Histopathological Changes of Renal Tubules in Metformin and *A. Crassna* Leaf Extract treated STZ-induced Diabetic Rats

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Abstract

This research is part of the project to study and assess the anti-diabetes effects of *A. Crassna* leaf extract treatment for STZ-induced diabetic rats, to evaluate tissue damage amelioration. To determine the side effects of Diabetes Mellitus (DM)-treated rats, metformin (a common anti-hyperglycemic medicine) and *A. Crassna* crude extract were tested on kidney tissue from the diabetic rats. We evaluated the changes in renal tubular histology in the kidneys of the diabetic rats that were given metformin and *A. Crassna* crude extract. Paraffin blocks with kidney samples embedded were prepared for each group: 1) control group, 2) diabetic group, 3) metformin-treated diabetes group, 4) *A. Crassna* leaf extract-treated diabetes group by 300 ml/kg (K300), and 5) 1000 ml/kg (K1000). The tissue sections were stained with routine hematoxylin and eosin and a special staining technique for glycogen accumulation identification, periodic acid-Schiff (PAS) reagent, to identify tubular pathology. In hyperglycemic conditions, as shown in the DM model in the metformin-treated group, and K300 and K1000 *A. Crassna*-treated investigations, subnuclear vacuolizations (Armanni-Ebstein lesions) were discovered to have increased by approximately 19.09% (p=0.01) in metformin-treated diabetic rats. However, tubular lesions were also observed in the DM, K300, and K1000 groups. The distal tubule is often targeted for tubular anomalies that do not damage the juxtaglomerular structure. The study demonstrates the renal tubular toxicity of metformin and *A. Crassna* leaf extract in acute hyperglycemic rats, possibly providing a renal side effect for diabetic treatment.

Keywords: *A. Crassna*, Diabetic nephropathy, Metformin, STZ-induced diabetic rats, PAS

Introduction

Diabetes mellitus (DM) is a metabolic-related illness that affects the glucose regulation pathway. Diabetic nephropathy (DN) is a complication of high blood glucose that affects several microvasculature vital organs (Kashihara, Haruna, Kondeti, & Kanwar, 2010). DN has been classified into different phases according to albumin presentation in urine and the severity of chronic kidney disease (Cao & Cooper, 2011). Hyperglycemia leads to an increase in advanced glycosylation end products (AGEs) and reactive oxygen species (ROS) production which play an important role in inducing and stimulating proinflammatory cytokine release in DN development (Forbes, Cooper, Oldfield, & Thomas, 2003). It has been identified by the increased urinary albumin with subsequence to glomerulosclerosis, a major DN pathology, which is characterized by the observation of mesangial expansion, glomerular basement membrane thickening, and extracellular matrix accumulation (Vinod, 2012; Soetikno et al., 2011; Zelmanovitz et al., 2009). The most notable acute

pathological lesion is an accumulation of glycogen granules in renal tubular epithelium with subnuclear vacuolization appearance known as the "*Armanni-Ebstein* lesion (AE)," which emerges from a glucose control problem in the circulation (Kang, Dai, Yu, Wen, & Yang, 2005) and it has been reported to be related to diabetic ketoacidosis (Ebstein, 1882). The proximal renal tubular cells are the major site of lesions accumulating glycogen and fat (Kock & Vestergaard, 1994; Nielsen, Thomsen, Kristensen, & Ottosen, 2003; Ritchie & Waugh, 1957).

