Original Article

Evaluation the Effects of *Cissus modeccoides* Hot Aqueous Extract on Alloxan-induced Diabetic Rats

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Abstract

Introduction: In this study, the hypoglycemic effects and the safety of *Cissus modeccoides* (CM) were assessed on alloxan-induced diabetic rats. **Methods:** Various concentrations of CM hot aqueous extract were orally administered to alloxan-induced diabetic rats for 30 days. Blood glucose level, hematological and biochemical parameters, and gene expression level were evaluated. **Results:** After CM treatment, diabetic rats presented nonreduced blood glucose level and unimproved body weight. Increased blood urea nitrogen was observed in CM-treated groups as well. Although hematological parameters and cholesterol level revealed nonsignificant effects from CM, decreased expression levels of the insulin receptor in the pancreas and insulin receptor substrate 2 and glucose transporter 2 in the liver were demonstrated in CM-treated groups. Nephrin in the kidney of CM groups was highly expressed. **Conclusion:** The results of this study revealed adverse effects and toxicity of CM extracts in diabetic rats.

Keywords: *Alloxan, blood glucose, Cissus modeccoides, gene expression, toxicity*

Introduction

Diabetes mellitus is a common metabolic disorder found in 382 million people around the world.[1] Hyperglycemia is the main characteristic of this disease. This condition is a result of an absolute deficiency of insulin secretion (type 1 diabetes) or abnormal pancreatic β-cell function affecting insulin secretion, including insulin resistance (type 2 diabetes). This disorder also causes various complications in other systems, causing many diseases such as coronary heart disease, hypertension, nephropathy, retinopathy, and peripheral neuropathy.[2,3] Elevated blood glucose level also induces the production of free radicals, causing oxidative stress that injures the liver and leads to carbohydrate metabolism disorder.

Although insulin administration is available as a basic remedy for diabetes, insulin injection before meals every day is not desirable. Oral drug administration, another diabetic treatment, has been reported to have some adverse side effects after long-term use. Therefore, maintaining blood glucose level during long-term treatment without (or with fewer) undesirable effects, the ultimate goal for diabetes therapy, should be investigated further.

Medicinal plants are alternative treatments which are widely used in many countries. In Thailand, various traditional medicinal plants capable of curing diabetes are available, such as ivy gourd (*Coccinia grandis* (L.) Voigt), garlic (*Allium sativum* L.), holy basil (*Ocimum tenuiflorum*), and bitter cucumber (*Momordica charantia* L.).[4-7] Some plants in *Cissus* genus, woody vine, such as veld grape (*Cissus quadrangularis*), princess vine (*Cissus sicyoides*), *Cissus multistriata*, *Cissus cornifolia,* and *Cissus rotundifolia* have been reported having antidiabetics effects also.[8-12] *Cissus modeccoides* (CM) Planch. is a woody vine that is distributed throughout the parts of Thailand, Cambodia, Vietnam, and China.[13] This plant is utilized to treat bone and joint pain, headache, swelling, and boils and also as laxative agent.[14] In Lamphun Province, Northern Thailand, this plant is used in folk medicine as an antidiabetic agent. However, little is known about the effects of this plant on the animal and human body with no scientific evidence supports the medicinal use. Therefore, the objective of this study was to find out the effects of CM aqueous extract on blood glucose level, in parallel with the toxicity of this plant to the pancreas, liver, and kidney in alloxan-induced diabetic rats.

How to cite this article: Pradit W, Khumpook T, Saenphet K, Saenphet S, Chomdej S. Evaluation the effects of *Cissus modeccoides* hot aqueous extract on alloxan-induced diabetic rats. J Rep Pharma Sci 2019;8:85-90.

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Access this article online

Website: www.jrpsjournal.com

DOI: 10.4103/jrptps.jrptps_19_18

Quick Response Code:

Materials and Methods

Water extract preparation

CM samples (leaves and stems) were collected from Lamphun, Thailand, identified and confirmed by the taxonomist, Dr. Angkana Inta. The voucher specimen was deposited at Queen Sirikit Botanical Garden (WP6390). The water extract of CM was prepared by weighing dried plants (120, 180, and 240 mg) and boiling in water (1 L) for 1 h to produce various concentrations of the extracts. After boiling, the missing volume of the extract was then added by hot water. The extracts were filtered and cooled to room temperature. Fresh extracts were prepared daily.

