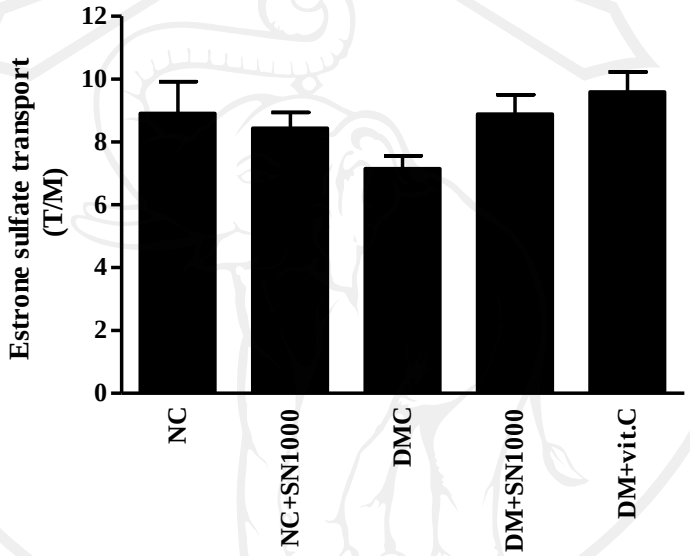

Figure 10 Estrone sulfate transport mediated by rOat3 using renal cortical slices. Each slices were incubated in the buffer in the presence of 20 µM glutarate and 50 nM [^{3}H]ES for 30 min at room temperature. Data are expressed as tissue to medium ratios (T/M), i.e. tissue content (DPM/mg) ÷ medium (DPM/µl). Each experiment was performed from separate animals using 5 slices from each animals (n=6).

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Effect of *Spirogyra neglecta* extract on insulin stimulated Oat1 & 3-mediated PAH transport

To determine regulatory function of rOat1 and 3, renal slices were pre-incubated in the absence or presence of insulin at the concentration of 30 μg/ml in the incubation buffer for 30 min at the room temperature and subsequently incubated in the incubation buffer containing 20 μM of glutarate and 5 μM [^{3}H]PAH for another 30 min. As shown in Figure 11, the results showed that pre-incubation with insulin in NC renal slices significantly increased [^{3}H]PAH uptake compared to that of the slices without insulin (170.1±12.9% of control), indicating that up-regulation of rOat1 and 3 by insulin stimulated PAH uptake was reproducible. On the other hand, insulin pre-incubation did not stimulate [^{3}H]PAH transport in NC+SN1000 and DMC (120.5±12.9 and 105.3±6.2% of control, respectively). Similar to NC, insulin stimulated PAH uptake were demonstrated in DM+SN1000 and DM+vitC (160.9±19.1 and 175.6±16.3% of control, respectively), suggesting that SN extract and vitamin C were able to restore insulin stimulated PAH transport in T2DM rats.

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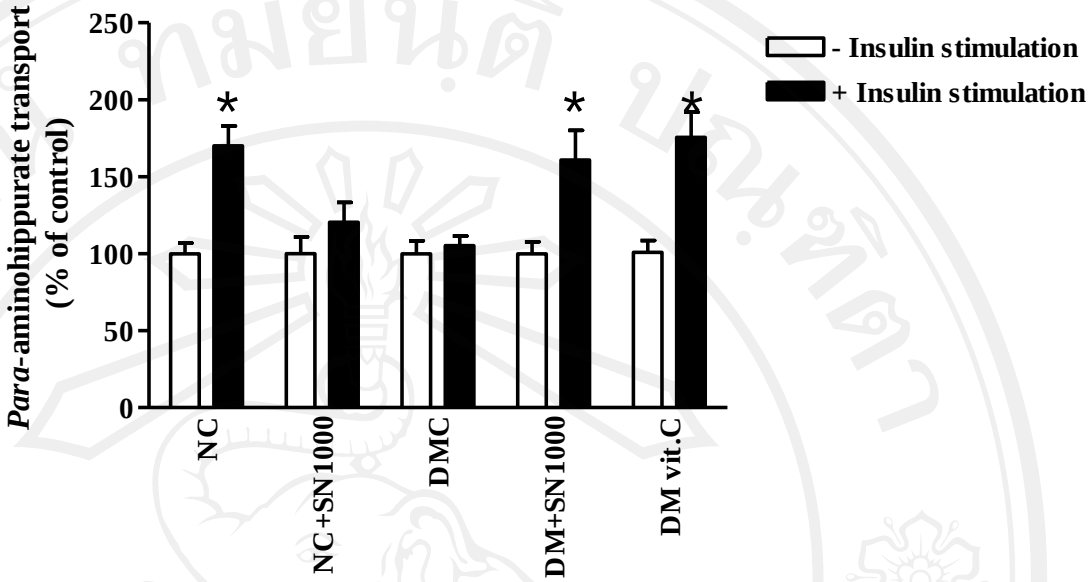

Figure 11 Effect of *Spirogyra neglecta* extract on insulin stimulated Oat1 and 3-mediated PAH transport. Rat renal cortical slices were pre-incubated for 30 min in the presence or absence of 30 µg/ml insulin and followed by incubation with 5 uM [^{3}H]PAH for 30 min. Data are expressed as tissue to medium ratios (T/M), i.e. tissue content (DPM/mg) ÷ medium (DPM/µl), and represented as percentage of control (without insulin) as mean ± S.E.M. Each experiment was performed from separate animals and at least 5 renal slices were used in each condition (n=6). *p < 0.05 indicates significant differences from the slices incubated with buffer alone.

