HE014

In vitro Free radical scavenging activity and anti-hyperglycemic effect of Tiliacora *triandra* (Colebr.) Diels in type 2 diabetic rats

Tipthida Pasachan[1]
Atcharaporn Ontawong[2]
Acharaporn Duangjai[3]
Oranit Boonphang[4]
Anchalee Pongchaidecha[5]
Doungporn Amornlerdpison[6]
Chutima Srimaroeng[7]

Abstract

Tiliacora triandra (Colebr.) Diels (TT) is a native plant of Southeast Asia and widely use in northeastern Thai cuisines known as “Yanang”. Several studies have shown that leaves of TT extract (TTE) has various pharmacological effects, such as, anti-inflammatory, anti-cancer, anti-aging, anti-oxidant, anti-hypercholesterolemia, and inhibition of intestinal cholesterol absorption. However, the beneficial effects of TTE on hyperglycemia is limited. This study investigated the effects of TTE on antioxidant activity and anti-hyperglycemia against diabetes in rats. Free radical scavenging activities of TTE were determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH•) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+) assays. Oral glucose tolerance test was used to screen anti-hyperglycemic effects in normal rats. Type 2 diabetic rats were induced by high-fat diet and a low-single dose of streptozotocin and supplemented with 250, 500, 1000 mg/kg BW of TTE daily for 12 weeks. Plasma biochemical parameters were analyzed. The mRNA expression of genes involved in oxidative stress including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) was evaluated using quantitative real-time PCR. Results show TTE exhibited free radical scavenging activities against DPPH• and ABTS•+. Acute anti-hyperglycemic effect

[1,2,4,5,7]Department of Physiology, Faculty of Medicine, Chiang Mai University
[2,3]Division of Physiology, School of Medical Sciences, University of Phayao
[6]Faculty of Fisheries Technology and Aquatic Resources, Maejo University

of TTE was shown at 1000 mg/kg BW in normal rats. TTE also exerted anti-hyperglycemia, anti-hypercholesterolemia and improved glucose intolerance in type 2 diabetic rats. These TTE effects were corresponding to the significant reduction in mRNA expression of antioxidant genes (SOD, CAT and GPx). These findings suggest that TTE has anti-diabetic and anti-oxidant effects which could potentially be promoted into nutraceutical product for diabetes.

Kewords : Tiliacora triandra (Colebr.) Diels; Hyperglycemia; Free radical scavenging activity, diabetes

Introduction

Diabetes Mellitus (DM) describes as a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates, and proteins which increased risk of complications including cardiovascular diseases and non-alcoholic fatty liver disease (Ross and Kasum 2002, Bugianesi, McCullough et al. 2005, Petersmann, Nauck et al, 2018). Due to the liver is the primary organ susceptible to the effects of hyperglycemia, this may lead to increased oxidative stress and inflammation resulting in liver tissue injury (Palsamy, Sivakumar et al, 2010). Thus, complications associated with DM could be delayed or prevented through proper blood glucose management, reducing long-term microvascular and macrovascular complications and maintaining the overall quality of life (Maji, 2004). At the present, traditional medicine, especially, plant-based medicines have raised to be an alternative option in health-care system (Bernardini, Tiezzi et al. 2018). Moreover, the polyphenol compounds that widely distribute in several plants have gained attention and implicated in human health due to their antioxidant activity with the less adverse side effects (Ross and Kasum, 2002).

Tiliacora triandra (Colebr.) Diels leaves extract (TTE) or Yanang is a native plant of Southeast Asia that widely use in northeastern Thai cuisines. Several studies have shown that TTE has various pharmacological effects, such as, anti-inflammatory, anti-cancer, anti-aging, anti-oxidant, anti-hypercholesterolemia, and inhibition of intestinal cholesterol absorption with no toxicity (Seewaboon Sireeratawong et al. 2008, Ali Ahmeda and Ismail, 2009., Nanasombat and Teckchuen 2009, Singthong, Ningsanond et al. 2009, Singthong, Oonsivilai et al. 2014, Duangjai and Trisat 2015). Among TTE's beneficial effects, antioxidant of TTE

which exerts anti-diabetic effects have not investigated yet. Thus, this study examined antioxidant and anti-hyperglycemia using *in vitro* and *in vivo* studies.