Animals and diabetes induction

Male Wistar rats (4 weeks old, 200–250 g) were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. All rats were kept in a room under controlled temperature (25°C ± 2°C) and light (from 6 am to 6 pm) with *ad libitum* access to water and food. The experiment was ethically conducted and approved (Re 002/09) by the Institutional Animal Care and Use Committee, Department of Biology, Faculty of Science, Chiang Mai University, Thailand. Diabetic rats were induced by intraperitoneal injection of alloxan (Sigma, USA) at 200 mg/kg body weight.[15] Twenty-four hours after alloxan injection, rats with blood glucose level ranging from 250 to 300 mg/dL were utilized in this study as diabetic rats.

Experimental design

Alloxan-induced diabetic rats were randomly divided into five groups with five rats in each group. The rats were orally treated with 1 mL of CM aqueous extract once daily for 30 days. In parallel, water was given to both the normal and diabetes control groups. The experimental groups were as follows:

- Group I: Normal control; given water
- Group II: Diabetes control; given water
- Group III: Diabetic rats; given CM extract at 120 µg/mL/day
- Group IV: Diabetic rats; given CM extract at 180 µg/mL/day
- Group V: Diabetic rats; given CM extract at 240 µg/mL/day.

At the end of the study, overnight-fasted rats were sacrificed. Whole blood samples were collected by cardiac puncture method. Liver, pancreas, and kidney tissue samples were collected and stored at −80°C for gene expression analysis.

Blood parameters

Blood glucose level was measured from serum using *o*-toluidine reagent.[16] Whole blood samples were evaluated for complete blood count including hematocrit, hemoglobin (Hb) concentration, red blood cell (RBC) count, and white blood cell (WBC) count. The blood chemical profile for blood urea nitrogen (BUN) and cholesterol was examined at the Division of Clinical Chemistry, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University.

Real-time reverse transcription polymerase chain reaction

Six genes related to diabetes were chosen in this study to determine the expression levels in three different tissues (pancreas, liver, and kidney). For the pancreas, insulin 2 (*Ins2*) and insulin receptor (*InsR*) genes were selected. Insulin receptor substrate 2 (*Irs2*) and glucose transporter 2 (*Glut2*) expression were measured in liver tissue. Detection of nephrin (*Nphs1*) and transforming growth factor-beta 1 (*TGF-β1*) expression levels in the kidney was performed. Total RNA was isolated using an innuPREP DNA/RNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol. cDNA was synthesized by RevertAid Reverse Transcriptase (Fermentas, USA) using 1 µg of total RNA of each sample. Reverse transcription was carried out using a two-step protocol by annealing 10 nmol of oligo dT_{16} with total RNA at 65°C for 5 min. Then, the reaction was added to a mixture containing 1X buffer, 1 mM dNTPs, 40 units RiboLock RNase Inhibitor (Fermentas, USA), and 200 units RevertAid Reverse Transcriptase to make a final volume of 20 µL. The mixture was incubated at 37°C for 5 min and at 42°C for 90 min. The cDNAs were then subjected to a mixture of 1X ThermOne Real-Time Premix with SYBR Green (RBC Bioscience, Taiwan), 0.2 µM of each forward and reverse primer [Table 1], and 20 ng of cDNA sample with a final volume of 25 µL. Amplification of the target genes was conducted using a MyCycler™ Thermal Cycler (Bio-Rad, USA) with the following thermal conditions: predenaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 30 s, annealing at different temperatures [Table 1] for 1 min, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. Fluorescent signals were measured after each extension step. After polymerase chain reaction (PCR) amplification, melting temperatures were generated to verify the quality of PCR products. The threshold cycle (C_T) of each sample was calculated and further used for relative expression by the $\Delta\Delta C_T$ method.[17] Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was selected as an endogenous control gene.

Statistical analysis

One-way analysis of variance was used to evaluate the differences among the treatments, followed by Tukey's multiple comparison posttest. Values were presented as mean ± standard error. Statistical significance was considered as $P \leq 0.05$.

