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Research article

The standardized *Jamu pahitan*, an Indonesian antidiabetic formulation, stimulating the glucose uptake and insulin secretion in the *in-vitro* models

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ABSTRACT

Jamu pahitan is a polyherbal formulation commonly used for the traditional management of diabetes in Indonesia and is mainly prepared from *Andrographis paniculata* (Burm.f.) Nees. It is widely varied in herbal composition for every region has their own plant component addition to the formulation. A version of the formulation used in the greater Surakarta area contained five plant constituents. This study evaluated the *in-vitro* glucose uptake and insulin secretion stimulatory activities of *Jamu pahitan* to provide scientific evidence on its efficacy and safety of use. The water and ethanol extracts of three *Jamu pahitan* formulations were prepared. The total phenolic content (TPC) of the extracts was evaluated by the standard Folin-Ciocalteau method. Their effects on the viability of L6 skeletal muscle and RIN-m5F pancreatic cells were evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The glucose utilized by L6 myotubes treated with *Jamu pahitan* was assessed indirectly by the glucose oxidase method. The insulin secreted by RIN-m5F treated with the formulation extracts was analyzed by the enzyme-linked immunosorbent assay (ELISA). The correlation between TPC and the profile of safety and efficacy of the formulation was statistically evaluated. The water extracts of *Jamu pahitan* were safe and exerted significant glucose uptake and insulin secretion stimulatory activity in L6 and RIN-m5F, respectively. The ethanol extracts showed more potent effects than their water counterpart, albeit they exerted cytotoxic effects on the cells at the higher tested concentrations. The formulations at lower concentrations stimulated the proliferation of RIN-m5F. In addition, the TPC was strongly correlated with the glucose uptake and insulin secretion stimulatory activities and also the IC_{50} of the cells in positive manner. The present study supported the use of *Jamu pahitan* for the traditional management of diabetes in Indonesia by stimulating glucose uptake in the muscle cells and improving insulin secretion in β-cells.

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1. Introduction

Diabetes is a complex metabolic disorder affecting 6.2% of adults and is one of Indonesia's top three death-causing diseases. More than 90% of diabetes worldwide are type-2 diabetes mellitus (T2DM), commonly treated with oral hypoglycemic agents. These drugs mainly ameliorate the insulin secretion by pancreatic β-cells and improve insulin resistance in the peripheral tissues [1]. The prolonged uses of oral hypoglycemic drugs might cause undesirable adverse effects, i.e., fluid retention, heart failure, hypoglycemia, and osteoporosis. The use of herbal medicines might help benefit with lower side effects [2]. At some points, the use of herbal medicines concurrently with oral hypoglycemic medications or individually was reported among Indonesian people with diabetes [3].

A polyherbal formulation contains a more complex constitution profile that is likely to interact with multiple receptor targets to dynamically generate better overall pharmacological effects [4]. This polyvalence paradigm might generate a favorable outcome in diabetes treatment, as the hyperglycemia in T2DM resulted from eight pathophysiological processes collectively called the ominous octet. The decreased glucose uptake, insulin secretion, and incretin effect, along with increased lipolysis, glucose reabsorption, hepatic glucose production, and glucagon secretion, as well as neurotransmitter dysfunction, lead to hyperglycemia in T2DM [5]. Further, a polyherbal formulation may benefit the patients with a lower dose needed to achieve desirable blood glucose levels and convenience in ingestion, leading to better adherence and eventually a better therapeutic effect [6].

Jamu pahitan is a polyherbal formulation popularly used for traditional prevention and treatment diabetes in Indonesia, particularly in Java [7–9]. It is mainly prepared from *Andrographis paniculata* (Burm.f.) Nees. and locally added with various bitter plants and Zingiberaceae rhizomes [10]. Hence, there are many variations of the formulation, i.e., species and ratio of each plant constituents, nationwide. Some variations of *Jamu pahitan* are included in the science-based *jamu* development program, and hence, their safety and antidiabetic effects in human are available [11,12]. However, such data for other variations are still limited, include one used in greater Surakarta area. The Surakarta-originated *Jamu pahitan* consisted *Andrographis paniculata* aerial parts, *Tinospora crispa* (L.) Hook. f. & Thomson stems, *Orthosiphon aristatus* (Blume) Miq. aerial parts, *Carica papaya* L. leaves, and *Curcuma aeruginosa* Roxb. rhizomes [13]. *Jamu pahitan* is traditionally consumed as a decoction. This preparation has been used in the greater Surakarta area for a long time and is empirically safe. However, the cytotoxicity study is carried out to ensure the formulation's safety on the targeted tissues. Since the taste of *Jamu pahitan* decoction is inappreciably bitter, this study evaluated the *in-vitro* glucose uptake and insulin secretion stimulatory activities of both water and ethanol extracts of formulations to develop it into a more convenient preparation with a better efficacy and safety profile. The *in-vitro* approaches used in this study gave insights on the mechanism of actions of the formulation in decreasing blood glucose levels.

2. Results

2.1. TPC

Both Formula I (FI) and Formula II (FII) exerted the statistically comparable TPC in water and ethanol extracts, which were lower than Formula III (FIII). Ethanol extracts (Fig. 1B) showed superior TPC than their water counterparts (Fig. 1A).

