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Author(s)	Lahrita, Lucy; Kato, Eisuke; Kawabata, Jun
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Uncovering potential of Indonesian medicinal plants on glucose uptake enhancement and lipid suppression in 3T3-L1 adipocytes

Lucy LAHRITA, Eisuke KATO*, Jun KAWABATA

Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan

*Corresponding author. Tel./fax: +81 11 706 2496.

Email address: eikato@chem.agr.hokudai.ac.jp (E. Kato).

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Abstract

Ethnopharmacological relevance

As obesity is a key factor in the development of type 2 diabetes, lowering lipid accumulation in adipose tissues is as important as increasing insulin sensitivity in diabetic patients. The selected plant extracts used in this screen have been traditionally used in Indonesian medicine for the treatment of diabetes and its complications.

Aim of the study

To investigate the ability of the selected plants to both increase insulin sensitivity through the enhancement of glucose uptake after insulin induction in adipocytes and suppress lipid production in the same target cells.

Materials and methods

Dried Indonesian medicinal plants were extracted with 50% (v/v) aq. methanol. The extracts were dissolved in 50% DMSO when tested on 3T3-L1 adipocytes. The screening platform consists of insulin-induced glucose uptake, lipid accumulation, and cell viability. Initially, an enzymatic fluorescence assay was designed to demonstrate the enhancement of 2-deoxyglucose (2-DG) uptake after insulin induction. Different concentrations of the extracts that enhanced glucose uptake were subjected to lipid accumulation assay using Oil Red O staining. Potential extracts based on lipid suppression were subsequently assessed by CCK-8 cell viability assay to distinguish lipid reduction activity from cytotoxicity.

Results

Out of 59 plants, 13 plants demonstrated their ability to increase glucose uptake in 3T3-L1 adipocytes after insulin induction, and 4 of these plants' extracts suppressed lipid production of the cells. The CCK-8 assay results of those 4 plant extracts suggest that the lipid inhibition activity of *Eurycoma longifolia* Jack (root) and *Piper nigrum* L. (fruits) extracts is not attributed to their cytotoxicity in the adipose cells. Both of the plant extracts increased glucose uptake by more than 200% at 50 µg/mL and suppressed lipid accumulation in a concentration-dependent manner.

Conclusions

Screening of selected Indonesian medicinal plants has uncovered the potentials of *Eurycoma longifolia* Jack (root) and *Piper nigrum* L. (fruits) with dual active functions, increasing insulin sensitivity through the enhancement of glucose uptake and reducing lipid accumulation in

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adipose cells. These findings suggest that the ability of both plants to suppress lipid production would provide additional benefits in the treatment of diabetes.

Keywords

Diabetes, Obesity, *Eurycoma longifolia* Jack, *Piper nigrum* L., Tropical plants

Abbreviations

2DG, 2-deoxyglucose; BSA, bovine serum albumin; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; G6PDH, glucose 6-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; IDF, International Diabetes Federation; KRPH, Krebs–Ringer–phosphate–Hepes; PBS, phosphate buffered saline; TEA, triethanolamine.

1. Introduction

In 2014, the International Diabetes Federation (IDF) reported that 387 million of the world's population was living with diabetes, and this number is anticipated to rise beyond 592 million within the next two decades (International Diabetes Federation, 2014). As a complex metabolic disorder, type-2 diabetes mellitus (T2DM) is associated with impaired insulin release and insulin resistance, which result in high levels of glucose in the blood. Meanwhile, obesity involves an abnormal accumulation of adipose tissue, characterized by an increasing adipocyte number (hyperplasia) and size (hypertrophy). The association between obesity and T2DM, which has

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been well-recognized for decades, is mainly due to the insulin resistance of target cells, which affects glucose utilization and energy production.

Insulin sensitizers are commonly prescribed to treat insulin resistance, the primary clinical issue in T2DM. Thiazolidinediones (TZDs) reduce insulin resistance by activating peroxisome proliferator activated receptor gamma (PPARγ). Clinical studies undertaken by Mayerson et al. (2003) and Virtanen et al. (2003) reported that TZDs reduce plasma insulin and increase glucose uptake. However, these improvements result in weight gain and undesirable lipid profiles. Therefore, there is on-going debate as to whether it is appropriate to use TZDs for the treatment of obesity associated with T2DM. In agreement with the reported results of TZDs, non-TZD PPARγ agonists from natural compounds have the capacity to activate PPARγ (Yang et al., 2007; Choi et al., 2009; Han et al., 2006). However, one consequence is the promotion of adipogenesis. For this reason, there is a growing need to identify novel bioactive compounds from local medicinal plants that have the capacity to increase insulin sensitivity and reduce lipid production at the same time.

