

Active fraction of Ciplukan (*Physalis angulate* L.) increases phosphorylation on IRS-1 Tyr-612 and Akt Ser-473 in 24-hour incubation time

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ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disorder marked by elevated blood glucose levels due to insufficient insulin production or action. This condition has become a major health concern globally, including in Indonesia. The increasing prevalence of DM in Indonesia poses significant health challenges, prompting exploration of traditional medicinal plants as alternative treatments. Ciplukan (*Physalis angulata* Linn) is known for its antidiabetic effect in Indonesians. Previous studies showed that the active fraction (AF) increased glucose absorption in C₂C₁₂ cells. This study aims to investigate the effect of *P. angulata*'s AF on insulin receptor substrate-1 (IRS-1) Tyr-612 and Akt Ser-473 phosphorylation in insulin-resistant C₂C₁₂ cells. Treatment of well-differentiated C₂C₁₂ cells with palmitate was conducted to induce insulin resistance. The insulin-resistant C₂C₁₂ cells were divided into four groups: the negative control and the treated groups each with 4-, 12- and 24-hour incubation time. Expression of p-IRS-1 Tyr-612 and p-Akt Ser-473 in insulin-resistant C₂C₁₂ cells was measured by a western blot analysis. The AF of *P. angulata* appeared to have an antidiabetic effect by increasing phosphorylation of IRS-1 Tyr-612 and Akt Ser-473 in insulin-resistant C₂C₁₂ cells after 24 hours of incubation ($p < 0.05$).

INTRODUCTION

Diabetes mellitus (DM) is a worldwide concern due to its high prevalence. According to the International Diabetes Federation data in 2021, Indonesia ranks 5th with the highest number of residents suffering from DM from all over the world. Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose, which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves [1]. Type

2 diabetes mellitus (T2DM) is the most common type of DM. The pathophysiology underlies that T2DM is insulin resistance, which involves various tissues, mainly muscle, adipose, and liver tissues [2]. The insulin resistance condition itself affects glucose absorption, especially the muscle tissue, which has an 80% role in the uptake of blood glucose after a meal [3].

GLUT4 is a transporter that helps the internalization of glucose into the cells. In order for GLUT4 to have this function, the insulin signaling pathway needs to be activated. The insulin signaling pathway starts when insulin binds to its receptor on the cell membrane, causing its downstream protein, insulin receptor substrate-1 (IRS-1) is phosphorylated on its tyrosine 612 residue, causing its full activation. The activated IRS-1 then binds to *phosphatidylinositol 3 kinase* (PI3K), which phosphorylates *phosphatidylinositol 4,5-bisphosphate*

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(PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) and eventually phosphorylates Akt/PKB on its serine 473 residue to be fully activated. This fully activated Akt/PKB then activates AS160, which is responsible for the GLUT4 translocation from cytosol to the cell membrane [4].

IRS-1 is crucial for regulating the intracellular insulin signaling cascade, as it is indeed the first step in dispersing the direction of insulin signaling by activating many other downstream proteins [5]. IRS-1 phosphorylation on serine residue inhibits the insulin signaling cascade as it interferes with the interaction between IRS-1 and PI3K, thus leading to an insulin resistance condition [6]. Akt/PKB activation is also needed to maintain the normal insulin signaling cascade. Disruption of this protein will result in decreasing GLUT4 translocation to the cell membrane, diminishing glucose uptake in insulin-sensitive tissues [7].

In Indonesia, many traditional herbs have been used by the locals to control blood sugar levels. One of the traditional herbs that has long been known for controlling blood sugar levels is Ciplukan (*P. angulata*). Several studies have proved that *P. angulata* contains bioactive components that have antidiabetic effects, such as withangulatin-A [8], quercetin [9], and rutin [10]. *In vivo* study about the effect of isolated compound of *P. angulata*, which was withangulatin-A on diabetic rats, showed that this compound was able to reduce blood sugar significantly [8]. In a separate study, quercetin was shown to improve glucose uptake in C_2C_{12} insulin-resistant cells via IRS-1/Akt pathway [11]. Meanwhile, in other research, rutin was proven to exhibit glucose-lowering activities by inhibiting hepatic glucose production via Akt activation [12]. In a previous study regarding glucose absorption in the C_2C_{12} cell line, the active fraction (AF) of *P. angulata* obtained from bioassay-guided fractionation was proved to increase the glucose absorption level by 26.47% [13]. Another study about the activity of this AF was conducted on insulin-resistant C_2C_{12} cells, which showed that the optimal concentration to increase glucose absorption was at 100 $\mu\text{g/ml}$ [14].