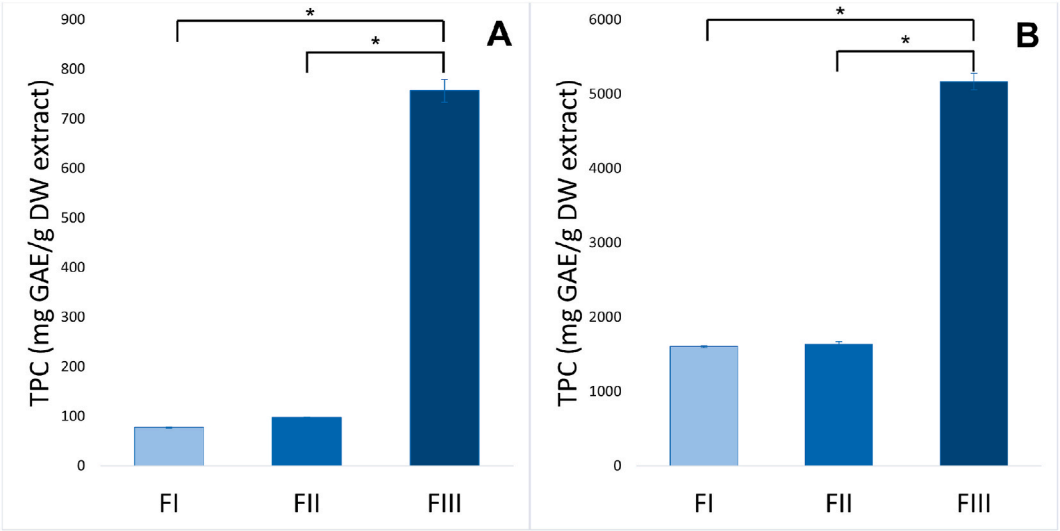

Fig. 1. TPC of the water (A) and ethanol (B) extracts of the formulation evaluated by the standard Folin-Ciocalteau method. The water extract of FIII showed higher TPC than those of FI and FII, which were comparable. The similar pattern was observed in the ethanol extracts of the formulations. Asterisk between bars represented a significant difference (one-way ANOVA, $p < 0.001$, $N = 3$/group).

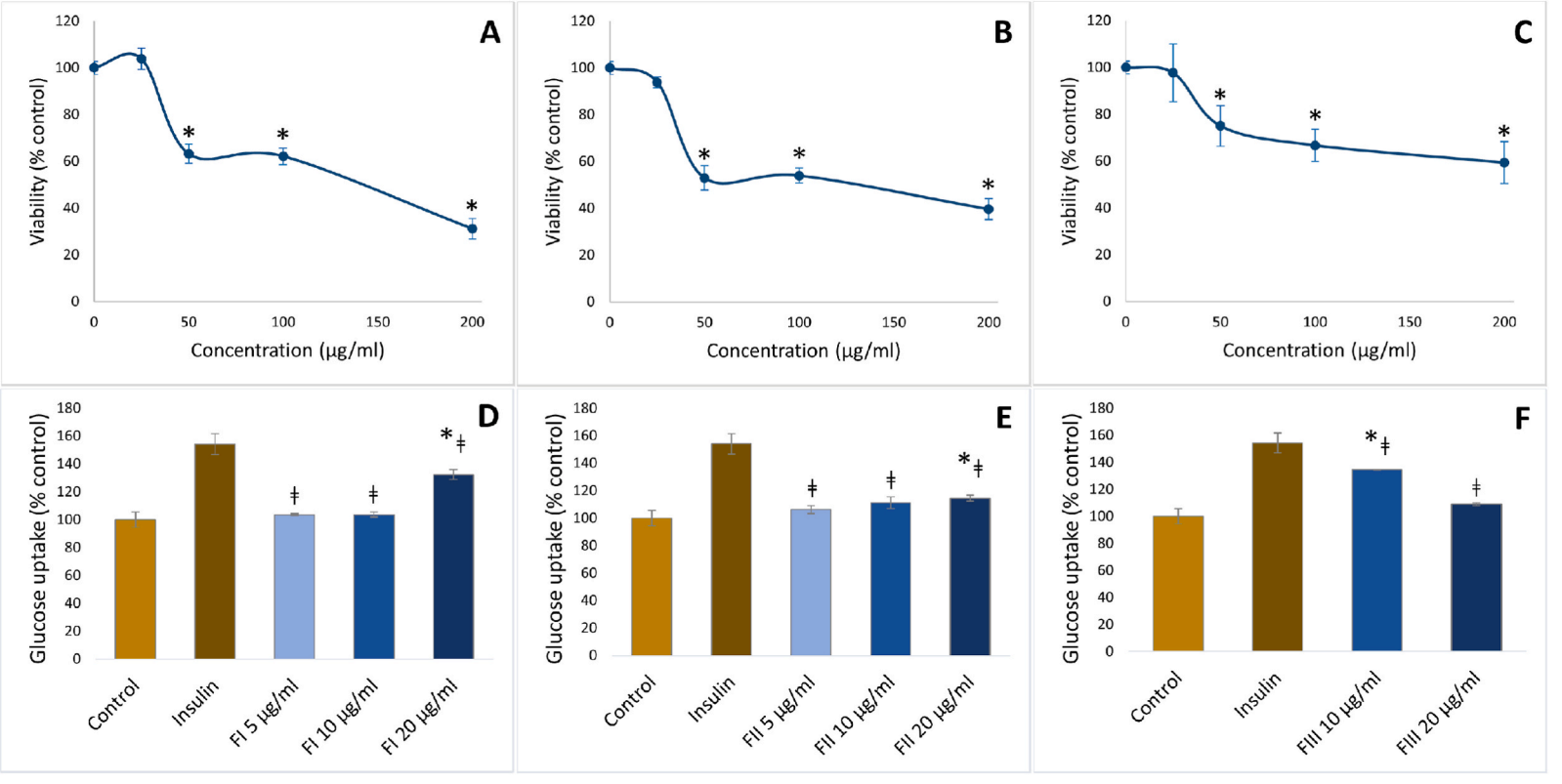

Fig. 2. The profile of cell viability of L6 treated with various concentrations of FI (A), FII (B), and FIII (C) water extracts evaluated by MTT test; and their respective glucose uptake stimulatory activity profile (D–F) on the skeletal muscle cell evaluated by the indirect glucose oxidase method. Extracts started to show toxic effects on the cells at 50 μg/ml, with FIII's extract showed the least effects on the cell viability. Asterisk on the line chart represented a significant difference in viability compared to control (one-way ANOVA, $p < 0.001$, $N = 3$/group), asterisk and ‡ on the bar chart represented a significant difference in glucose uptake compared to control and insulin group (one-way ANOVA, $p < 0.001$, $N = 3$/group), respectively.