Streptozotocin (STZ) is commonly used to develop diabetes conditions in animal models via the mechanism of nitric oxide-induced pancreatic β-cell apoptosis. Furthermore, STZ is harmful to numerous organ tissues as a result of ROS production (Szkudelski, 2001). Metformin is the most commonly used oral anti-diabetic drug for the first line of type II diabetes mellitus therapy (Inzucchi et al., 2012). The mechanism of action is considered to be adenosine monophosphate-activated protein kinase (AMPK) activation (Hasanvand et al., 2016; Kawashima & Kirito, 2016). Diarrhea, nausea, and vomiting are some of metformin's side effects (Bailey & Turner, 1996; Davidson & Peters, 1997). Furthermore, the rare incidence of the side effect called metformin-associated lactic acidosis (MALA) is still reported with mortality rates of 30-50% (Eppenga et al., 2014; Richy, Sabido-Espin, Guedes, Corvino, & Gottwald-Hostalek, 2014).

The Asian traditional medicinal herb *Aquilaria Crassna* Pierre ex Lecomte (Thymelaeaceae) or *A. Crassna,* has been widely used in China and Southeast Asia, including Thailand, as a prescribed treatment for cardiac diseases, inflammatory illnesses, and infectious disorders, according to several studies (Dahham et al., 2015), and has been used as an anti-hyperglycemic agent (Pranakhon, Pannangpetch, & Aromdee, 2011). The major compounds found in the crude extract consist of iriflophenone 3,5-C-β-diglucoside, iriflophenone 3-C-β-glucoside mangiferin, iriflophenone 2-O-α-rhamnoside, genkwanin 5-O-β-glucoside, and genkwanin 4′-methyl ether 5-O-β-primeveroside (Hara et al., 2008; Ito et al., 2012). Recently, mangiferin, a potent antioxidant, and anti-hyperglycemic compound was identified in *A. Crassna* leaves (Ray, Leelamanit, Sithisarn, & Jiratchariyakul, 2014). Several reports showed the use of mangiferin in diabetes treatment (Dineshkumar, Mitra, & Manjunatha, 2010; Ganogpichayagrai, Palanuvej, & Ruangrungsi, 2017; Ichiki et al., 1998). However, the microscopic effects of standard anti-diabetic medication and *A. Crassna* crude extract on renal tubular epithelium have yet to be determined. The kidney tissues of STZ-induced diabetic rats were thus examined histopathologically, comparing them with healthy rats, DM rats, DM-treated with metformin rats, and DM-treated with *A. Crassna* crude extract (300 and 1,000 mg/kg body weight) rats.

Methods and Materials

A. Crassna leaf extract

An aqueous crude extract was prepared from young leaves of *A. Crassna* sourced in Phitsanulok province. The extract was kindly provided by Assoc. Prof. Kornkanok Ingkaninan of the Faculty of Pharmaceutical Sciences, Naresuan University, Thailand. The biological activities such as antioxidant, antiinflammation, vasodilation effect, and plant fingerprint have been studied recently (Wisutthathum et al., 2019; Wongwad et al., 2019).

Kidney samples

Kidney-embedded paraffin tissues were obtained from Assist. Prof. Rachanee Chanasong, Department of Anatomy, Faculty of Medical Science, Naresuan University. All protocols were conducted following the guidelines of and were approved by, the Center for Animal Research Naresuan University, Phitsanulok, Thailand (NUAE 591029). Briefly, male Wistar rats (200-250 g) were divided into 5 groups (n=3 in each group) including the normal group (CRT), diabetic rats (DM), diabetic rats treated with metformin (MET), 300 mg/kg of *A. Crassna* leaf extract (K300), and 1000 mg/kg of *A. Crassna* leaf extract (K1000), respectively. Diabetic rats were induced to hyperglycemia by streptozotocin (STZ) 60 mg/kg single dose injection until a blood glucose level $\geq$ 250 mg/dl was achieved. The DM rats were treated by gavage feeding with 500 mg/kg metformin, 300 mg/kg, and 1000 mg/kg *A. Crassna* crude extract. After 6 weeks, the rats' kidneys (n=15 in total samples) were harvested and fixed in 10% neutral formalin, embedded in paraffin, and sectioned with 3 μM thickness. To evaluate the morphology of kidney tissue sectioning, the ribbon was processed by deparaffinization and tissue staining.