This study aims to observe the optimal incubation time for the AF of *P. angulata* at 100 $\mu\text{g/ml}$ to increase the phosphorylation of IRS-1 Tyr-612 and Akt Ser-473.

MATERIALS AND METHODS

Materials

Materials used in the study were DMEM (Gibco, Lot: 2512444), Fetal Bovine Serum (FBS, SIGMA, Lot: 001671877), Horse Serum (HS, SIGMA, Lot: 22B441), antibiotics (Elabscience, Lot: GY1142RN8913), BSA (Elabscience, Lot: DI02260R2362), GOD PAP (DSI, Lot: 60159945), p-IRS-1 Tyr-612 antibodies (Invitrogen, Lot: 2350459), p-Akt Ser-473 antibodies (ABClonal, Cat No: AP0140), and β -actin antibodies (ABclonal, Cat No: AC050). An AF of *P. angulata* was gained from the Department of Pharmacology and Therapy, Faculty of Medicine, Public Health, and Nursing, UGM.

Cell culture and differentiation

C_2C_{12} cells were obtained from the Department of Pharmacology and Therapy, Faculty of Medicine, Public Health, and Nursing, UGM. The passage of the cells was 50. C_2C_{12} cells were cultured in DMEM supplemented with 10% FBS at

37°C and 5% CO_2 . Differentiation of C_2C_{12} cells was induced by replacing the media with DMEM containing 2% HS [15]. Experiments were conducted after 6–8 days of differentiation.

Validation of insulin resistance

Insulin resistance condition was obtained by treating cells with palmitate (PA). Palmitate acts by reducing the phosphorylation of IRS-1 Tyr-612 and Akt Ser-473 [16,17]. An optimal concentration of palmitate was needed to induce insulin resistance in C_2C_{12} cells. Therefore, we conducted validation of insulin resistance.

Validation of insulin resistance on C_2C_{12} cells was done by the GOD PAP method. The 3×10^3 cells were seeded in a 96-well plate and then differentiated. The well-differentiated cells were divided into five groups: negative control, PA 0.25, PA 0.5, PA 0.75, and PA 1, which were treated without palmitate, with 0.25, 0.5, 0.75, and 1-mM PA, respectively. After 16 hours of incubation [18], the media was tested with the GOD PAP method [19], read by an ELISA reader, and the result was analyzed by quantifying glucose concentration in the media.

Induction of insulin resistance and treatment with AF of *P. angulata*

On a 6-well plate, 2.5×10^5 of C_2C_{12} cells were seeded. After differentiation, they were divided into four groups: K1, P1, P2, and P3 (Table 1 shows the distribution of groups). The optimal concentration of PA achieved from the validation's result was used to treat the well-differentiated C_2C_{12} cells. After 16 hours of PA incubation, the cells had become insulin-resistant and ready to be treated [18]. Treatments were done by replacing the media with 100- $\mu\text{g/ml}$ AF of *P. angulata* and incubating each group according to its incubation time. This concentration was used as it was the optimal concentration for AF to increase glucose absorption in insulin-resistant C_2C_{12} cells within the previous study [14].

Insulin induction and protein isolation

Induction of insulin was done to start the insulin signaling pathway of the C_2C_{12} cells. Ten minutes before the end of induction time, each group was treated with 100 nM insulin [20]. After treatment with insulin, the media was retrieved and the cells were scrapped. The collected cells were stored at -80°C. Protein isolation was conducted to measure the expression of p-IRS-1 Tyr-612 and p-Akt Ser-473 with the western blot method [21].

Measuring expression p-IRS-1 Tyr-612 and p-Akt Ser-473

Western blot analyses were conducted to detect p-IRS-1 Tyr-612 and p-Akt Ser-473. After the isolation, proteins

Table 1. Distribution of groups.