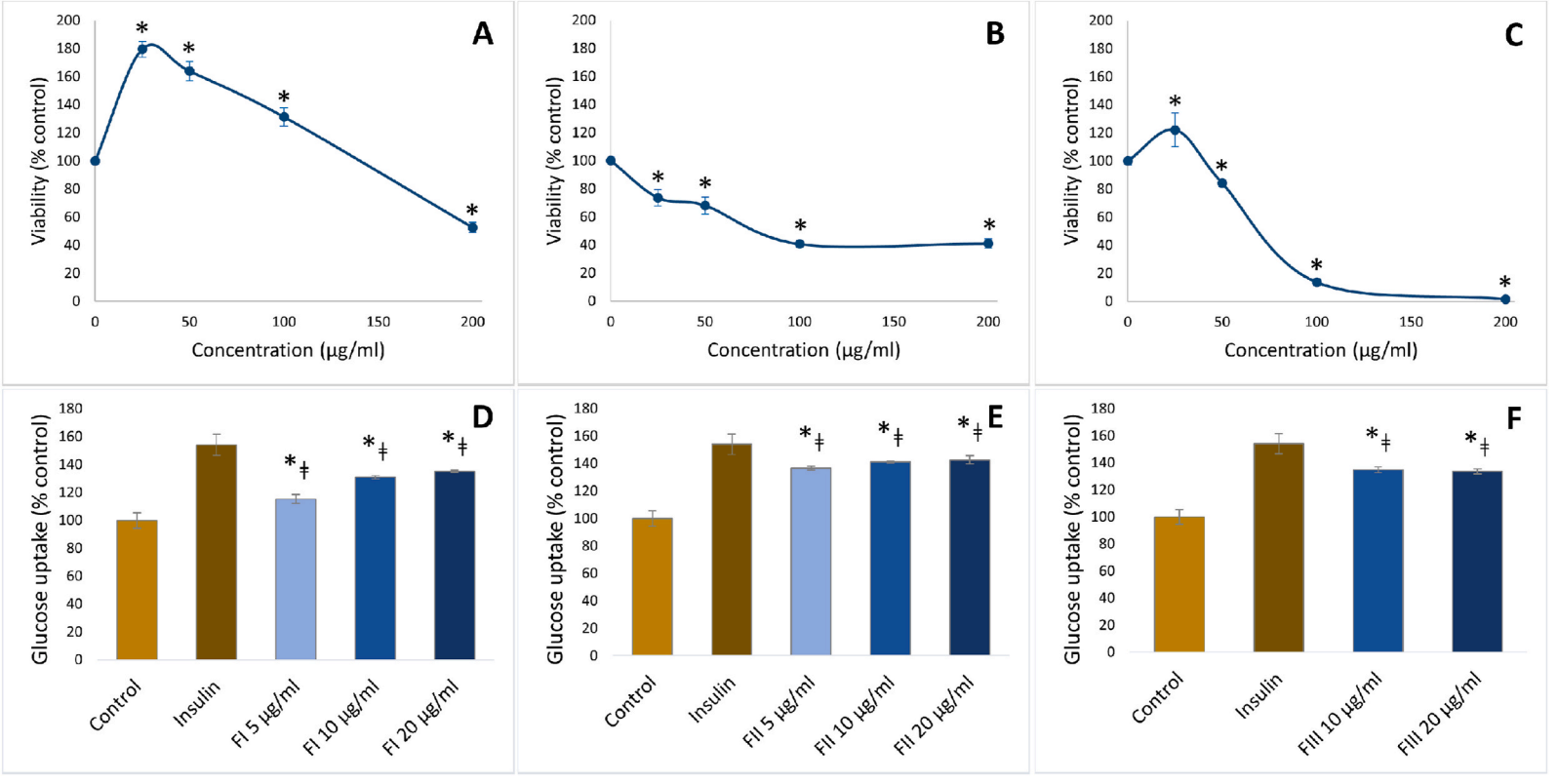

Fig. 3. The profile of cell viability of L6 treated with various concentrations of FI (A), FII (B), and FIII (C) ethanol extracts evaluated by MTT test; and their respective glucose uptake stimulatory activity profile (D–F) on the skeletal muscle cell evaluated by the indirect glucose oxidase method. Extracts started to show toxic effects on the cells at 25 µg/ml. Asterisk on the line chart represented a significant difference in viability compared to control (one-way ANOVA, $p < 0.001$, $N = 3$/group), asterisk and ‡ on the bar chart represented a significant difference in glucose uptake compared to control and insulin group (one-way ANOVA, $p < 0.001$, $N = 3$/group), respectively.