Histological study

Basic morphological alterations were randomly identified using hematoxylin and eosin (H&E) staining, and glycogen granule accumulation in the renal tubular epithelium was investigated using periodic acid-Schiff (PAS) staining. Briefly, the paraffin-embedded kidney tissue blocks were washed and rehydrated with xylene, absolute alcohol, and tap water. Hematoxylin and eosin dyes were used to stain the nuclear and cytoplasm for morphological observation. For glycogen accumulation identification, the periodic acid-Schiff reagent was applied to tissue slides. Under light microscopy, the renal cortex and medullary structures including renal corpuscle, renal tubule, and juxtaglomerular apparatus were evaluated for pathological changes. Randomized renal sections from each group were captured, and the point of interest (POI) for the overall renal area and the area of the subnuclear vacuolization were measured using the ImageJ program. The percentage of the renal tubular lesion in the rat cortex was calculated using the formula below, which was based on systemic randomization of tissue sectioning:

$$\%\ Tubular\ lesion = [\frac{Tubular\ lesion\ area}{Total\ tubular\ area}] \times 100$$

Statistical analysis

Three different experiments were used to calculate the mean, standard error of the mean, and SEM. GraphPad Prism software was used to evaluate differences between treatment groups using a one-way ANOVA followed by Dunnett multiple comparisons (GraphPad Prism, San Diego, California, USA) where $p<0.05$ was considered statistically significant.

Results

After 6 weeks of intragastric feeding, kidneys were harvested and fixed in neutral buffer formalin before sectioned tissue ribbons were stained with H&E and PAS to characterize the morphology of renal cortical structures.

Histology of renal cortex in STZ-induced diabetic rats

To assess the overall structural alterations in the renal cortex, H&E staining was used to examine the histological structures in the renal cortex, including the renal corpuscles and renal tubules. The renal cortex consisted of renal corpuscles (glomerulus and its apparatus), renal tubules, and vascular structures. The control group showed no change under low-power microscopy, while the diabetes, metformin, 300 mg/ml, and 1,000 mg/ml *A. Crassna*-treated groups revealed tubular lesions (Figure 1). To assess the glomerular and juxta-glomerular structures, high magnification images of the tissue samples were captured, and the renal corpuscles evaluated.