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Effect of *Spirogyra neglecta* extract on insulin stimulated Oat3-mediated ES transport

To specific determine regulatory function of rOat3, the slices were pre-incubated in the absence or presence of insulin at the concentration of 30 μg/ml in the incubation buffer and subsequently incubated with 20 μM of glutarate and 50 nM [^{3}H]ES for 30 min. Similar to PAH, pre-incubation with insulin significantly stimulated [^{3}H]ES transport in NC slices (165.4±13.8% of control) whereas the effect of insulin on [^{3}H]ES uptake was attenuated in NC+SN1000 and DMC slices (113.6±13.8 and 114.3±5.8% of control, respectively) (Figure 12). Interestingly, insulin stimulation in DM+SN1000 and DM+vitC were markedly increase ES uptake (146.0±7.4 and 170.2±19.6 % of control, respectively). Therefore, this result indicated that SN extract and vitamin C improved the impairment of insulin stimulated ES transport mediated by rOat3 in T2DM rats.

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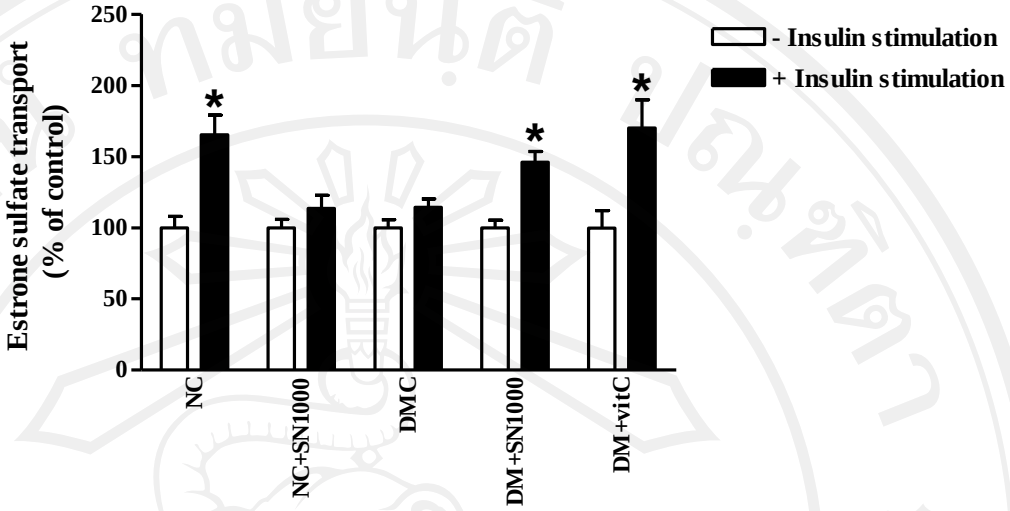

Figure 12 Effect of *Spirogyra neglecta* extract on insulin stimulated Oat3 mediated ES transport. Rat renal cortical slices were pre-incubated for 30 min in the presence or absence of 30 µg/ml insulin and followed by incubation with 50 nM [^{3}H]ES for 30 min. Data are expressed as tissue to medium ratios (T/M), i.e. tissue content (DPM/mg) ÷ medium (DPM/µl), and represented as percentage of control (without insulin) as mean ± S.E.M. Each experiment was performed from separate animals and at least 5 renal slices were used in each condition (n=6). *$p < 0.05$ indicates significant differences from the slices incubated with buffer alone.

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Effect of *Spirogyra neglecta* extract on rOat3 protein expression

To clarify whether insulin up-regulated rOat1 and 3, as seen in previous experiments, were due to the SN extract increased renal transporter protein expressions, and because none of the commercially available anti-rOat1 antibody could be detected, therefore, only rOat3 protein expression was obtained using western blotting analysis. Consistent with basal uptake of ES, there was no difference in relative rOat3 protein expressions at the MW 130 kDa in total cell lysates, membrane and cytosolic fractions among these experimental groups compared to that of its reference genes, β-actin. The preparation of membrane fractions was also detected by anti-Na^+-K^+-ATPase antibody at the MW 112 kDa (Figure 13). Therefore, this result indicated that restoration of up-regulated ES uptake through insulin stimulation in DM+SN1000 rat kidneys were not cause by increased rOat3 expression.