Materials and Methods

TTE extract preparation

Tiliacora triandra (Colebr) Diels aqueous extract (TTE) was identified for species and a voucher specimen (number 003800) has been deposited at the herbarium of the PNU herbarium, Faculty of Science, Naresuan University, Phitsanulok, Thailand. Briefly, 400 g of TT leaves was extracted by 1 L of boiling water at 100 °C for 1 h and the TT solution was subsequently filtered through fabric filter 2 times. TTE solution was dried using spray dryer (Euro Best Technology Co., Ltd, Bangkok, Thailand). TTE was stored at -20 °C for further experiments.

Stability of free radical scavenging on $ABTS^{\bullet+}$ and $DPPH^{\bullet}$

Free radical scavenging activity of extract was assessed in TTE from 3 months storage compared to fresh extraction using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) cation decolorization assay according to previous study (Re, Pellegrini et al, 1999). In brief, TTE was incubated with $ABTS^{\bullet+}$ reagent at room temperature for 6 min. The absorbance of the sample was then determined at 734 nm using a Synergy™ HT microplate reader (Biotek, VT, USA). The unit of total antioxidant activity is defined as the concentration of Trolox having equivalent antioxidant activity expressed as μg/mL extract. The half maximal effective concentration (EC_{50}) value of the extract which means the effective concentration of extract at which $ABTS^{\bullet+}$ radical was scavenging by 50% was also calculated.

Similarly, free radical scavenging activity of TTE was also confirmed using the 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging ($DPPH^{\bullet}$) assay according to the method from previous study (Gamez, Luyengi et al., 1998). Briefly, TTE was incubated with 100 μM $DPPH^{\bullet}$ solution at room temperature for 30 min. The absorbance of the sample was then determined at 517 nm using a Synergy™ HT microplate reader (Biotek, VT, USA). The EC_{50} value of extract was calculated similar to mentioned above.

Animals

Male Wistar rats weighing 170-190 g each were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. The animal facilities and protocols were approved by the Laboratory Animal Care and Use Committee at Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. All rats were housed in a room maintained at 25±1 ℃ on a 12:12 h

dark–light cycle. For anti-hyperglycemia screening, 4 groups of normal rats were received the following solutions: distilled water (control), TTE at 500 and 1000 mg/kg BW, respectively, and metformin at 30 mg/kg BW for 30 min by oral gavage. Subsequently, the oral glucose tolerance test (OGTT) was performed and blood glucose level was determined using enzymatic colorimetric assays kit obtained from Erba Lachema (Brno, Czech Republic). For type 2 diabetic rat model, set of animals were separated from anti-hyperglycemia screening protocol. Briefly, normal-diet fed rats were consumed commercial available normal chow diet (C.P. Mice Feed Food no. 082, Bangkok, Thailand), containing 19.77 % of fat of total energy in the diet (%E) while the high-fat diet rats were received high-fat diet containing approximately 59.28% energy of fat, ad libitum. After 2 weeks of dietary manipulation, the rats were induced to be experimental type 2 diabetic models using the modified method as previously described (Srinivasan, Viswanad et al., 2005). Briefly, a single dose of 40 mg/kg BW of streptozotocin (STZ) (Sigma Aldrich, MO, USA) dissolved in 0.1 M citrate buffer was intraperitoneal injection (i.p). The normal-diet group was given vehicle citrate buffer, i.p, respectively. Fourteen-days later, the rats with fasting blood glucose level exceed 250 mg/dL were considered type 2 diabetes (DM) (Srimaroeng, Ontawong et al. 2015). Subsequently, the animals were randomly divided into 5 groups: normal diet or control, DM, and DM supplemented with TTE at the dose of 250, 500, and 1000 mg/kg BW. The rats were allowed to continue feeding for 12 weeks. Three days before sacrifice, the animals were fasted overnight and OGTT was conducted. At the end of the experiment, the animals were sacrificed, blood and tissue samples were collected for further experiments.

Biochemical assays

To determine biochemical parameters, the quantitative total plasma glucose and total cholesterol were determined using commercial enzymatic colorimetric assay kits obtained from Erba Lachema (Brno, Czech Republic).