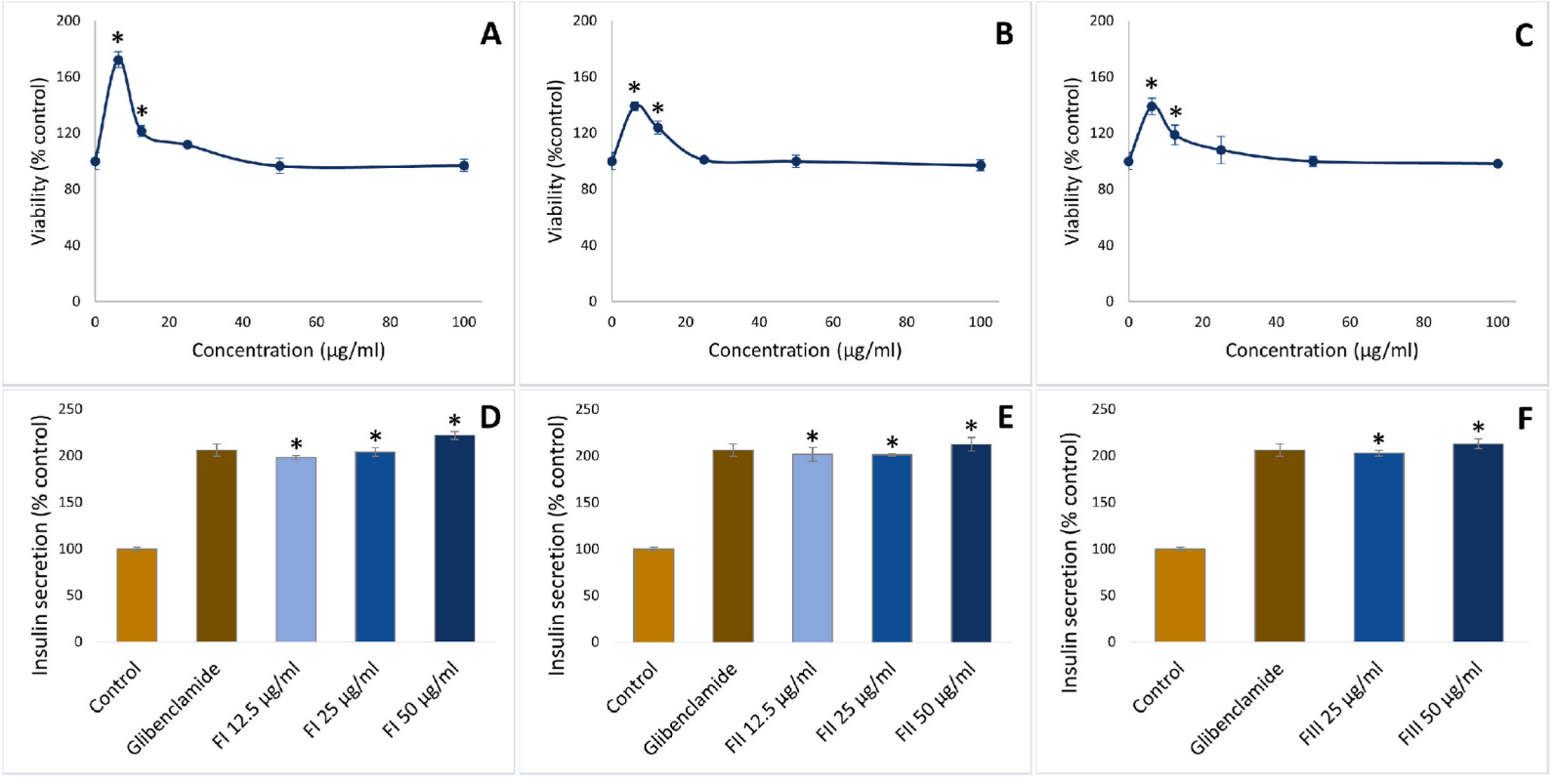

Fig. 4. The profile of cell viability of RIN-m5F treated with various concentrations of FI (A), FII (B), and FIII (C) water extracts evaluated by MTT test; and their respective insulin secretion stimulatory activity profile (D–F) on the pancreatic cells evaluated by the enzyme-linked immunosorbent assay (ELISA). Extracts exerted proliferation stimulation at the lower concentrations and were nontoxic to the cell at the higher ones. Asterisk on the line chart represented a significant difference in viability compared to control (one-way ANOVA, $p < 0.001$, $N = 3$/group), while it on the bar chart represented a significant difference in insulin secretion compared to control group (one-way ANOVA, $p < 0.001$, $N = 3$/group), respectively.