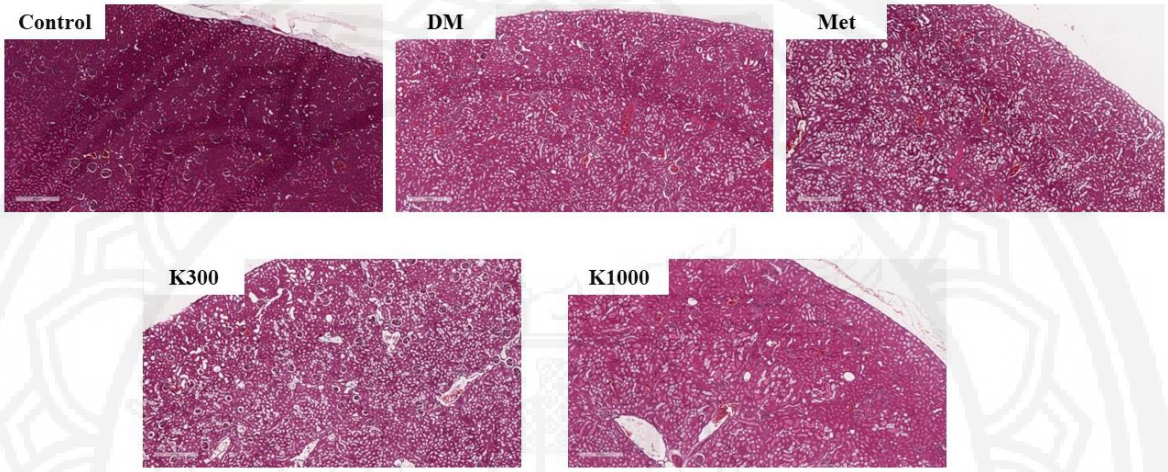

Figure 1 Rat renal cortex stained with hematoxylin & eosin showing the renal corpuscles and renal tubules with branches of cortical vessels obtained from the control, diabetic (DM), metformin-treated (Met) diabetic rats, *A. Crassna* 300 mg/kg rat body weight treated diabetic rats (K300), and *A. Crassna* 1000 mg/kg rats body weight treated diabetic rats (K1000) (*hematoxylin & eosin ×100*)

Histopathological characteristic of renal corpuscle in hyperglycemia

Renal corpuscle, renal tubule, and juxtaglomerular components were clearly revealed under high magnification. Cytoplasmic eosinophilic staining with brushed lumen defined normal proximal tubules, whereas pale acidophilic staining without a brush border identified the distal tubules. In acute hyperglycemic conditions, the structures of the renal corpuscle, including glomerulus and bowman's structures, were not changed between the control and DM groups when examined under a light microscope. The sodium sensing structure, macular densa, seems to be normal in all experimental groups (Figure 2). However, high glucose level reveals renal tubular presenting with subcellular vacuolized accumulation that is exhibited in diabetic, Metformin, 300 mg/kg rat body weight, and 1,000 mg/kg rat body weight treated conditions. To investigate subnuclear vacuolization in the renal tubule, PAS staining was used to identify glycogen droplets inside renal tubular epithelial cells.

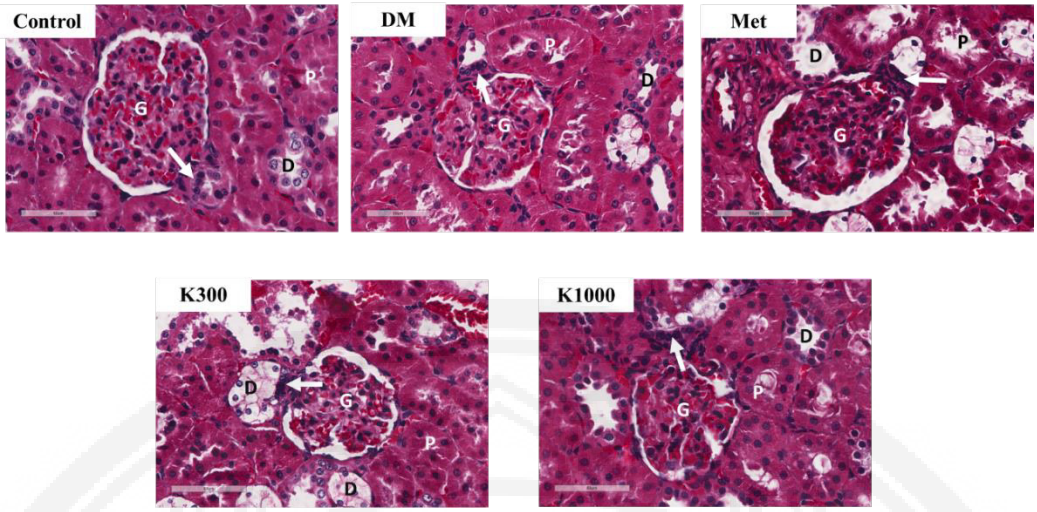

Figure 2 Renal cortex showing glomerulus (G) and renal tubules, proximal (P) and distal (D) tubules with macular densa (arrow) condensed by nucleic hematoxylin staining. Tubular lesions exhibited by subnuclear vacuolization in tubules of diabetic (DM), metformin-treated (Met) diabetic rat, *A. Crassna* 300 mg/kg rat body weight treated diabetic rat (K300) and *A. Crassna* 1000 mg/kg rat body weight treated diabetic rat (K1000) (*hematoxylin & eosin ×400*)

The subnuclear vacuolization of renal tubule in STZ-induced diabetic rat

To identify components of the renal tubular lesions, PAS staining was performed with a standard protocol (mentioned earlier). The impaired renal tubules in the hyperglycemic model groups clearly showed subnuclear vacuolization following STZ injection. The lesion had a condensed hematoxylin-stained luminal nucleus with a substantial vacuole beneath it (subnuclear vacuolization), which contained glycogen granules and fat droplets when stained using the PAS technique (Figure 3). However, further research is needed to determine the components found inside the vacuole.