Quantitative real-time PCR Analysis

Total RNA was purified from freshly isolated rat liver tissues using TRIzol reagent (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instruction. The first strand cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad, CA, USA) and qPCR was performed using SYBR real-time PCR master mix (Bioline, London, UK) on ABI 7500 (Life Technologies, NY, USA). Forward

and reverse primers were used as previous published (Ontawong, Saowakon et al., 2013) and purchased from Macrogen (Seoul, Korea). Gene expressions of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were normalized to GAPDH and mRNA level was reported as relative fold changes (RFC). qPCR amplification was performed in duplicate for each cDNA.

Statistical analysis

Data were expressed as means ± S.E.M. Statistical differences were assessed using one-way ANOVA followed by LSD post hoc test using statistical package for the social sciences (SPSS) version 11.5 (SPSS Inc., IL, USA). *P* value < 0.05 was considered to be significant.

Results and Discussion

The effect of TTE on $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging

The stability of free radical scavenging activities of TTE from 3 months storage was compared to fresh extraction. As shown in Table 1, the antioxidant property of TTE after storing for 3 months was show against $ABTS^{\bullet+}$ radical. The calculated EC_{50} of TTE from 3 months storage on $ABTS^{\bullet+}$ radical scavenging activity was 297.4 ± 3.32 µg/mL which was not different from fresh extraction (337.5 ± 2.73 µg/mL).

Likewise, the effect of TTE against $DPPH^{\bullet}$ radical scavenging TTE from 3 months storage was also compared to fresh extraction. As shown in Table 1, the antioxidant property of TTE after storing for 3 months was present against $DPPH^{\bullet}$ radical. The calculated EC_{50} of TTE stored for 3 months on $DPPH^{\bullet}$ radical scavenging activity were 18.98 ± 0.10 µg/mL which was not different when compared with fresh extraction (22.8 ± 0.9 µg/mL). This result demonstrated that TTE has high ability to capture $DPPH^{\bullet}$ free radicals in micromolar range which represents as a strong antioxidant. The result of TTE against $ABTS^{\bullet+}$ radical was also in agreement with which obtained by the $DPPH^{\bullet}$ radical method, suggesting strong antioxidant property of TTE lasting-long at least 3 months. Previous study demonstrated that phenolic and flavonoid compounds were responsible for acting as antioxidant activity of plants (Spiridon, Bodirlau et al., 2011). Moreover, *Passiflora alata* Curtis leave extract had showed a high antioxidant activity confirmed by scavenging $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals and acted as anti-inflammatory agent which was able to improve diabetic condition in mice (Colomeu, Figueiredo et al. 2014). In addition, anti-radical and anti-

diabetic effects of *Syzygium mundagam* bark methanol and water extract were also demonstrated by improving hyperglycemia and insulin resistance in diabetic rats (Chandran, Parimelazhagan et al., 2017). Thus, TTE has high antioxidant against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ free radicals which may act as anti-diabetic effect.

Table 1. The effect of TTE on $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging

EC_{50} of TTE extracts (µg/mL)			
$ABTS^{\bullet+}$		$DPPH^{\bullet}$	
Fresh extraction	3 months storage	Fresh extraction	3 months storage
337.5 ± 2.73	297.4 ± 3.32	22.8 ± 0.9	18.98 ± 0.10

Acute anti-hyperglycemic effect of TTE in normal rats

To determine whether TTE has a potential to have anti-diabetic effect, oral glucose tolerance test (OGTT) in normal rats was carried out. As shown in Figure1A, plasma glucose level in control group was significantly increased at 30 and 60 min and tended to return to normal level at 120 min after 2 g/kg BW of glucose loading similarly to that of the animals from pre-treatment with 500, 1000 mg/kg BW of TTE. However, plasma glucose level of the rats pretreated with TTE at the dose of 1000 mg/kg BW was reduced at every time point after glucose loading as shown by the total are under the curve (TAUC) when compared with control (Figure 1B). This effect was similarly to that of metformin, an antihyperglycemic drug, indicating that TTE has acute anti-hyperglycemic effect which may apply in diabetic condition.