Results

Blood glucose and body weight

At the end of the experiment, blood glucose of all experimental rats was investigated. As shown in

Table 1: DNA sequences of real-time polymerase chain reaction primers

Gene	Sequences	Annealing temperature (°C)	PCR product (bp)	Accession number
GAPDH	F: TCTCCTGCGACTTCAACA	54	178	NM_008084.2
	R: TGGTCCAGGGTTTCTTACT			
InsR	F: AGTCGAGCCCTAGCTCCCGC	70	403	NM_010568.2
	R: TGGGACCCCGGCGATCAGAG			
Ins2	F: AAGCCTATCTTCCAGGTTATT	55	212	NM_008387.4
	R: TGGGTCCTCCACTTCACG			
Glut2	F: TGCTGGACGAAGTGTATC	63	208	NM_031197.2
	R: TAGGCCAAGTAGGATGTG			
Irs2	F: GGGGCGAACTCTATGGGTA	56	139	NM_001081212.1
	R: GCAGGCGTGGTTAGGGAAT			
Nphs1	F: CCTGAAGGACCCTACTGAG	58	277	NM_019459.2
	R: AAATCGGACAACAAGACG			
TGF-β1	F: TCCCTCAACCTCAAATTATTCA	59	493	NM_011577.1
	R: GCGGTCCACCATTAGCAC			

GAPHD: Glyceraldehyde-3-phosphate dehydrogenase, *InsR*: Insulin receptor, *Ins2*: Insulin 2, *Glut2*: Glucose transporter 2, *Irs2*: Insulin receptor substrate 2, *Nphs1*: Nephrin, *TGF-β1*: Transforming growth factor-beta 1, PCR: Polymerase chain reaction, bp: Base pair

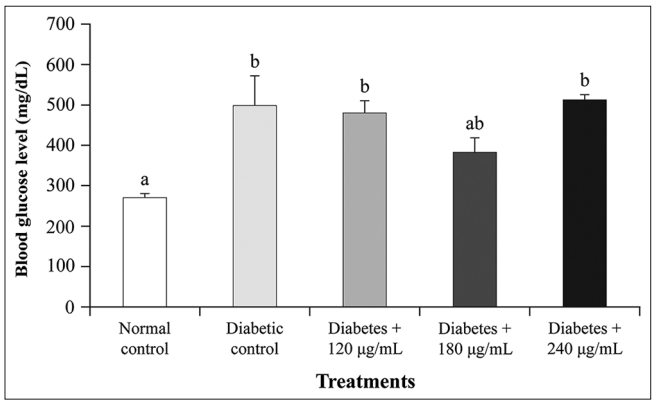

Figure 1: Blood glucose level in normal and diabetic rats induced by alloxan, with or without *Cissus modeccoides* extracts. The bar graph represents mean ± standard error (*n* = 5). The letters (a, b) indicate significant differences at *P* ≤ 0.05

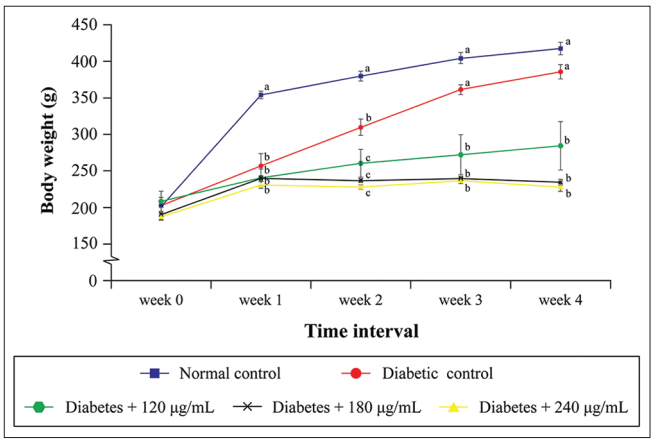

Figure 2: Body weight of normal and diabetic rats induced by alloxan, with or without *Cissus modeccoides* extracts, before and after the experiment. The graph represents mean ± standard error. The letters (a, b, and c) indicate significant differences at *P* ≤ 0.05

Figure 1, CM extracts at different concentrations (120, 180, and 240 µg/mL) did not decrease the blood glucose level of diabetic rats compared with the diabetic control group. However, 180 µg/mL CM extract tended to reduce the blood glucose level when compared with other CM extract concentrations.