Group	PA	Insulin	AF of <i>P. angulata</i> 100 $\mu\text{g/ml}$	Incubation time
K1	+	+	-	0 hour
P1	+	+	+	4 hours
P2	+	+	+	12 hours
P3	+	+	+	24 hours

were normalized and subjected to SDS-PAGE. The stained proteins were loaded into SDS-PAGE gel and transferred to the PVDF membrane. After blocking with blocking buffer, the PVDF membrane was immunoblotted with a specific antibody (p-IRS-1 Tyr-612, p-Akt Ser-473, and β -actin) and the proteins were revealed by a chemiluminescence method [21]. The results were quantified with ImageJ and analyzed statistically.

Statistical analysis

Analysis of insulin resistance and western blot data was performed using GraphPad Prism version 9.3.1 on the Windows operating system. The data were evaluated through one-way ANOVA with a 95% confidence interval, followed by Tukey's post hoc test for multiple comparisons, and the results were expressed as geometric mean $\pm$ standard deviation (SD). Statistical significance was defined as $p < 0.05$ [22].

RESULTS AND DISCUSSION

Cell culture and differentiation

Before treatment with AF of *P. angulata*, C2C12 myoblasts were differentiated into myotubes. Morphology changes of C₂C₁₂ cells during the differentiation process are shown in Figure 1. Fully differentiated myotubes were seen on day 8.

Validation of insulin resistance

In order to present a diabetic state, these cells needed to undergo palmitate treatment to make them insulin-resistant. Palmitate is a saturated fatty acid that is often linked to insulin resistance. Palmitate is able to inhibit the phosphorylation of IRS-1 Tyr-612 and Akt Ser-473 through the formation of