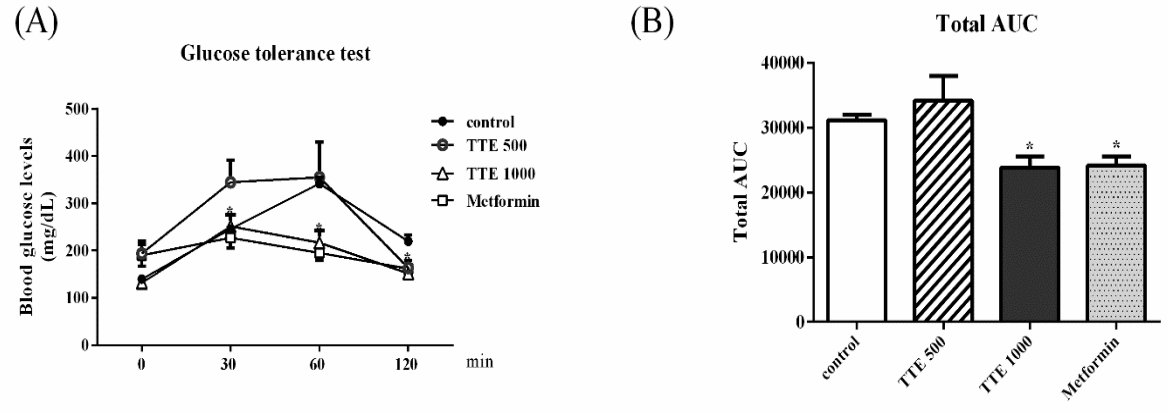

Figure 1 The acute anti-hyperglycemic effect of TTE in normal rats. Oral administration of 500 and 1000 mg/kg BW of TTE and 30 mg/kg BW of metformin was given to normal rats for 30 min. Subsequently, 2 g/kg BW of 50% glucose was administered into each rat. Blood samples were individually collected and blood glucose level was determined. (A). Blood glucose level in each time point (B) Total area under the curve calculated from (A). Data are expressed as means ± S.E.M (n=4-5), *, $p<0.05$ indicates significant differences from blood glucose level in control group.

The effect of TTE on body weight and plasma parameters in T2DM rats.

As shown in Figure 2A, DM rats have increased in body weight (BW) when compared to normal-diet rats or control group. However, BW of the rats from TTE supplementation at the highest dose (1000 mg/kg BW) was significantly reduced when compared with that DM while BW in TTE at 250 and 500 mg/kg BW supplemented rats did not different when compared with DM. Similarly, plasma glucose and total cholesterol were increased in DM group when compared with control. On the other hand, these levels were significantly reduced in DM rats supplemented with TTE at 1000 mg/kg BW when compared with DM group (Figure 2B and C). Consistently, DM rats exhibited glucose intolerance as indicated by markedly increasing of total area under the curve (TAUC) when compared with control. Again, the effective dose of TTE at 1,000 mg/kg BW, but not TTE at 250 and 500 mg/kg BW, significantly improved glucose intolerance (Figure 2D). These data suggest that TTE exerted anti-hyperglycemia, anti-hypercholesterolemia and anti-insulin resistance. Previous study reported that increased plasma triglyceride and low-density lipoprotein levels, and decreased high-density lipoprotein levels are common characteristics of dyslipidemia that can be observed in diabetic and steatosis patients (Moscatiello, Manini et al. 2007, Ui 2009). This was due to insulin resistance induced lipolysis which resulting from the accumulation of fatty acids and the β-oxidation disturbance in hepatic mitochondria leading to further infiltration of fats (Moscatiello, Manini et al. 2007). Thus, changing in hepatic lipid metabolism could be a major cause of hepatic oxidative stress which primarily contributes for hepatic complications (de Andrade, Moura et al. 2015).

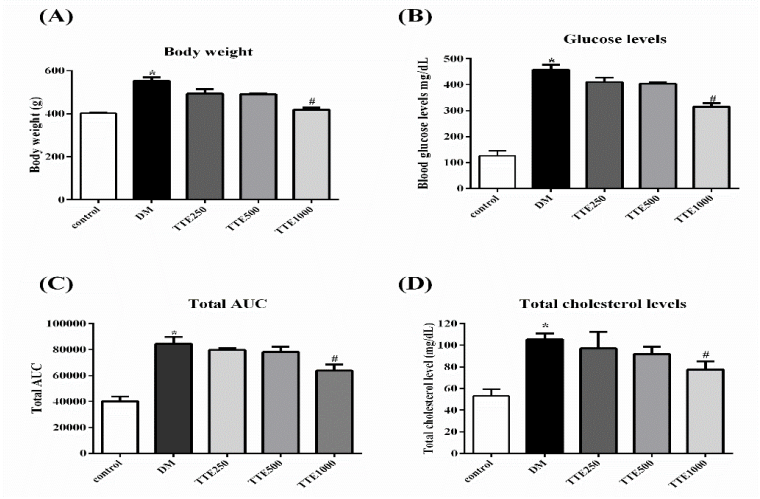

Figure 2.The effect of TTE on body weight and plasma parameter in T2DM rats.