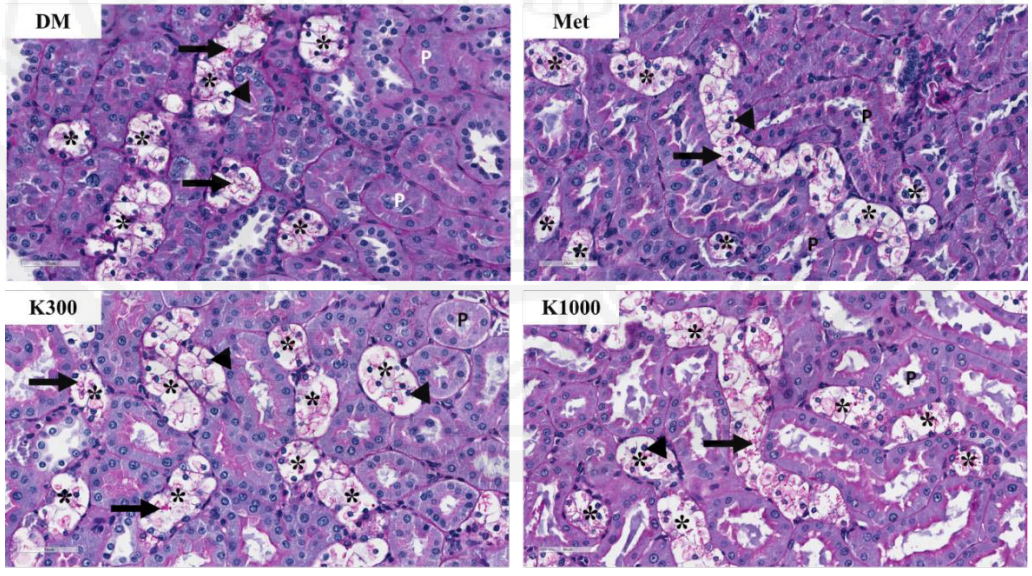

Figure 3 A lesion called *Armani-Ebstein* (AE) presents in STZ-induced diabetic rat of diabetic (DM), metformin-treated (Met) diabetic rat, *A. Crassna* 300 mg/kg rat body weight treated diabetic rat (K300), and *A. Crassna* 1000 mg/kg rat body weight treated diabetic rat (K1000) showing subnuclear vacuolization (arrow) located in the distal tubule (asterisk) with condensed hematoxylin-stained nuclei (arrowhead), while normal proximal tubules (P) were observed (*PAS ×400*)

The percent of tubular lesion in metformin-treated Wistar rats

The hyperglycemic rats that were given 500 mg/kg body weight of metformin had the highest percentage of tubular lesions (19.09% of the mean) with a significant increase ($p<0.01$). The percentage of DM rats treated with *A. Crassna* leaf extract that had tubular lesions was 2.34%, with 5.17% of the rats treated with K300 rats and 7.42% of the rats treated with K1000 also having tubular lesions whereas the control group showed no tubular lesions (Figure 4). These findings imply that metformin at 500 mg/kg of body weight causes renal tubular damage in acute hyperglycemic rats.