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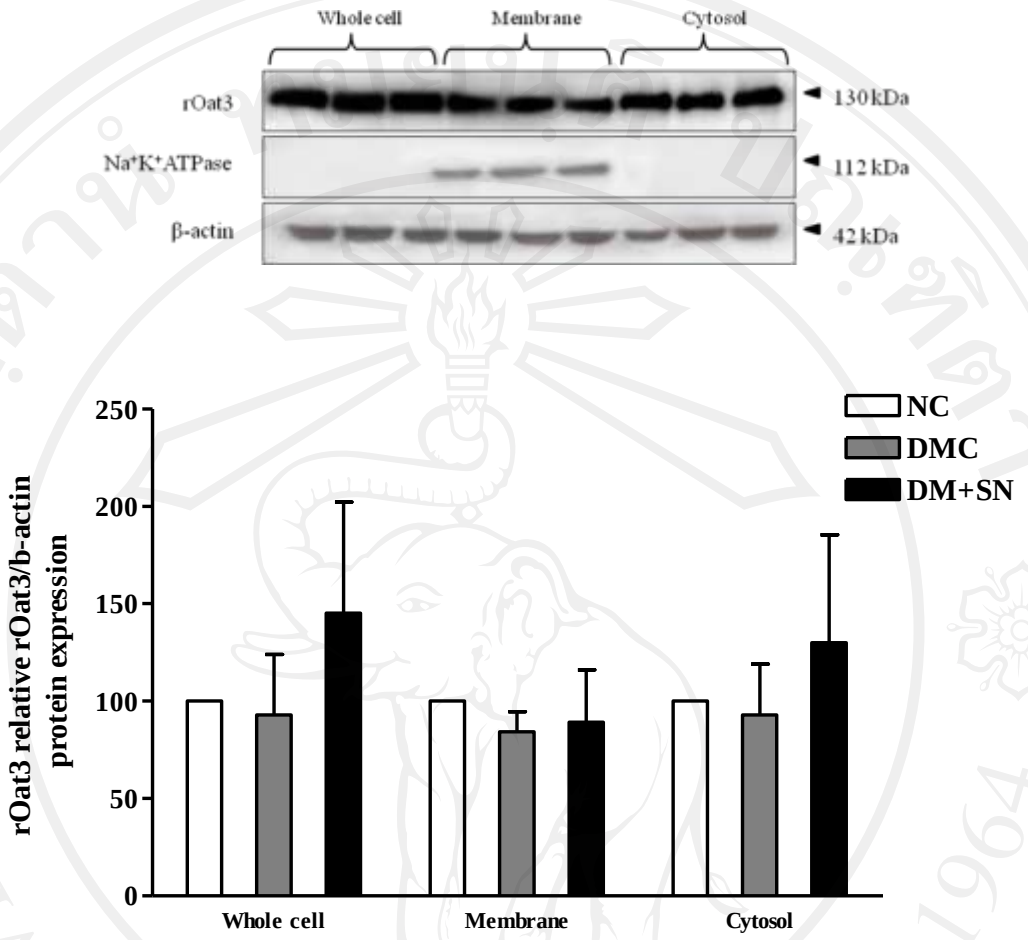

Figure 13 Western blot analysis of rOat3 protein expression in rat kidneys. Whole cell lysate, membrane and cytosolic fractions were extracted from rat renal cortical tissues. The protein samples were then separated using electrophoresis and western blotting. Anti-rOat3 antibody was subsequently detected at the 130 kDa whereas anti-Na^+-K^+-ATPase antibody at 112 kDa and anti-β-actin antibody at the 42 kDa were also detected as a membrane marker and loading control, respectively. The data are expressed as mean ± S.E.M. and repeated from separate animals (n=6). A representative blot of rOat3 protein expression is shown in *top* and quantification of relative protein expression in each fraction is presented in b*ottom*.

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Optimized conditions for semi-quantitative polymerase chain reaction for measurement of antioxidant gene marker mRNA expressions

To quantitate mRNA expression, the numbers of PCR cycles were optimized for each antioxidant gene marker. As shown in Figure 14, the linear phases of PCR amplification from normal renal tissues for RT-PCR product of glutathione peroxidase (GPx) and Cu-Zn superoxide dismutase (SOD) were fall in the optimal cycles at 29 cycles (Fig 7A and B, respectively) whereas catalase and β-actin were shown at 31 cycles (Fig 7C and D, respectively). Therefore, these numbers of the cycles were subsequently used for RT-PCR analyses in renal cortical tissues.

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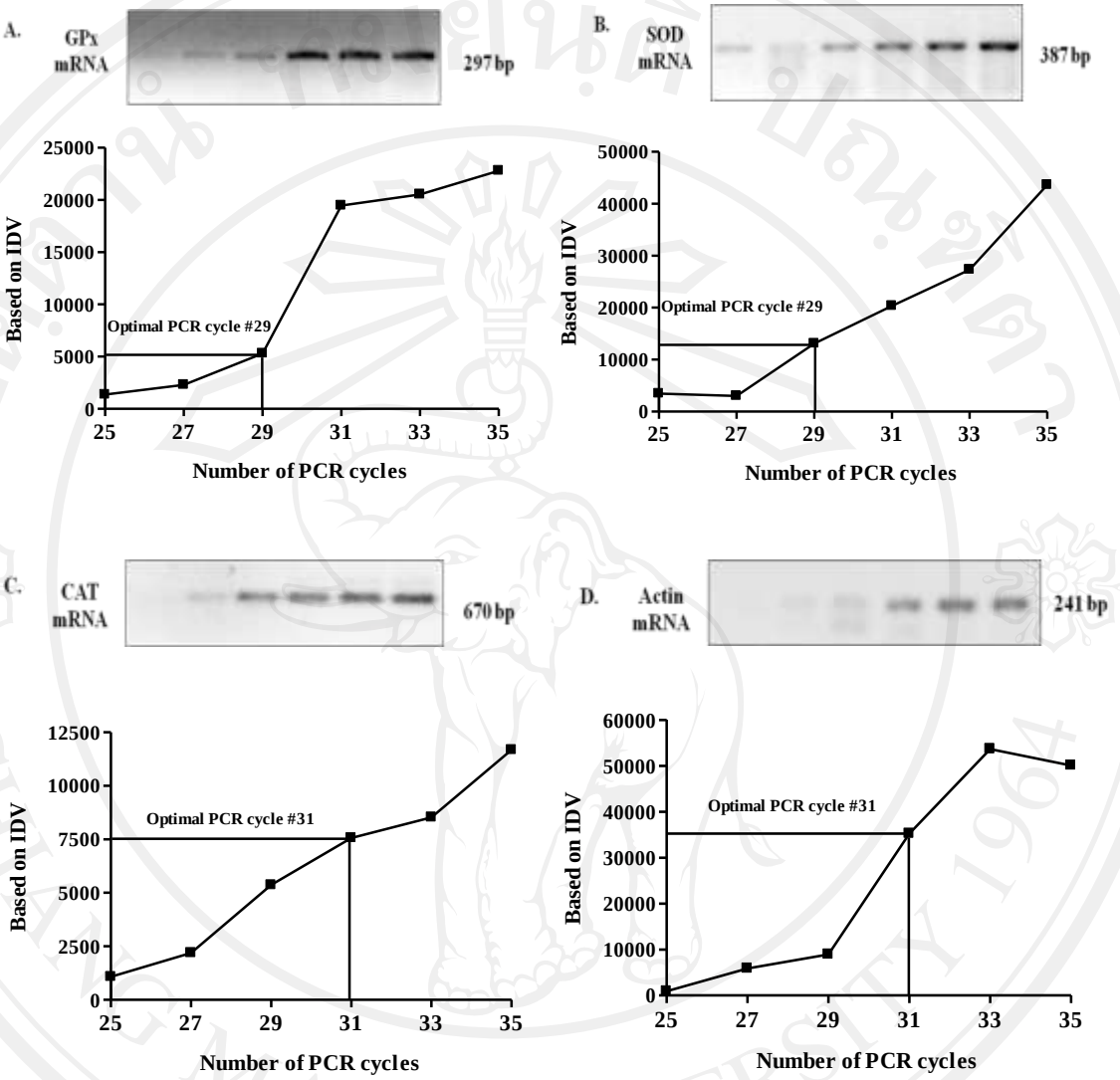