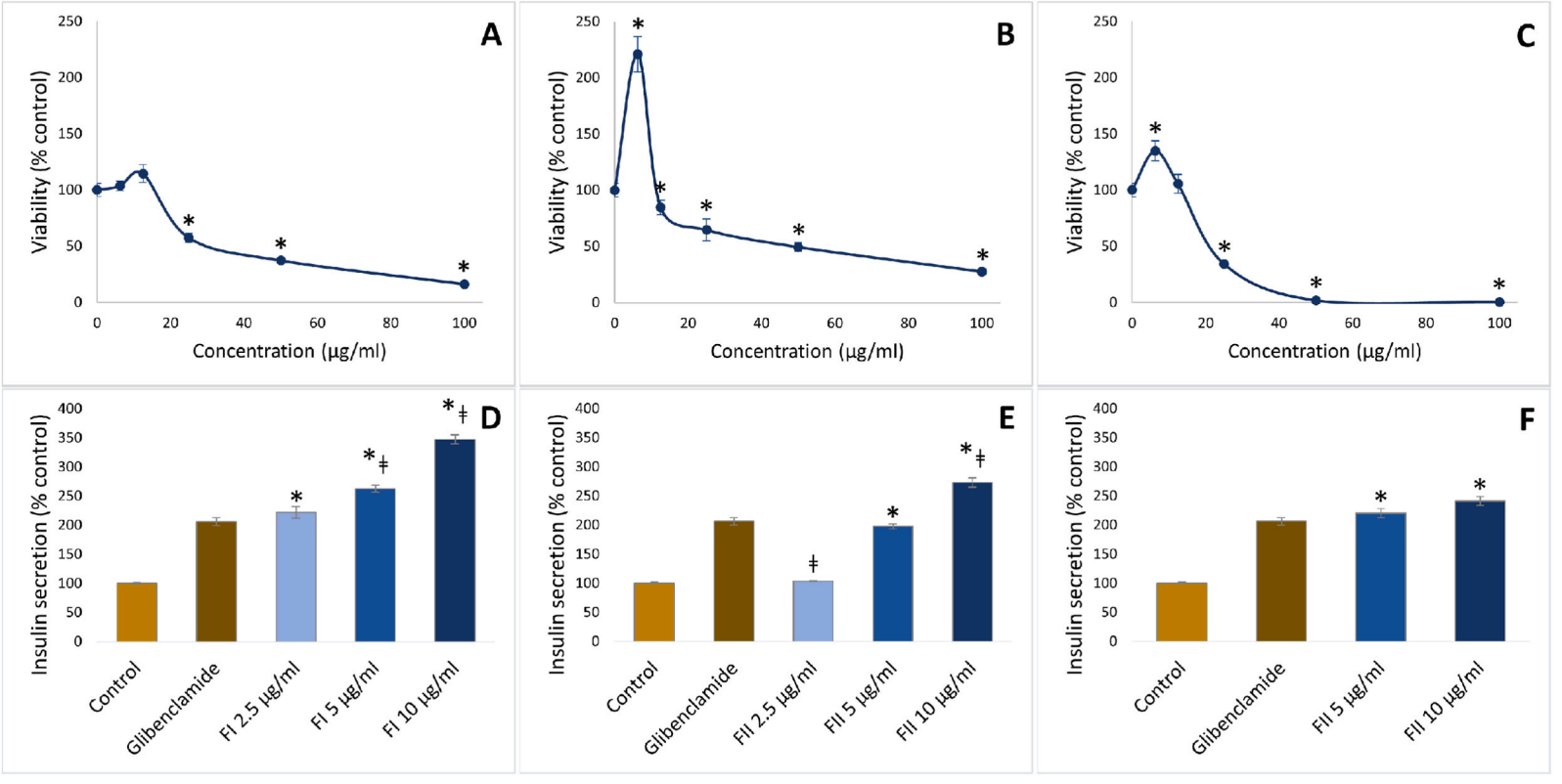

Fig. 5. The profile of cell viability of RIN-m5F treated with various concentrations of FI (A), FII (B), and FIII (C) ethanol extracts evaluated by MTT test; and their respective insulin secretion stimulatory activity profile (D–F) on the skeletal muscle cell evaluated by ELISA. Extracts of FII and FIII exerted proliferation stimulation at the lower concentrations, while all extracts reduced cell viability at 25 μg/ml and higher concentrations. Asterisk on the line chart represented a significant difference in viability compared to control (one-way ANOVA, $p < 0.001$, N = 3/group), asterisk and ‡ on the bar chart represented a significant difference in insulin secretion compared to control and glibenclamide group (one-way ANOVA, $p < 0.001$, N = 3/group), respectively.

2.2. Glucose uptake stimulatory activity of the formulations

2.2.1. The effects on L6 viability

The safe concentration of the water extracts on L6 cells was 25 μg/ml, with the IC_{50} value of FI, FII, and FIII was 137.43 ± 6.92, 136.99 ± 9.93, and 223.95 ± 16.32 μg/ml, respectively (Fig. 2A–C). All water extracts showed a strong positive correlation between their TPC and IC_{50} on L6 cells, with an R-value of 0.904–0.976.

The safe concentration for each formulation's ethanol extract was varied. The ethanol extracts of the lower tested concentrations of FI and FIII showed a proliferation stimulatory activity, while all the tested concentrations of FII exerted a toxic effect on the cells (Fig. 3A–C). The respective IC_{50} value of ethanol extract of FI, FII, and FIII was 205.94 ± 2.97, 123.19 ± 11.51, and 101.70 ± 3.11 μg/ml. Like in the water extracts, all ethanol extracts showed a strong positive correlation between their TPC and IC_{50} on L6 cells, with a significant correlation was observed in the ethanol extract of the FIII.

2.2.2. Glucose uptake stimulatory activity

The water extracts of FI and FII at a safe concentration of 20 μg/ml stimulated glucose uptake in L6 with the efficacy of 85.87 and 74.32% of insulin, respectively (Fig. 2D–E). A similar effect was shown by FIII water extract, with efficacy of 87.32% (Fig. 2F). On the other hand, all the tested concentrations of ethanol extracts of the formulations showed the glucose uptake stimulatory activity at 76.56–92.59% of the positive control (Fig. 3D–F). The best stimulatory activity was exerted by ethanol extract of FII, albeit lower than insulin response (Fig. 3E). The correlation between TPC with the glucose uptake stimulatory activity at the highest tested concentration of the water and ethanol extracts was strong in a positive manner, with a significant correlation was observed in the ethanol extracts of FI and FII.

2.3. The insulin secretion stimulatory activity of the formulations

2.3.1. The effects on RIN-m5F viability

The water extracts of all formulations in all tested concentrations did not show the toxic effects on RIN-m5F. Furthermore, all three formulations showed a cell proliferation stimulatory activity at the lowest tested concentration (Fig. 4A–C). The IC_{50} value of water extracts of FI, FII, and FIII was 161.83 ± 4.53, 211.23 ± 7.40, and 225.17 ± 11.00 μg/ml, respectively. The ethanol extracts of FI and FIII started to show toxic effects at 25 μg/ml, while FII was at 12.5 μg/ml (Fig. 5A–C). Compared to those of water extracts, a much lower IC_{50} value was shown by the ethanol counterpart, i.e., 54.57 ± 1.97, 66.97 ± 2.00, and 42.72 ± 1.95 μg/ml. FIII ethanol extract showed a significant correlation between TPC to the IC_{50} value on RIN-m5F. In addition, all other extracts also showed a strong positive correlation.

2.3.2. Insulin secretion stimulatory activity

All the extracts showed the insulin secretion stimulatory activity on RIN-m5F cells, with equal or even better efficacy than glibenclamide's as the positive control, except the lowest concentration of FII ethanol extract. The stimulatory activity of water extracts of three formulations was similar and comparable to glibenclamide (Fig. 4D–F). On the other hand, their ethanol extracts at the highest concentration showed 117.86–169.70% efficacy of the positive control (Fig. 5D–F). The best stimulatory activity was exerted by ethanol extract of FI (Fig. 5D). Similarly, the strong positive correlation was observed between TPC and the insulin secretion stimulatory activity at the highest tested concentration of the formulation's water extract. The same trend was observed in the ethanol extracts of the formulation.

3. Discussion

FI and FII contain plants with rich phenolic compounds, i.e., *Orthosiphon aristatus* and *Carica papaya* [14,15]. The ratio of plant materials did not affect the TPC of *Jamu pahitan* as both FI and FII extracts contained a comparable TPC. Our data suggested that the type of extract highly affected TPC, as ethanol extracts contain remarkably high phenolic compounds. Other than the suitable polarity of the ethanol to extract the phenolic compounds, the excessive heat used to extract compounds and evaporate water might play a role in the decomposition of the extracted compounds in the water extract [16,17].