Body weights of the rats were also checked once a week. The results exhibited a significant decrease in body weight gain from the 1st week of the study in CM-treated groups when compared with the normal control group. The lower body weight in CM-treated rats was prolonged until the end of the experiment [Figure 2].

Hematological profiles

To examine the toxicity of CM extracts at three different concentrations (120, 180, and 240 µg/mL) in diabetic rats, hematological profiles were analyzed at the end of the study. It was found that the RBC counts of alloxan-induced diabetic rats were significantly lower than the normal control. In addition, the Hb concentrations of diabetic rats receiving 240 µg/mL CM extract were significantly higher than the normal control, while hematocrit was similar, as shown in Table 2. Total WBC count of every diabetic rat group was not different from the normal control. However, differences in the numbers of lymphocytes, neutrophils, eosinophils, and basophils were observed. Interestingly, alloxan-induced rats tended to have decreased numbers of lymphocytes, eosinophils, and basophils but increased neutrophils.

Cholesterol and blood urea nitrogen

As shown in Table 2, although cholesterol levels of every group were not significantly different, it was shown that CM extract probably increased the cholesterol level in a dose-dependent manner. To verify kidney function, BUN

Table 2: Effects of *Cissus modeccoides* extracts on the hematological and biochemical profiles in diabetic rats

Parameters	Groups				
	Control	Diabetes	Diabetes		
			+120 µg/mL	+180 µg/mL	+240 µg/mL
RBC count (×10^6 cells/mL) [5-10×10^6/µL]	10.4±0.5[a]	7.6±0.1[b]	8.3±0.2[b]	8.5±0.2[b]	7.8±0.3[b]
Hematocrit (%) [35%-57%]	45.8±2.0	47.6±1.6	48.4±1.6	48.8±1.2	50.2±0.9
Hb concentration (g/dL) [11-19 g/dL]	21.5±0.5[a]	21.1±0.7[a]	21.1±0.3[a]	22.5±0.3[ab]	24.0±0.3[b]
WBC count (×10^3 cells/µL) [3-17×10^3/µL]	6.3±0.6[ab]	6.2±1.3[ab]	7.4±0.2[b]	5.8±0.5[ab]	4.1±0.4[a]
Lymphocytes (%) [65%-83%]	84.5±1.6[a]	82.0±2.1[ab]	75.4±4.8[ab]	70.9±2.9[b]	74.7±4.1[ab]
Neutrophils (%) [13%-26%]	4.3±0.5[a]	13.3±2.5[ab]	17.5±4.2[b]	19.3±2.2[b]	13.2±3.1[ab]
Monocytes (%) [0%-4%]	6.0±1.2	5.0±0.6	6.4±1.0	5.6±1.0	7.8±1.1
Eosinophils (%) [0%-4%]	2.9±0.7[a]	0.8±0.3[ab]	0.5±0.2[b]	2.2±0.7[ab]	2.6±0.6[ab]
Basophils (%) [0%-1%]	2.3±0.6[a]	0.4±02[b]	0.2±0.2[b]	1.3±0.5[ab]	1.4±0.5[ab]
Cholesterol (mg/dL) [-]	66.7±6.7	70.0±4.0	71.7±9.2	93.0±7.4	99.6±12.8
BUN (mg/dL) [11-23 mg/dL]	24.3±0.9[a]	29.3±3.7[ab]	33.0±7.5[ab]	47.6±3.5[b]	45.3±4.3[b]

Data are presented as mean±SE (n=5). Superscript letters (a, b) indicate significant differences at $P \leq 0.05$. Reference blood values in square brackets were obtained from Sharp and Villano[24]. SE: Standard error, RBC: Red blood cell, WBC: White blood cell, BUN: Blood urea nitrogen, Hb: Hemoglobin

was examined. The results showed that BUN was elevated after treatment with CM extracts at 180 and 240 µg/mL. A dose-dependent increase of BUN was also observed.