Figure 14 The amplification curve of antioxidant gene marker mRNA expression in renal tissues. Total RNAs were extracted from normal renal cortical tissues. Complementary cDNAs were then obtained and RT-PCR products were amplified. Optimal PCR cycles were determined by plotting amplification curve for glutathione peroxidase (GPx) and Cu-Zn superoxide dismutase (SOD), catalase and β-actin, respectively. IDV; integrated density value

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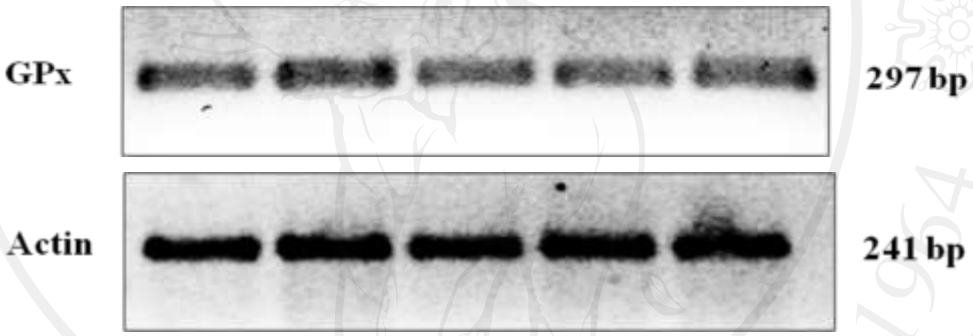
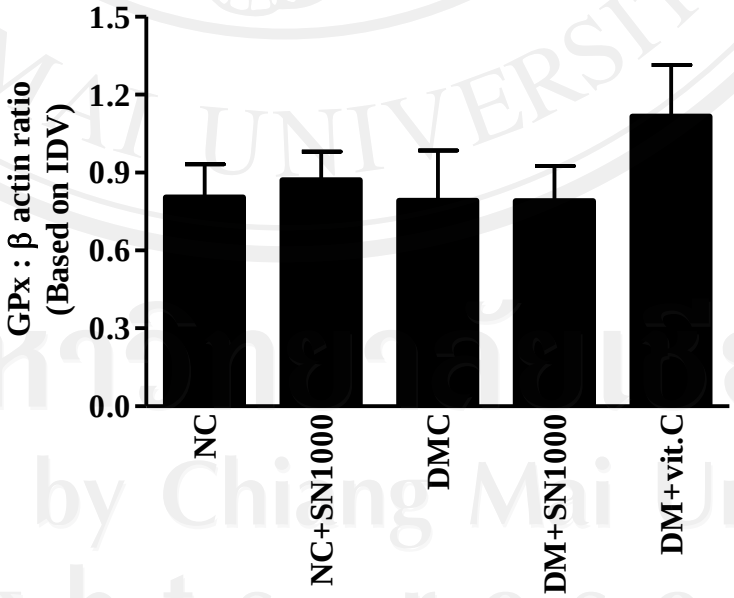
Effect of *Spirogyra neglecta* extract on antioxidant gene marker mRNA expressions in renal cortical tissues.

As shown in Figure 15, the relative RT-PCR products of GPx (A), Cu-Zn SOD (B), and catalase (C) were not different among experimental groups, including NC, NC+SN1000, DMC, DM+SN1000 and DM+vit.C. Therefore, this result indicated that T2DM rat model did not affect antioxidant gene transcriptions in renal cortical tissues and supplementation of SN extract at the dose of 1000 mg/kg BW/day for 12 weeks might not be able to increase antioxidant system at this transcriptional level.

A.