Skeletal muscle plays a crucial role in postprandial glucose control and is one of the main target sites for insulin action [18]. Both FI and F II water extracts showed an equal IC_{50} that underlined their safety on the muscle cells. FII ethanol extract showed the most toxic profile to L6 cells than any extracts of the *Jamu pahitan*. It might be related to the higher proportion of *Carica papaya* in FII, which contained highly toxic carpaine (IC_{50} = 21.5 μM) [19]. Herbal preparation's use, particularly as a polyherbal formulation, is expected to exert polyvalence effects that result in better overall efficacy or less toxicity [4]. Albeit not apple to apple comparable, the relatively higher IC_{50} of *Jamu pahitan* extracts over carpaine might indicate the polyvalence of *Jamu pahitan*.

The strong positive correlation between TPC and IC_{50} of the extracts to L6 indicated a higher level of phenolic compounds associated with less toxic effects on the cells. The possible negative regulation of toxicity by phenolic compounds was previously reported in *Chroogomphus rutilus* extracts on various cell lines [20]. The phenolic compounds are likely not the sole principles responsible for the toxicity of the formulation on the tested cells. Ethanol extracts showed higher TPC, but also contained higher level of other human cell toxic compounds, such as carpaine and andrographolide, than in the water extracts [19,21]. Both compounds have a better solubility in ethanol.

Both glucose uptake and insulin secretion stimulatory activities of *Jamu pahitan* extracts were evaluated in their respective safe

concentrations. Albeit a similar viability profile, FI water extracts, particularly at the highest tested concentration, showed a better glucose uptake stimulatory activity than FII. This better activity might be attributed to the presence of more *Andrographis paniculata* and *Tinospora crispa* in FI. Previous studies reported that the deoxyandrographolide of *Andrographis paniculata* and the water extract of *Tinospora crispa* stimulated glucose uptake in L6. Both plants enhanced the glucose uptake in the presence of insulin [22,23]. Andrographolide, the main compound of *Andrographis paniculata*, was also reported to increase the glucose uptake of C2C12 myoblast cells [24]. Interestingly, the methanol-water extracts of *Andrographis paniculata* and *Tinospora crispa* stimulated the glucose uptake on 3T3-L1 adipocyte cells. At the same time, *Orthosiphon aristatus, Carica papaya,* and *Curcuma aeruginosa* did not show stimulatory activity [25].

In addition to the potential glucose uptake stimulatory activity of *Andrographis paniculata, Curcuma zanthorrhiza* and *Curcuma longa* might contribute to the FIII's stimulatory capacity. As previously reported, ethyl acetate extracts of *Curcuma longa* showed a glucose uptake stimulatory activity [26]. Curcumin, the main compounds of *Curcuma longa* and *Curcuma zanthorrhiza*, also stimulated glucose uptake in L6 cells [27,28]. Although somewhat lower than that of insulin, the glucose uptake stimulatory activity of *Jamu pahitan* is an appealing subject to be further studied. At the efficacy of 92.59% of insulin, it is possible to increase the activity by bioactivity-guided fractionations, as previously demonstrated in the bioactive fractions of *Punica granatum* L. and *Gnetum africanum* Welw [29,30]. In addition, the oral route for the preparation is generally preferable to the injection required for insulin, which might benefit diabetic patients with better medication adherence.

RIN-m5F is a suitable cell line for studying insulin secretion control in response to the presence of glucose [31]. Interestingly, all tested extracts but FI ethanol extract stimulated proliferation of the pancreatic cell line. Further studies are needed to evaluate whether *Jamu pahitan* can be exploited to regenerate β-cell mass loss commonly seen in diabetes. Restoration of β-cell is considered the focal therapeutic target in the treatment of diabetes [32]. Our result, however, is consistent with a previous report that mentioned that andrographolide of *Andrographis paniculata* promoted differentiation of human pancreatic ductal PANC-1 cells into insulin-producing cells by increasing the expression of pancreatic and duodenal homeobox1. The ethanol extract of *Carica papaya* leaves also stimulated the regeneration of the β-cells in diabetic rats [33,34].

The similar profile of safety and glucose uptake and insulin secretion stimulatory activities between FI and FII underlined the safety and efficacy of the traditional uses of the formulation. Our result was in agreement with the insulin secretion stimulatory activity of the individual plant constituents of *Jamu pahitan*. The water extracts of *Andrographis paniculata*, *Tinospora crispa*, and *Orthosiphon aristatus* were previously reported to show a substantial stimulation of insulin secretion in BRIN-BD11 and isolated rat pancreatic cells [35–37].

On the other hand, *Jamu pahitan* potentially developed into a more potent insulin secretion stimulatory agent as their ethanol extracts showed significantly stronger effects. As previously reported, Borapetol B isolated from *Tinospora crispa* and the hexane extract of *Orthosiphon aristatus* improved the insulin secretion from isolated Langerhans islets and INS-1 insulinoma cells, respectively [38,39]. Hence, FI's better insulin stimulatory activity over FII ethanol extract might be related to the higher fraction of both plants in FI. In addition, the chloroform extract of *Carica papaya* also improved the basal insulin secretion in the isolated rat pancreatic cells [40]. As the ethanol extract of both formulations showed a similar safety profile on RIN-m5F, FI might be more appealing to be further studied for the insulin secretion stimulating agent. Compared to the FIII, ethanol extracts of FI and FII showed better insulin secretion stimulatory effects. Besides *Orthosiphon aristatus*, *Curcuma longa* might also contribute to its activity, as shown in the isolated rats' pancreatic tissues under basal and hyperglycemic conditions [41].

The better stimulatory effects of *Jamu pahitan* ethanol extracts than that of insulin likely resulted from the polyvalence. Numerous compounds bind to multiple targets and result in better overall effects. Glibenclamide increased insulin secretion by blocking potassium adenosine 5'-triphosphate (ATP)-dependent channels, specifically by binding to SUR1 receptors in the membranes of the beta cells [42]. Our study did not evaluate the mechanisms of stimulation of insulin secretion by *Jamu pahitan*. However, the available data suggested the insulin secretion stimulatory activity of *Andrographis paniculata* and *Tinospora crispa* was mediated by affecting the function of potassium ATP-dependent channels and modulating the concentration of Ca^{2+} in beta cells [37,43]. Hence, the compounds of *Jamu pahitan* extracts might act in multiple ways to stimulate insulin secretion from the pancreatic cells.