Expression level of genes involved with diabetes

Three different tissues (pancreas, liver, and kidney) were kept for evaluation of the expression level of genes related to diabetes. In pancreas, the messenger RNA (mRNA) levels of *Ins2* increased in a dose-dependent manner and significantly differed from the normal control when treated with CM extract at >180 µg/mL. However, a nonsignificant difference in *Ins2* levels between the normal and diabetic control groups was demonstrated in this study. In addition, all concentrations of CM extract did not elevate the expression level of *InsR* in the pancreas [Figure 3a]. In liver tissue, the diabetic controls demonstrated a significant increase of *Irs2* and *Glut2*. After treatment with CM extract, the mRNA levels of *Irs2* and *Glut2* declined significantly, as shown in Figure 3b. Moreover, elevation of *Nphs1* in the kidney was found in a dose-dependent manner in all diabetic rats with and without CM extract treatment. However, a significant difference of *Nphs1* compared with the control group was found only in the 240 µg/mL treated group. As shown in Figure 3c and d, depletion of *TGF-β1* mRNA level in the kidney was observed in diabetic control rats, including diabetic rats treated with 120 and 240 µg/mL CM extracts.

Discussion

The present study is the first report concerning the pharmacological effects of CM hot aqueous extract, which was employed to imitate the extraction method of CM in folk medicine. After treatment with CM extract at various concentrations in alloxan diabetic rats, a nonsignificant reduction of blood glucose compared with diabetic rats was revealed. These results were in accordance with unimproved body weight of diabetic rats treated with CM extract at 120–240 µg/mL. Although a nonsignificant difference of cholesterol levels in plasma of the CM-treated diabetic rats was shown, cholesterol levels tended to increase in a CM dose-dependent manner. These may be a result of the low expression of *InsR* in pancreatic cells as well as *Irs2* and *Glut2* in hepatic cells. Although elevated levels of *Ins2* mRNA were found after CM extract treatment at various concentrations, low levels of *InsR* existed. This may affect insulin secretion because of inadequate and/or abnormal insulin signaling in β-cells, resulting in high blood glucose as found in this study. A decline in expression levels of *Irs2* and *Glut2* in the liver of diabetic rats after treatment with CM extract may cause high blood glucose level by the depletion of β-cell mass regulator (*Irs2*) and glucose transporter in hepatic cells (*Glut2*). Although this circumstance was similar to the decrease of *Irs2* and *Glut2* by hyperinsulinemia,[18,19] hyperglycemia and low body weight were detected in this study.

Various complications, for example, cardiovascular diseases, retinopathy, nephropathy, and peripheral neuropathy, typically occur in diabetic patients.[3,20] Thus, a blood test was performed in this study to determine the effects of CM extract on some pathophysiological features associated with diabetes such as anemia and low immune system.[21-23] The nonsignificant differences in RBC count, hematocrit and Hb concentration between the CM-treated groups and the diabetic group as well as the normal ranges of RBC count and hematocrit[24] were revealed. This result suggested that CM extract may not lead to anemia, a condition of RBC and/or Hb depletion found in diabetic patients.[25] Moreover, no effects of CM extract on immunity of alloxan diabetic rats was suggested by the nonsignificant differences of WBC count between the CM-treated groups and the diabetic control group.

As for the removal of metabolic wastes in blood, an examination of renal aberrations to determine the effects