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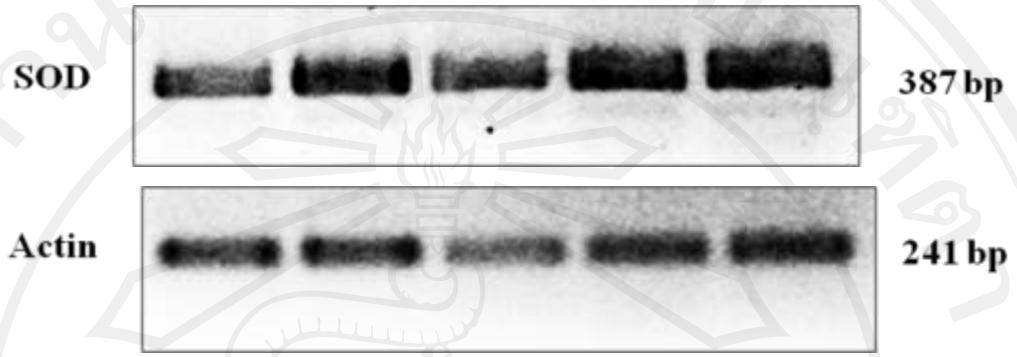

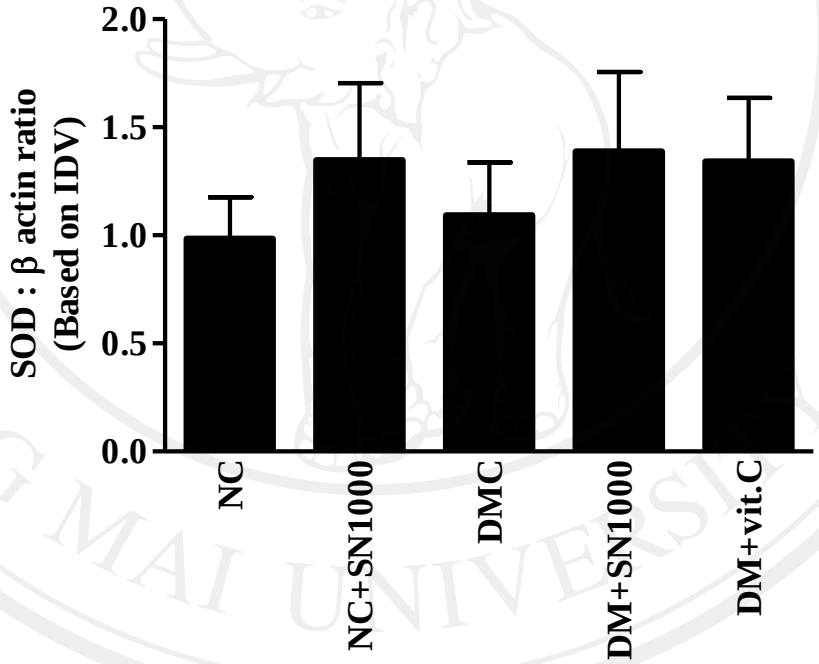

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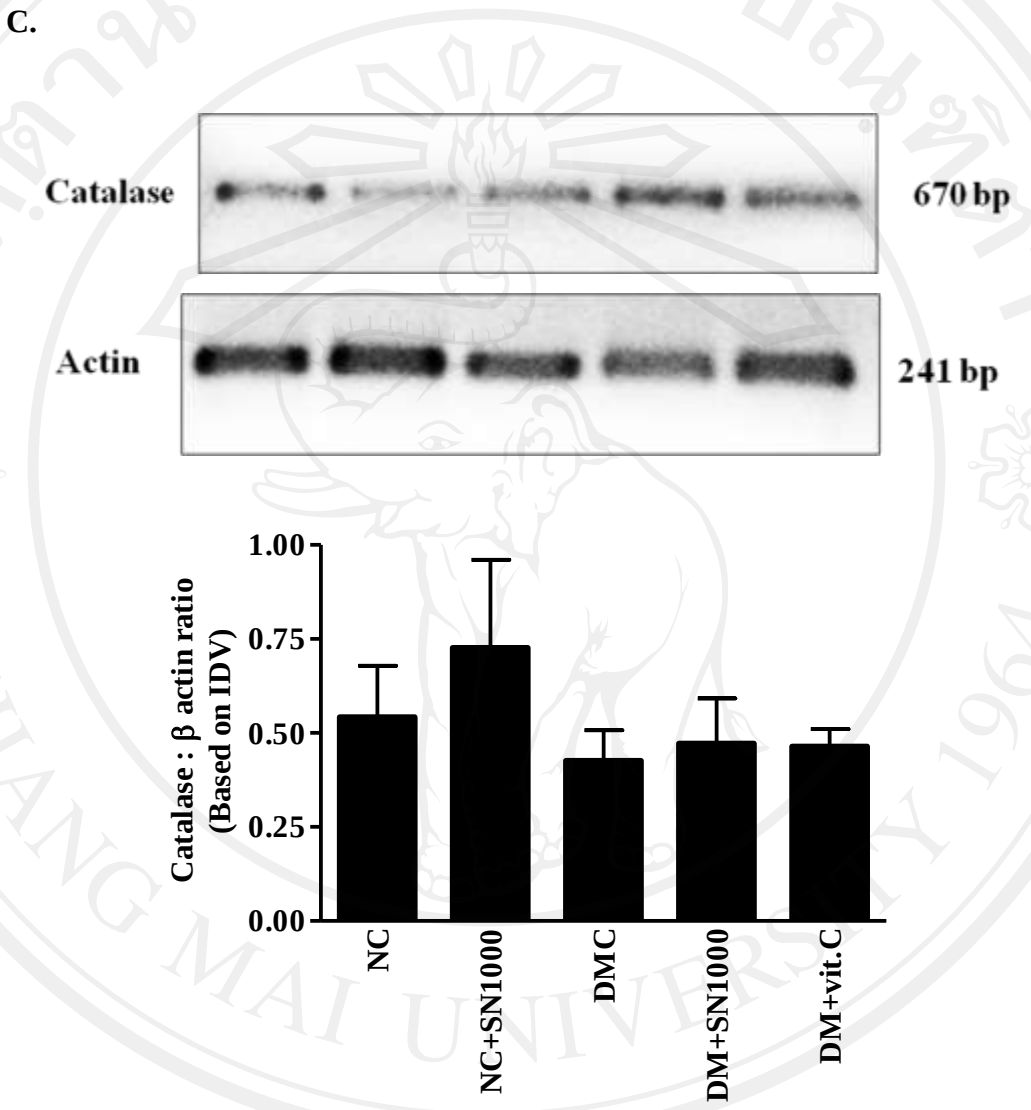