(A) body weight, (B) glucose level, (C) total cholesterol level and (D) total area under the curve from oral glucose tolerance test in normal-diet rats or control, diabetes (DM) and DM treated with TTE at the dose of 250, 500, and 1,000 mg/kg BW, respectively. Data are expressed as means ± S.E.M., (n=3-5), *, $p<0.05$ compares with control group, #, $p<0.05$, compares with DM group.

Effect of TTE on genes related hepatic antioxidant enzymes in T2DM rats.

To determine whether TTE has protective effect as anti-oxidant in liver tissues, antioxidant genes: Cu-ZnSOD, GPx and CAT, mRNA expression were determined using qPCR. As shown in Figure 3, DM group had significant increase in mRNA expression of all three genes when compared with control group, indicating a compensatory response of antioxidant genes against oxidative stress under hyperglycemia and hypercholesterolemia. In contrast, the expression of Cu-ZnSOD, GPx and CAT genes were significant decreased in the rats supplemented with the highest dose of TTE when compared with DM group. Consistently, previous studies reported that quercetin, a constituent in TT, had potential as agent for both prevention and treatment of hepatic steatosis (Vidyashankar, Sandeep Varma et al. 2013). Quercetin could induce an increase in the activity of cellular antioxidants such as CAT, GPx and SOD and increase in reduced-glutathione level (Vidyashankar, Sandeep Varma et al. 2013). Likewise, rutin, an active compound found in TT, attenuated cellular oxidative stress induced by oleic acid through rising SOD, GPx and CAT protein levels (Wu, Lin et al. 2011). Thus, antioxidant property of TTE

that contains both quercetin and rutin (Phunchago, Wattanathorn et al., 2015) may help to relieve oxidative stress, prevent free radicals damaged to biomolecules such as proteins, DNA and lipids in the liver tissues resulting in protection of hepatic complications due to diabetes

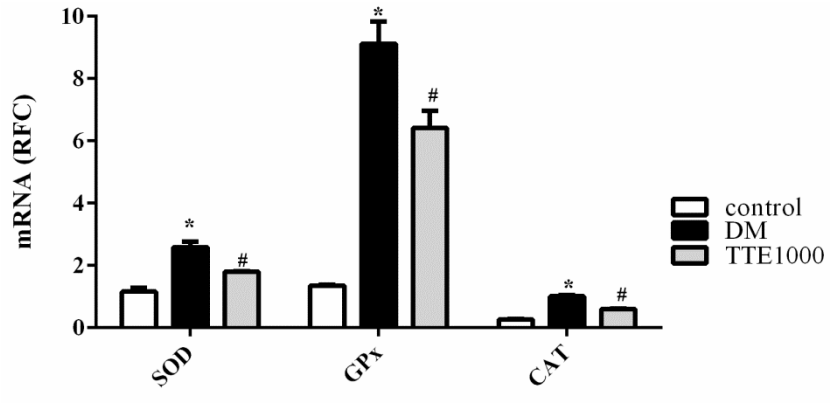

Figure 3. Antioxidant of TTE on genes related hepatic antioxidant enzymes in T2DM rats.

The expression of mRNA levels of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) in normal-diet or control, diabetes (DM) and DM treated with TTE at 1,000 mg/kg BW. Data are expressed as mean ± S.E.M (n=3-5), *, $p<0.05$ compares with control group, $^{\#}$, $p<0.05$, compares with DM group.

Conclusion

These findings suggest that TTE has high ability as free radical scavenger by capturing $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free radicals in micromolar range, indicating that TTE is a strong antioxidant. Moreover, TTE exerted anti-hyperglycemia,anti-hypercholesterolemia, and improved glucose intolerance. Such effects were contributed to normalize hepatic oxidative stress which could prevent hepatic steatosis and other complications. Thus, TTE could potentially be developed to nutraceutical product for prevention of risk of diseases induced by diabetic. Further studies are still required to elucidate the protective mechanisms involvement of TTE in diabetes.

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