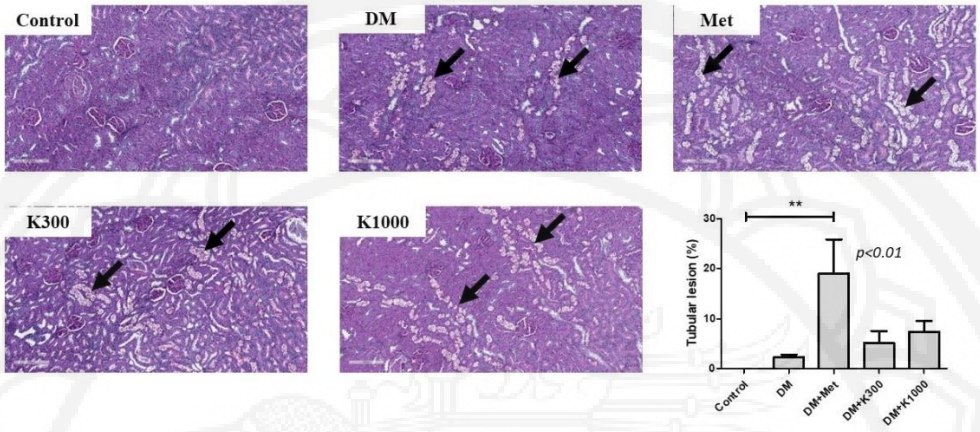

Figure 4 The renal cortex of the control rat group, diabetic (DM), metformin-treated (Met) diabetic rat, *A. Crassna* 300 mg/kg rat body weight treated diabetic rat (K300), and *A. Crassna* 1000 mg/kg rat body weight treated diabetic rat (K1000) showing subnuclear vacuolization within renal tubules (Arrow) (*PAS ×100*) and the graph of the percentage of tubular lesions comparing with healthy group (***p-value<0.01*)

The target of subnuclear vacuolization in renal tubule

At a high magnification field, PAS staining revealed *Armanni-Ebstein* lesions in the distal convoluted tubule, but the macula densa appeared as tall columnar cells with normal morphology at the glomerulus' vascular pole (Figure 5). Furthermore, there were no structural differences between groups in the proximal tubule, collecting tubule, or renal corpuscle.

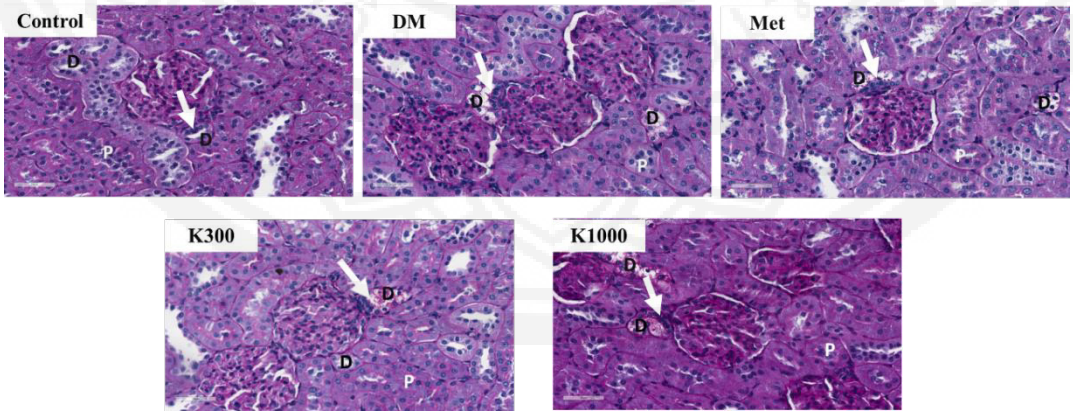

Figure 5 PAS staining shows distal convoluted tubules (D) were affected by subnuclear vacuolization. However, no alteration of macula densa between the group of treatment (arrow) including the control group, diabetic (DM), metformin-treated (Met) diabetic rat, *A. Crassna* 300 mg/kg rat body weight treated diabetic rat (K300), and *A. Crassna* 1000 mg/kg rat body weight treated diabetic rat (K1000). Proximal tubules (P) showed normal histology. (*hematoxylin & eosin ×400*)