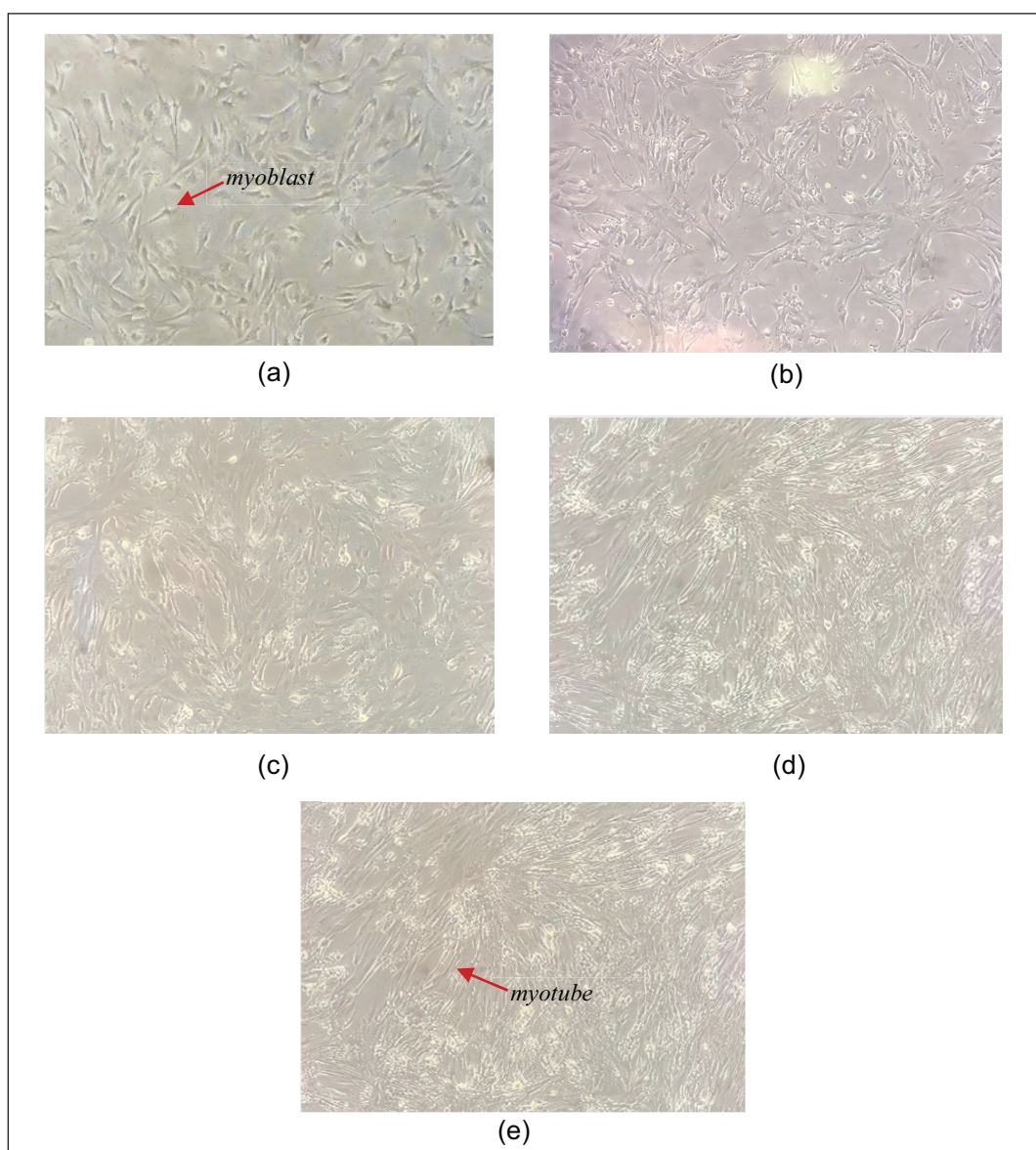


Figure 1. Differentiation of C₂C₁₂ cells. (a–e) 0, 2nd, 4th, 6th, and 8th day differentiation.

ceramide and diacylglycerol, which are metabolites derived from palmitate [16,17].

Validation of insulin resistance was conducted by observing the glucose concentration in media of the treatment group compared with the control group. The insulin-resistant cells were not able to absorb the glucose in the media, and therefore, the group with a significantly higher glucose concentration compared to the control group is considered insulin-resistant.

Figure 2 shows the result of the glucose consumption test with the GOD PAP method. According to the result, PA with a concentration of 0.75 mM could induce an insulin-resistant state in C₂C₁₂ myotubes (*p* < 0.05). Therefore, PA at this concentration was used to induce insulin resistance in C₂C₁₂ myotubes before treatment with AF of *P. angulata*.

Effect of AF of *P. angulata* on the expression of p-IRS Tyr-612 on insulin-resistant C₂C₁₂ cells

The effect of AF of *P. angulata* was observed in every group. As seen in Figure 3, the highest phosphorylation of IRS-1 Tyr-612 was achieved in P3 (24-hour incubation time group), with a significant difference *p* < 0.05 compared with K1, the negative control group. Phosphorylation of IRS-1 Tyr-612 in P1 was also seen slightly higher than K1, but without any significant difference compared with the negative control (*p* > 0.05). Although the graph shows that P2 has the lowest expression of IRS-1 Tyr-612, it had no significant difference compared with P1 (*p* > 0.05).

IRS-1 is a downstream protein of insulin receptor. To be fully activated, IRS-1 needs to be phosphorylated on the right residue. In a resistant insulin model, increased phosphorylation of certain serine residue of IRS-1 is found. This corresponds to inhibition of insulin signaling pathway by preventing the interaction between IRS-1 and its downstream protein, which is PI3K [6]. On the other hand, phosphorylation on tyrosine residue of IRS-1 leads to the internalization of glucose through activation of insulin signaling pathway. Phosphorylation of IRS-1 on tyrosine 612 is very important to activate PI3K as its downstream protein [23].

Effect of AF of *P. angulata* on the expression of p-Akt Ser-473 on insulin-resistant C₂C₁₂ cells

The result of ImageJ quantification of p-Akt Ser-473 is presented in Figure 4. The highest expression of p-Akt Ser-473 is seen in the P3 group, with 24-hour incubation time (*p* < 0.05). P1 and P2 are shown to have higher expression of p-Akt Ser-473 than K1, the negative control, but with no significant difference (*p* > 0.05). Although P2 seems to have a slightly higher expression of p-Akt Ser-473 than P1, it had no significant difference (*p* > 0.05). Therefore, P1 and P2 can be considered to have the same level of expression of p-Akt Ser-473.