Figure 15 Antioxidant gene marker mRNA expressions in renal cortical tissues. (A) mRNA expression levels of glutathione peroxidase, (B) Cu-Zn superoxide dismutase and (C) catalase from NC, NC supplemented with SN extract, DMC, DM supplemented with SN extract, and DM supplemented with vit.C. Total RNA was extracted from rat renal cortical tissues, complementary cDNAs were then obtained, and RT-PCR products were amplified. mRNA levels were analyzed using semi-quantitative RT-PCR. The results are expressed as mean ± S.E.M and repeated from separate animals (n=6).

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Effect of *Spirogyra neglecta* extract on stress-sensitive protein expressions in renal cortical tissues

I. p65NFκB expression in renal cortex.

To further identify the effects of SN extract on the activity of stress-sensitive protein NFκB , the phosphorylation of NFκB (p65NFκB) was detected using western blotting analysis. As shown in Figure 16, there was no different in p65NFκB protein expression at 65 kDa in whole cell and cytosolic fractions among NC, DMC and DM+SN1000 rat kidneys. However, p65NFκB was significantly increased in nuclei fraction of DMC and DM+SN1000 compared to that of control. The expression of NFκB was markedly decreased in nuclei fraction of DM+SN1000 relatively to DM rat kidneys. The preparation of nuclei fractions was also detected by anti-lamin B1 antibody, which is nuclear protein marker at the MW 66 kDa. This data indicated that oxidative stress in T2DM occurred through NFκB activation, and SN extract could de-activate this protein in DM rat kidneys.

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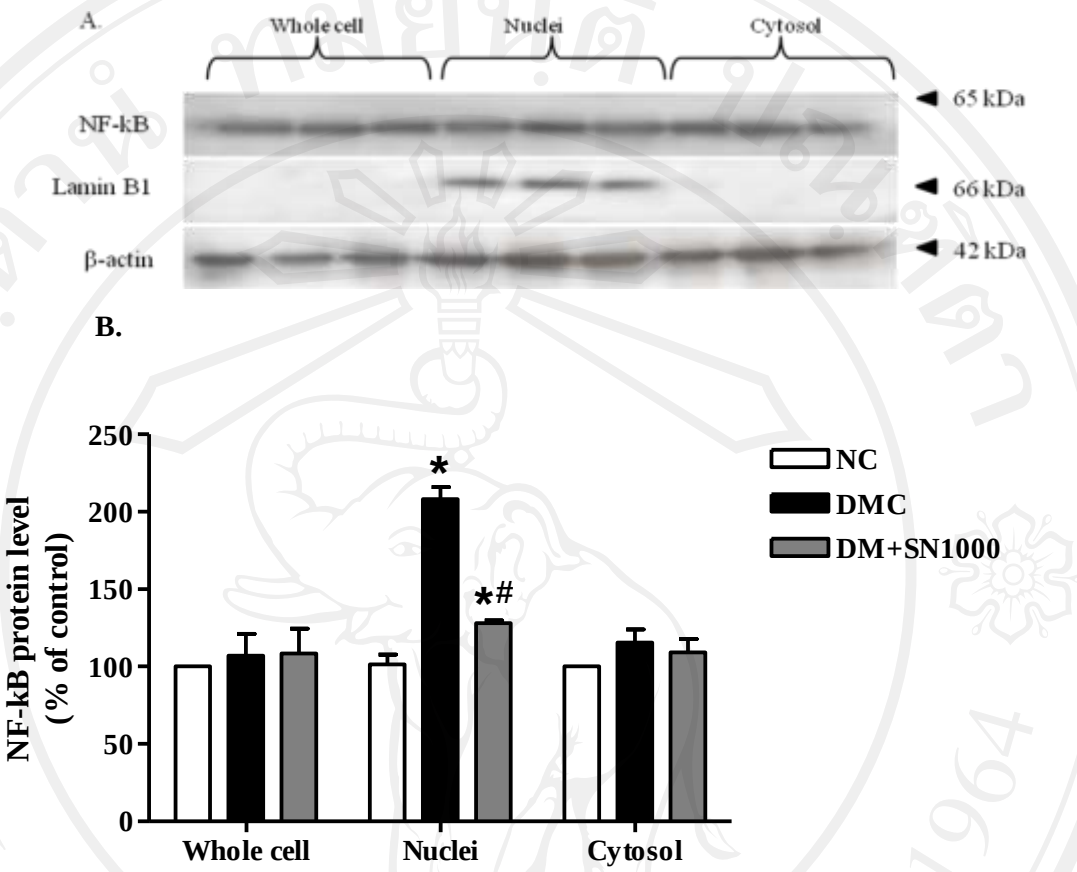