The main difference between the ethanol and water extracts is their polarity. The water extracted the highly polar compounds of the formulation, while the ethanol extract contained the less polar compounds. The better stimulatory activities toward glucose uptake and insulin secretion of ethanol extracts than the water counterparts likely related with the extracted chemical constituents in each extract. The compounds with proven glucose uptake stimulation activity, i.e., deoxyandrographolide and andrographolide of *Andrographis paniculata*, are semi polar compounds that are better extracted in ethanol than in the water [22,24]. Similarly, borapetol B of *Tinospora crispa*, the known compound with profound insulin secretion stimulatory activity, also has a better solubility in the ethanol [39]. Hence, deoxyandrographolide, andrographolide, and borapetol B are active compounds of the formulation with known antidiabetic mechanisms. Also, among five plant components, *Andrographis paniculata* and *Tinospora crispa* are likely the main antidiabetic active components. However, polyvalence effect in a polyherbal formulation is believed to exert better overall biological effects than individual components. From this point of view, all plant constituents might be attributable to the glucose uptake and insulin secretion stimulatory activity of *Jamu pahitan*. The ethanol extract of FI showed the best overall *in-vitro* antidiabetic activities in this study. This formulation contained 23% of *Andrographis paniculata* and *Tinospora crispa* extracts.

Overall, our result suggested that the traditional uses of *Jamu pahitan* as water extracts effectively enhance glucose uptake in muscle cells and insulin secretion in pancreatic cells and are safe for both cells. However, the better efficacy but the higher toxicity of ethanol extracts might limit the practical use of the result of this study. Further studies on the fractionation of the ethanol extracts might be beneficial to obtain the optimal glucose uptake and insulin secretion stimulatory activities that are less toxic to the targeted tissues are needed. Also, this study could not provide the information on the overall hypoglycemic effect of the formulation. The *in-vivo* studies on diabetic animals are required to validate the *in-vitro* findings of this study. Previous study reported that FIII water extract decreased

blood glucose and HbA1c levels in the diabetic patients [44]. Hence, this study provides the underlying mechanisms of the antidiabetic activity of the formulation. As there were no reports on the pharmacological evaluations of FI and FII available yet, this study is the first one to provide the information.

4. Conclusion

This study predicted the mechanism of antidiabetic actions of the *Jamu pahitan* by stimulation of glucose uptake in the muscle cells and improvement of insulin secretion in β-cells. The water extracts showed a safer profile and still had a considerable glucose uptake and insulin secretion stimulatory activities. Tested at the safe concentrations to the cells, the ethanol extracts exerted stronger *in-vitro* antidiabetic activities than the water counterparts. Both safety and efficacy of the cells were strongly correlated with TPC in a positive manner.

5. Materials and Methods

5.1. Chemicals, cell lines, and plant materials

Folin-Ciocalteu reagent, gallic acid, dimethyl sulfoxide (DMSO), methanol, and ethanol were purchased from Sigma Aldrich (USA). Dulbecco's modified eagle medium (DMEM), RPMI 1640, fetal bovine serum (FBS), Penicillin-Streptomycin, and other cell culture supplies were obtained from Thermo Fisher Scientific (USA). MTT Assay Kit, Glucose Assay Kit, and Human Insulin ELISA Kit were from Abcam (UK). Glucose (ANB Laboratories, Thailand), insulin (Humulin® Eli Lilly, USA), and glibenclamide (Siam Bheasach, Thailand) were also used in this study.

L6 (CRL1458™, passage number of 27–33) and RIN-m5F (CRL-2058™, passage number of 19–24) were from ATCC® (USA). They were cultured in DMEM and RPMI 1640, respectively, supplemented with 10% heat-inactivated FBS and 0.1% Penicillin-Streptomycin (10,000 U/mL). Cells were maintained in an incubator (Thermo Scientific, USA) at 37 °C and 5% CO_2.

The plant name has been checked with and in accordance with their respective accepted name in the World Flora Online (http://www.worldfloraonline.org/) database. The crude drugs of *Andrographis paniculata, Tinospora crispa*, and *Orthosiphon aristatus* were purchased from Vejpong Pharmacy, Bangkok, Thailand with batch number of FGTH0321A, FGTH0703A, and FGTH6621A, respectively. They were authenticated by comparing their macroscopic and microscopic morphology to the authentic plant materials kindly provided by Sireeruckhachati Nature Learning Park, Mahidol University, Nakhon Pathom, Thailand, and their monograph in Thai Herbal Pharmacopeia. *Carica papaya* leaves and *Curcuma aeruginosa* rhizomes were kindly provided by Sireeruckhachati Nature Learning Park and Chom-beung herbal plantation, Ratchaburi, Thailand, respectively. The identity of both plants was verified by the morphological characters described in the Malaysian Herbal Monograph. Specimens of *Carica papaya* and *Curcuma aeruginosa* are deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University with respective batch number of Hartanti004 and Hartanti005.

5.2. Preparation of the formulations

Formula I (FI) and Formula II (FII) of *Jamu pahitan* consisted of *Andrographis paniculata*, *Tinospora crispa, Orthosiphon aristatus*, *Carica papaya*, and *Curcuma aeruginosa* in a ratio of 3-3-3-2-2 and 1-1-1-1-1, respectively. The powdered crude drugs were individually weighed, homogenously mixed in the specified proportion, and packed in an air-tight container. Formula III (FIII) is a variation of *Jamu pahitan* purchased from *Klinik Wisata Kesehatan Jamu,* Tegal, Indonesia. It is currently prescribed for patients with diabetes seek medication in the clinic. It is in the form of mixture of the crude drugs of *Andrographis paniculate* aerial parts, *Orthosiphon aristatus* aerial parts, *Syzygium polyanthum* (Wight) Walp. leaves, *Cinnamomum burmanni* (Nees & T. Nees) Blume barks, *Curcuma zanthorrhiza* Roxb. rhizomes, *Curcuma longa* L. rhizomes, and *Phyllanthus niruri* L. aerial parts in a ratio of 5-3-5-7-15-10-5. It is in the ready-to-use package, weighed 50 g, for a day use.