Akt, also known as PKB, is a crucial protein that has functions related to cell survival and glucose metabolism. Similar to IRS-1, Akt also needs to be activated on its right residue to be fully activated. When Akt is fully activated, it will cause AS160 to translocate GLUT4 in the cytosol to the

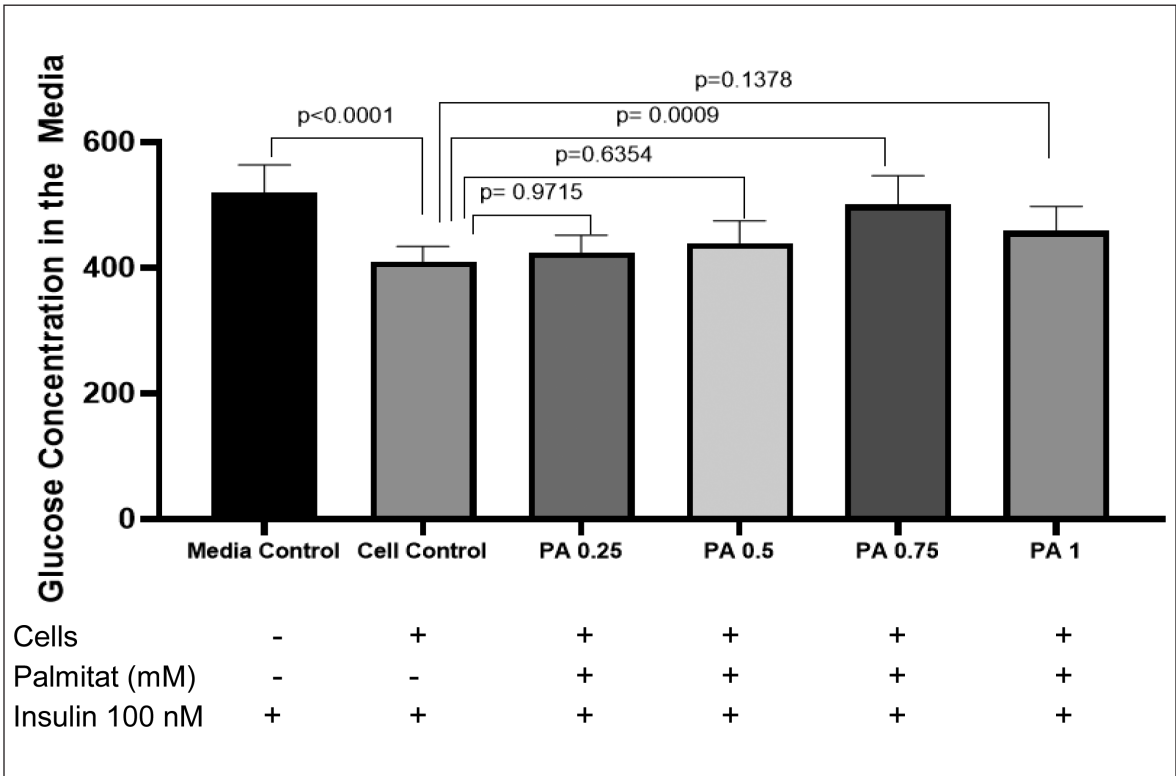


Figure 2. Glucose concentration in the media. PA 0.75 shows a significantly higher level of glucose concentration in media compared with negative control group (*p* < 0.05) (*n* = 5, One way ANOVA, post hoc Tukey).