Figure 16 Effect of *Spirogyra neglecta* extract on the expression of p65NFκB in renal cortex. Whole cell lysate, nuclei and cytosolic fractions from rat renal tissues were extracted. The protein samples were then separated by Western blotting. (A) Anti-p65 NFκB antibody was detected at the MW 65 kDa in each cellular fraction of the rat kidneys. (B) Quantification of relative p65NFκB protein expression from cellular fractions in each experimental group. Anti-lamin B1 and β-actin antibodies were used as a nuclei marker and loading control, respectively. Densitometry was analyzed and expressed as mean ± S.E.M. The data was repeated from separate animals (n=6). *p<0.05 indicates the significant differences from normal (NC) and $^{\#}p$<0.05 indicates the significant differences from T2DM (DMC) rats.

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II. PKCα and p-PKCα protein expressions in renal cortex.

To identify whether the restoration of insulin up-regulated rOat1 and 3 by SN extract through stress sensitive pathway, the expression and activity of PKCα were evaluated. As shown in Figure 17, the expressions of non-phosphorylated PKCα at 80 kDa were not significant different in whole cell lysate, membrane and cytosolic fractions among NC, DMC and DM+SN1000. Interestingly, phosphorylated PKCα at 82 kDa were highly expressed in whole cell and membrane fractions whereas its expression was substantial decreased in cytosolic fraction in DMC compared to that of NC, indicating that PKCα was activated and subsequently translocated to the membrane under DM condition. In contrast, the significant decrease of p-PKCα in whole cell and membrane fractions was shown in DM+SN1000 relatively to that of DM rat kidneys, suggesting that SN extract restored the up-regulation of rOat1 and 3 functions by de-activation and decreased membrane expression of PKCα in DM rat kidneys.

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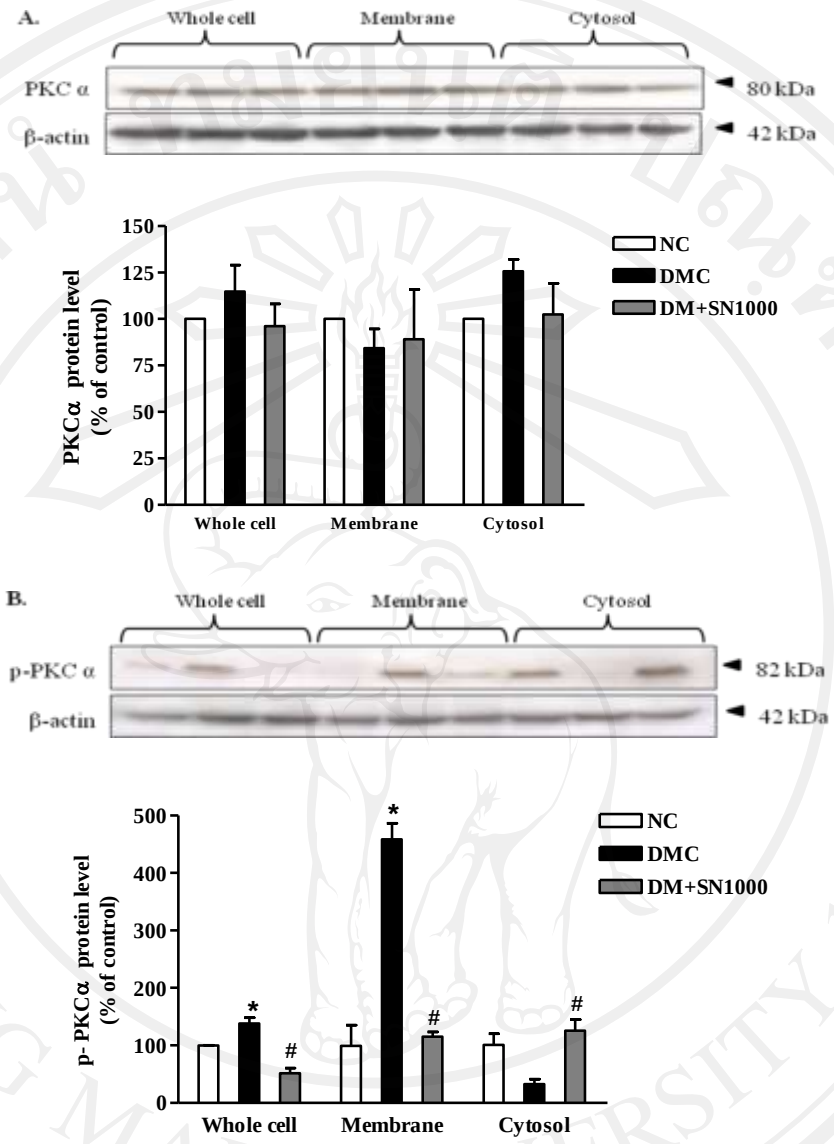

Figure 17 Effect of *Spirogyra neglecta* extract on PKCα expression and activation in renal cortical tissues. Whole cell lysate, membrane and cytosolic fractions from rat renal tissues were obtained. The protein samples were then separated by Western blotting. (A) Anti-PKCα and (B) p-PKCα antibodies were detected in NC, DMC and DM+SN1000 rat kidney tissues. Anti-β-actin antibody was used as a loading control. Densitometry was analyzed and expressed as mean ± S.E.M. and also repeated from separate animals (n=6). *p<0.05 indicates the significant differences from normal (NC) and $^{\#}p$<0.05 indicates the significant differences from T2DM (DMC) rats.