Discussion

Since the *Armanni-Ebstein* (AE) lesion was described in 1882 (Ebstein, 1882), it has been linked to hyperglycemia. The lesion affects several parts of the renal structures, including the proximal convoluted tubule and the ascending limbs of the loop of Henle (Ishizaki et al., 1987; Zhou, Yool, Nolan, & Byard, 2013). In this study, we found that under acute hyperglycemic conditions the *AE* lesion presented on the distal renal tubule but not on macular cells. Metformin, which is used to treat Type 2 diabetes and other metabolic diseases (Hardie, 2008; Saravi, Hasanvand, Shahkarami, & Dehpour, 2016), has been linked to a serious side effect called metformin-associated lactic acidosis (MALA) (Schwetz et al., 2017). Rats treated with metformin showed the highest percentage of tubular lesions, which may be the accumulation of glycogen granules or fat droplets, while *A. Crassna* crude extract exerted fewer side effects. In an *in vivo* study of ketone production by metformin treatment, rats fed a high-fat diet produced ketone accumulation in the blood, resulting in MALA (Tessari & Tiengo, 2008).

These findings suggest that metformin therapy in high-dose streptozotocin-induced diabetic rats can cause renal tubular lesions (AE), whereas *A. Crassna* crude extract may produce fewer renal lesions. This is the first report of the histopathological changes of *A. Crassna* crude extract treated diabetic rats. However, fasting blood sugar (FBS) was examined by another project group that showed metformin reduced FBS after 6 weeks of treatment while *A. Crassna* crude extract did not alter the blood glucose level compared to the baseline (unpublished results). A longer treatment period must be done to confirm the anti-hyperglycemic condition of *A. Crassna* crude extract.

In a recent study showing metformin relieved diabetic nephropathy in low-dose STZ-produced diabetic rats, the issue of metformin's effect on renal tubules remains unresolved (Zhang, Xu, Yu, Wu, & Sui, 2017). This suggests that renal status is a critical factor in MALA development and that more research and clarification in DM treatment are required. Furthermore, recent research has discovered that mangiferin, which is the principal active ingredient in *A. Crassna* crude extract, is effective as an anti-hyperglycemic agent and slows the progression of diabetic nephropathy in diabetic rats induced by STZ (Pal, Sinha, & Sil, 2014). While it has been reported that iriflophenone 3-C-ß-glucoside derived from agarwood reduces blood glucose levels by activating glucose absorption (Pranakhon, Aromdee, & Pannangpetch, 2015), our study demonstrated decreased renal damage, which is advantageous for anti-diabetic therapy. Other organ toxicity tests, particularly on smooth muscle cells, showed that *A. Crassna* leaf extract had no effect (Wisutthathum et al., 2019).

Conclusion and Suggestions

Diabetic nephropathy is the most common long-term hyperglycemic consequence. Metformin is still used to treat diabetes. However, the toxicity to essential organs, notably the kidneys, must be considered as a cause of distal tubular change. In addition to herbal medicinal therapy, *A. Crassna* crude extract can be administered as an anti-hyperglycemic treatment with reduced renal toxicity.

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Author Contributions

Sasiprapa Khunchai assisted in conducting the experiments, performed the statistical analysis and data visualization and wrote the manuscript. Rattanaporn Jarernsook and Thanapoom Monthathong performed the tissue staining, microscopic picture providing, statistical analysis, and data visualization. Krongkarn Chootip, Kornkanok Ingkaninan, Eakkaluk Wongwad, and Rachanee Chanasong provided crude extract and tissue in paraffin block preparation, Kitsaphon Kanamnouy was the pathological consultant. Sangkab Sudsaward designed and conducted all the experiments and wrote the manuscript. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declare that they hold no competing interests.

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