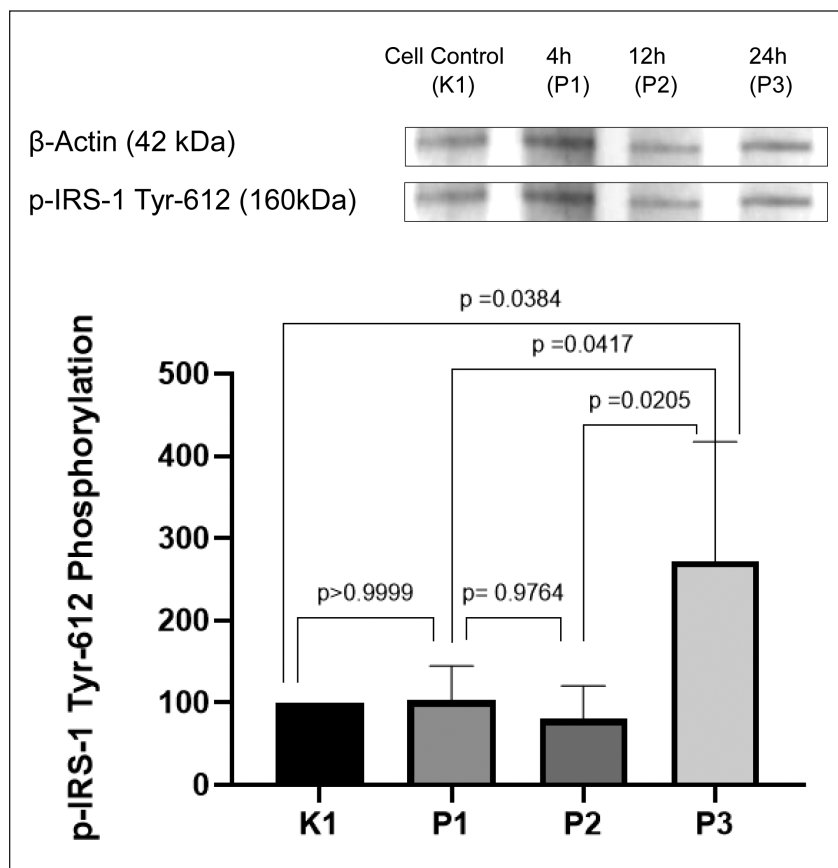


Figure 3. Expression of p-IRS-1 Tyr-612 on insulin-resistant C_2C_{12} cells after treatment with *P. angulata* AF. P3 has the highest expression of p-IRS-1 Tyr-612 ($p < 0.05$) ($n = 4$, One way ANOVA, post hoc Tukey).

membrane cell [24]. When Akt is phosphorylated in threonine 308 and serine 473, it will be fully activated [25].

The AF of *P. angulata* used in this research was the methanol-insoluble partitioned resulted from the chloroform extract of the *P. angulata* herb, and therefore, the predicted compositions were semi-polar to nonpolar secondary metabolites. The secondary metabolites with these properties include alkaloids, terpenoids, and flavonoids [26]. Several studies have discovered that these compounds exert antidiabetic effects. Hirsutine, an alkaloid isolated from *Uncaria rhynchophylla*, was able to enhance glucose uptake by activating the PI3K/Akt pathway in insulin-resistant HepG2 and H9c2 cells [27]. Along with this, another research also discovered that epiberberine, an alkaloid derived from *Rhizoma coptidis*, could increase GLUT4 protein expression and IRS-1/PI3K/Akt insulin signaling pathway in insulin-resistant HepG2 cells [28]. Eurocristatine, an alkaloid isolated from *Eurotium cristatum*, showed its antidiabetic effect also by increasing PI3K/IRS-1/Akt activation *in vivo* and *in vitro* [29].

Terpenoid constituent from *Pinus* spp. (*Phyllostachys nigra* and *Pinus radiata*), α -pinene, and essential oils derived from *P. nigra* could enhance the transcription of the GLUT4/Scl2a4 gene, leading to increased production of GLUT4 protein and its translocation to the plasma membrane in C_2C_{12} cells [30]. A study about other terpenoids involved a terpenoid-rich extract

from *Dillenia indica* L. bark, with findings indicating that the extract was able to enhance IRS-1/PDK1/Akt phosphorylation in insulin-resistant C_2C_{12} cells [31]. Triterpenoid saponins extracted from *Stauntonia chinensis* were observed to be able to increase glucose uptake in insulin-resistant HepG2 cells via AMP-activated protein kinase and IR/IRS-1/PI3K/Akt signaling pathway [32].

Flavonoid also has an antidiabetic effect, as seen in a study on kaempferol, flavonoids found *Bauhinia forficata* in HepG2 cells. This study indicated that kaempferol had the capability to increase AKT phosphorylation in these cells [33]. Research on a different type of flavonoid was also carried out using *Enicostema littorale*. One of the plant's flavonoid-rich fractions was able to upregulate glucose uptake rate via IRS-1/PI3K/Akt pathway in insulin-resistant HepG2 cells [34]. Poncirin, a flavone glycoside found in many citrus fruits, was proven to improve glucose uptake by activating the PI3K/Akt signaling pathway in insulin-resistant C_2C_{12} cells [35].