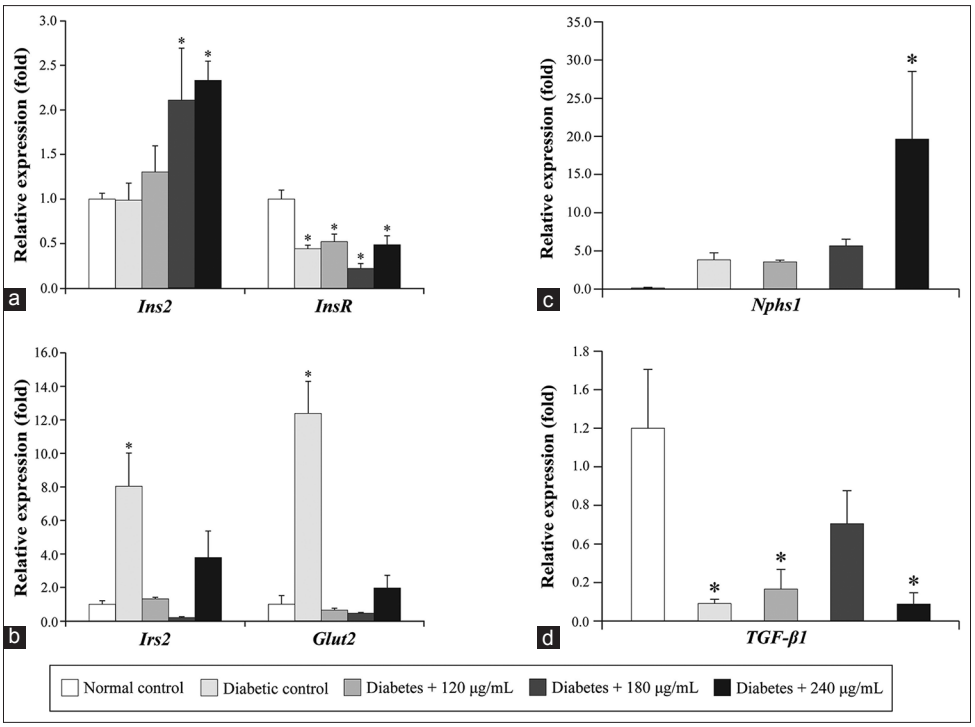

Figure 3: Expression levels of *Ins2* and *InsR* genes in the pancreas (a), *Irs2* and *Glut2* genes in liver (b) and *Nphs1* (c) and *TGF-β1* (d) genes in the kidney tissues of normal and diabetic rats induced by alloxan, with or without *Cissus modeccoides* extracts. The bar graph represents mean ± standard error, with *P* ≤ 0.05 (*) considered a statistically significant difference compared with the control group. *Ins2*: Insulin 2, *InsR*: Insulin receptor, *Irs2*: Insulin receptor substrate 2, *Glut2*: Glucose transporter 2, *Nphs1*: Nephrin, *TGF-β1*: Transforming growth factor beta 1

of CM extract was carried out. An elevation of BUN was exhibited. This phenomenon correlated with the increase of *Nphs1* mRNA levels in a dose-dependent manner. *Nphs1* is a gene-encoding nephrin, a critical transmembrane protein implicating the filtration of the kidney, resulting in protection against albuminuria.[26] The upregulation of *Nphs1* may restore the slit diaphragm structure of podocytes.[27,28] This implied that CM extracts may show some toxic effects on kidney characteristics. The nonsignificant difference of *Nphs1* between diabetic and normal control groups also supported this conclusion. In diabetic nephropathy, TGF-β plays a critical role in matrix production leading to glomerular matrix accumulation and loss of filtration in the kidney. TGF-β1 is a well-known cytokine responsible for fibronectin deposition in glomerular and tubulointerstitial compartments.[29] Upregulated *TGF-β1* mRNA have been found in diabetic nephropathy patients.[30] This was in contrast to the depletion of *TGF-β1* expression levels demonstrated in both diabetic control and CM-treated groups in this study. In 2014, Pourghasem *et al*.[31] observed early renal histological changes leading to diabetic nephropathy after 8 weeks of an experiment in alloxan diabetes using male Wistar rats. This could imply that inadequate duration of the experiment (4 weeks) may be a cause of the low expression levels of *TGF-β1* in alloxan diabetic rats. The results suggested that the CM extract did not affect the accumulation of the extracellular matrix. Likewise, similar levels of *Nphs1* in diabetic and control groups may have occurred due to inadequate duration, since the results were in contrast to the low expression level of *Nphs1* in diabetic nephropathy patients in previous reports.[32,33] Nevertheless, only preliminary results were obtained from this study; further studies of other parameters such as liver and kidney functions, histological changes of these organs, and mechanism of CM extract at the protein level should be evaluated to better understand the effects of CM extract on diabetic pathology.

Conclusions

The results showed that CM hot aqueous extract did not have a hypoglycemic effect. Moreover, it had adverse effects on some biochemical parameters which suggest the toxicity of CM extract in alloxan-induced diabetic rats.

Acknowledgments

We thank Ms. Piengchanok Faikear, Assistant Professor Salika Aritajat, and Associate Professor Dr. Korakot Nganvongpanit and Mr. Chakkrapong Kuensaen for their kind support.

Financial support and sponsorship

This study was financially supported by the National Research Council of Thailand.

Conflicts of interest

There are no conflicts of interest.

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