5.3. Extract preparation

The water extracts of *Jamu pahitan* formulations were prepared by accurately weighing and decocting the crude drugs in water in a ratio of 1:20 (w/v) for an hour. After filtration, the filtrate was evaporated over the water bath to dryness. The ethanol extracts were prepared by accurately weighing and extracting the crude drugs in ethanol at the ratio of 1:100 (w/v) under sonication for 60 min. A vacuum rotary evaporator was used to evaporate the solvent.

5.4. Total phenolic content analysis

A modified Folin-Ciocalteau method was used to determine each extract's TPC [45]. 0.5 ml of reagent was mixed with 0.1 or 0.25 ml of the extract solution (at 10 mg/ml) and 7.9 or 7.75 ml of water and stood for 5 min. Saturated sodium carbonate (1.5 ml) was further added, and the mixture was kept at room temperature. After 2 h, the absorbance was recorded by a spectrophotometer (Thermo Scientific, USA) at 764 nm. The calibration curve was prepared from gallic acid (0–1.2 mg/ml), and TPC was presented as mg gallic acid equivalents (GAE)/g dry weight (DW) crude extract.

5.5. Glucose uptake stimulatory assay

The cytotoxic effects of formulation extracts on skeletal muscle were evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay [46]. Cells were seeded in a 96-well plate at the density of 2×10^4 cells/well in DMEM enriched with FBS and Penicillin-Streptomycin and incubated for 24 h. Cells were further incubated in DMEM containing ethanol and water extracts of each formulation at concentrations of 25–200 μg/ml for 24 another h. DMEM with 0.5% DMSO was used as the control. MTT Assay Kit was used to evaluate the cell viability. According to the manufacturer's protocol, the absorbance was read in a microplate reader (BMG LABTECH, Germany) at 590 nm. The concentrations of extracts resulting in 50% of cell viability (IC_{50}) were mathematically calculated from the concentration-effect curves accordingly.

L6 myoblasts were seeded in a 24-well plate at a density of 2×10^4 cells/well in DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin in a working volume of 500 μl as previously reported [46]. Once the cells reached a confluence of 90%, the media was switched to the differentiate one (DMEM with 2% FBS and 1% Penicillin-Streptomycin). The media was changed every other day to allow cells to differentiate into myotubes for at least seven days. The myotubes were incubated in differentiate media containing extracts of *Jamu pahitan* in FI and FII at 5, 10, or 20 μg/ml or FIII at 10 and 20 μg/ml at 37 °C under 5% CO_2 for 24 h. Insulin at 100 nM was used as the positive control, while differentiate media containing 0.5% DMSO was the negative control. The aliquot of media was collected, and their volume was corrected to 500 μl ml with sterile water. Glucose Assay Kit was used to analyze the glucose level in the media, and the absorbance was measured at the wavelength of 570 nm according to the manufacturer's protocol. The glucose-uptake stimulatory activity of each extract was relatively calculated toward the control group.

5.6. Insulin secretion stimulatory assay

Cells were seeded in a 96-well plate at 4×10^4 cells/well in RPMI 1640 enriched with FBS and Penicillin-Streptomycin and incubated for 24 h. Cells were further incubated in RPMI 1640 containing ethanol and water extracts of each formulation at concentrations of 6.25–100 μg/ml for another 24 h. RPMI 1640 with 0.5% DMSO was used as the control. The cell viability was evaluated by MTT Assay Kit, and the IC_{50} was calculated accordingly [47].

Cells were seeded in a 24-well plate at 3×10^5 cells/well and incubated in RPMI 1640 with FBS and Penicillin-Streptomycin at 37 °C in a working volume of 500 μl for 72 h. After media removal, the cells were washed with PBS thrice. The cells were further pre-incubated in 1 ml of the Krebs Ringer Bicarbonate (KRB) buffer containing NaCl, KCl, $CaCl_2$, $MgSO_4$, KH_2PO_4, $NaHCO_3$, and HEPES at the pH of 7.4 as previously described for 40 min at 37 °C [47]. The cells were incubated in 0.5 ml KRB containing 1.1 mM glucose and extracts of FI and FII (water extracts at the concentrations of 12.5, 25, or 50 μg/ml; ethanol extracts at 2.5, 5, and 10 μg/ml) or FIII (water extracts at concentrations of 25 and 50 μg/ml; ethanol extracts at 5 and 10 μg/ml) at 37 °C under 5% CO_2 for 30 min. Glibenclamide (at concentrations of 10 μg/ml) and KRB with 0.5% DMSO were used as the positive and negative control. The aliquot of buffer was collected, and their volume was corrected to 500 μl with the fresh KRB. The insulin level in the buffer was analyzed by following the manufacturer's Human Insulin ELISA Kit protocol. The insulin secretion stimulatory activity of each extract was relatively calculated as the percentage of the negative control group.

5.7. Data analysis

TPC, cell viability, IC_{50}, glucose uptake, and insulin secretion stimulatory activities were expressed as the mean value ± standard error of the mean (SEM). Cell viability and stimulatory activities of glucose uptake and insulin secretion between concentrations and TPC and IC_{50} between extracts were compared by one-way ANOVA and Duncan's tests. The Pearson correlation test evaluated the correlations between the TPC and IC_{50} values, glucose uptake stimulatory and insulin secretion stimulatory activities of the extracts. The difference in value was considered significant at a p-value <0.05. Statistical analysis was conducted by the general procedures of IBM SPSS Statistics (IBM, USA).

Author contribution statement

Dwi Hartanti: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nutputsorn Chatsumpun; Worawan Kitphati: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Penchom Peungvicha; Wasu Supharattanasitthi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at https://doi.org/10.17632/tvjm454c3y.1.

Declaration of interest's statement

The authors declare no competing interests.

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Appendix A. Supplementary data

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