According to the results, the treatment of 100- μ g/ml AF of *P. angulata* was shown to have a positive effect on phosphorylation of IRS-1 Tyr-612 and Akt Ser-473 in 24-hour incubation time, which was inhibited beforehand with PA 0.75 mM. This effect worked in a time-dependent manner. The 24-hour incubation was considered the optimum time for compounds in AF of *P. angulata* to exert the antidiabetic effect.

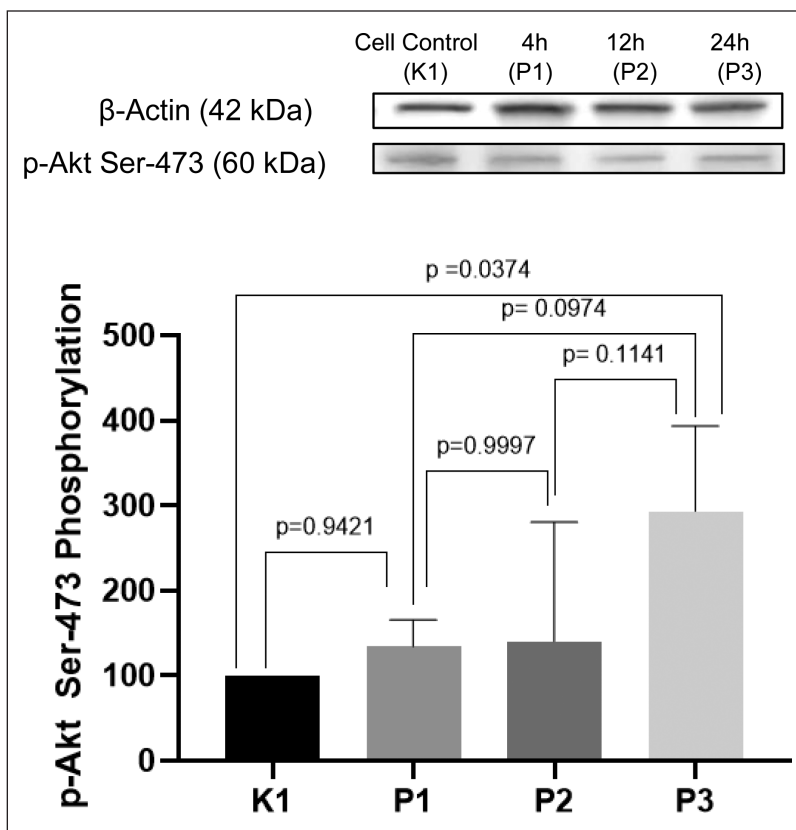


Figure 4. Expression of p-Akt Ser-473 on insulin-resistant C_{12} cells after treatment with *P. angulata* AF. P3 has the highest expression of p-Akt Ser-473 ($p < 0.05$) ($n = 4$, One way ANOVA, post hoc Tukey).

This effect was probably because the longer the incubation time, the better the absorption level of AF into the cells, so that it could cause a higher effect of phosphorylation.

In brief, this study showed that the AF of *P. angulata* increased IRS-1 Tyr-612 and Akt Ser-473 phosphorylation in 24-hour incubation time. This study could be further investigated by exploring the mechanism of action of *P. angulata*'s AF in other parameters of insulin signaling pathways, either using the same cell line or different types of cell lines. Furthermore, *in vivo* studies could be conducted to further investigate the potential of *P. angulata*'s AF to enhance understanding of this herb's potential as an alternative treatment for DM.

CONCLUSION

The results of this study demonstrate that 100- μ g/ml AF of *P. angulata* was able to increase phosphorylation of IRS-1 Tyr-612 and Akt Ser-473 in 24-hour incubation time. Since IRS-1/Akt phosphorylation is essential for addressing insulin resistance, as seen in DM, therefore this discovery offered insight into future alternative treatments for DM utilizing an AF of *P. angulata*.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The study protocol was approved by the Ethics Committee of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Indonesia (Approval No.: KE/FK/1731/EC/2023).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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