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Effect of *Spirogyra neglecta* extract on regulatory protein PKCζ and p-PKCζ expressions in renal cortex.

As previous study shown that up-regulation of Oat1 and 3 by insulin shared a common pathway through PKCζ activation, and PKCζ-rOat3 interaction plays a central role in controlling rOat3 function (Barros et al., 2009). We, therefore, further determine the mechanism of SN extract on rOat1 and 3 regulatory functions using western blotting analysis. In Figure 18A demonstrated that total PKCζ protein expression was significantly increased in whole cell and cytosolic fractions of DM+SN1000 rats compared to that of NC. However, there was no change in other fractions among experimental groups, suggesting that SN extract could stimulate PKCζ protein synthesis.

In addition, the activation of PKCζ indicated by p-PKCζ of DM rats was markedly increased in all fractions compared to that of control (Figure 18B). Interestingly, p-PKCζ in both whole cell and membrane fractions of DM+SN1000 were persistently increased compared to that of DMC whereas its expression was reduced in cytosolic fraction relatively to DMC. This result demonstrated that despite PKCζ was activated under DM condition, SN extract stimulated cellular PKCζ protein synthesis and activated PKCζ, leading to translocation of PKCζ from cytosol toward the membrane resulted in restoration of rOa1 and 3 regulatory functions.

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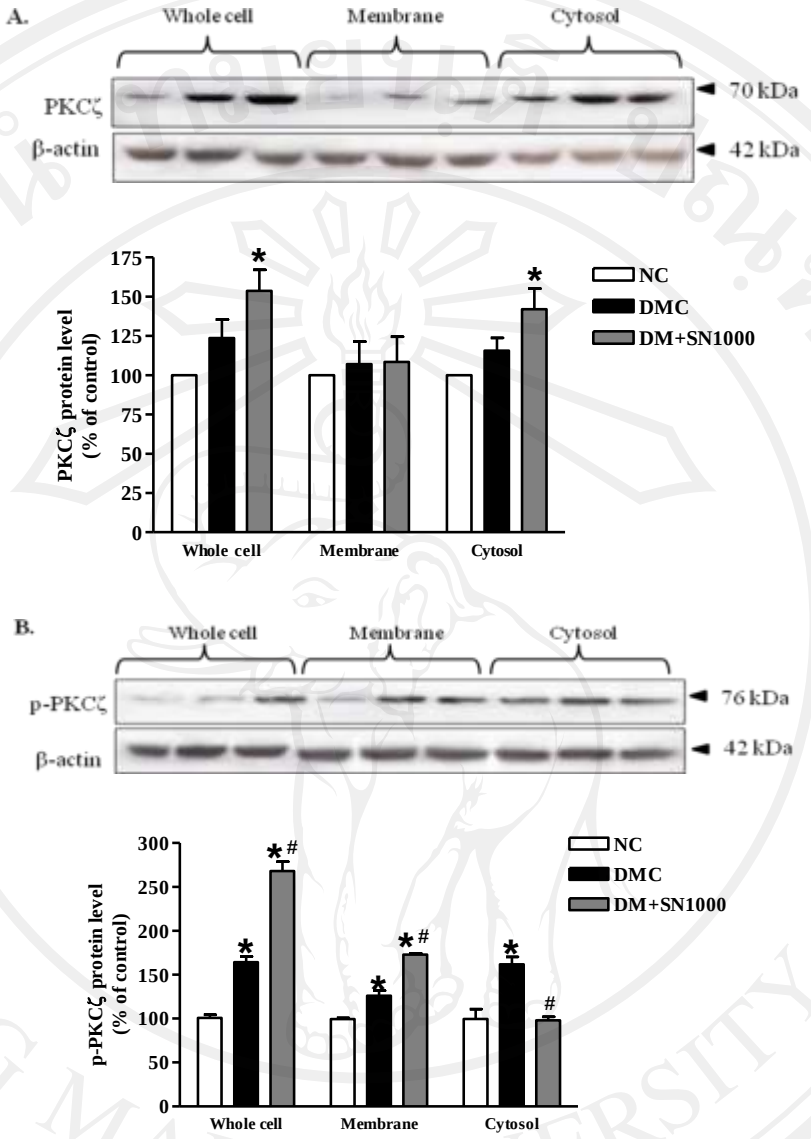

Figure 18 Effect of *Spirogyra neglecta* extract on the expression of PKCζ and p-PKCζ in renal cortical tissues. Whole cell lysate, nuclei and cytosolic fractions from rat renal tissues were obtained. The protein samples were then separated by Western blotting. (A) anti-PKCζ and (B) anti-p-PKCζ antibodies were detected in each cellular fraction. Anti-β-actin antibody was also used as a loading control. Densitometry was analyzed and expressed as mean ± S.E.M. The data was repeated from separate animals (n=6). *p<0.05 indicates the significant differences from normal (NC) and $^{\#}p$<0.05 indicates the significant differences from T2DM (DMC) rats.

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