This study investigates and identifies plants with dual active functions that could serve as anti-diabetes and anti-obesity agents. Those plants listed in Table 1 were selected for use in this study because they are commonly used by local herbal industries in Indonesia (Elfahmi et al., 2014). Other plants that are often used locally for curing diabetes complications were also selected (Dalimartha, 2000; Hariana, 2006; Wijayakusuma, 2006). Certain plants in this study were expected to have unique mechanisms that could increase glucose uptake, while simultaneously suppressing lipid production.

To the best of our knowledge, this work is the first to report the *in vitro* screening of Indonesian medicinal plants for insulin-induced glucose uptake enhancement and lipid-lowering

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activity in 3T3-L1 adipocyte model cells. The findings of the study were anticipated to corroborate the traditional application of Indonesian medicines, known as *jamu*, in treating T2DM and its complications, thus contributing to a more complete understanding of their efficacy.

2. Materials and Methods

2.1. Chemicals

For culture medium, DMEM (high glucose with *L*-Glutamine and phenol red), streptomycin sulfate, and gentamicin sulfate were purchased from Wako Pure Chemical (Osaka, Japan). FBS and penicillin G potassium salt were supplied by Hyclone Laboratories Inc. (Utah, USA) and Sigma-Aldrich (Tokyo, Japan), respectively. For the induction of differentiation, IBMX and DEX were provided by Sigma-Aldrich (Tokyo, Japan), while insulin was obtained from Wako Pure Chemical (Osaka, Japan). 2-Deoxyglucose (2-DG), hexokinase (from *Saccharomyces cerevisiae*), and G6PDH (from *Leuconostoc mesentriodes*) were purchased from Sigma–Aldrich (Tokyo, Japan). Resazurin sodium salt and triethanolamine hydrochloride (TEA) were obtained from Wako Pure Chemical (Osaka, Japan). Adenosine 5-triphosphate disodium salt (ATP), β-nicotineamide adenine dinucleotide phosphate (β-NADP+), and diaphorase (from *Clostridium kluyvery*) were provided by Oriental Yeast (Tokyo, Japan). Rosiglitazone hydrochloride was purchased from Takeda Pharmaceuticals (Osaka, Japan). The Quickstart protein assay was supplied by Bio-Rad Laboratories Inc., while Oil Red O was obtained from Sigma-Aldrich (Tokyo, Japan).

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2.2. Plants

Dried materials of Indonesian plants were purchased from Merapi Farma Herbal Co. (Yogyakarta, Indonesia), a local herbs supplier, in April 2014. The plants, collected from the Central Java region, were identified the company's herbalist. Their voucher specimens were deposited at the same company and each batch number is presented in Table 1.

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Table 1

List of selected Indonesian medicinal plants for the treatment of diabetes and its complications

Plant species	Batch number	Local name	Family	Part used	References	IiGUE* activity
Alpinia galanga (L.) Willd.	AG1314.073	Lengkuas	Zingiberaceae	Rhizome	Hariana (2006)	51.8 ± 7.6
Alstonia scholaris (L.) R. Br.	AS1314.004	Pule	Apocynaceae	Bark	Elfahmi et al. (2014)	66.4 ± 1.3
Andrographis paniculata (Burm.f.) Nees	AP1314.042	Sambiloto	Acanthaceae	Whole plant	Elfahmi et al. (2014)	129.2 ± 4.4
Areca catechu L.	AC1314.006	Pinang	Arecaceae	Fruits	Ghate et al. (2014)	143.2 ± 9.2
Averrhoa bilimbi L.	AB1314.070	Belimbing wuluh	Oxalidaceae	Leaves	Pushparaj et al. (2000)	78.9 ± 15.8
Azadirachta indica A.Juss.	AI1314.051	Mimba	Meliaceae	Leaves	Atangwho et al. (2012)	113.3 ± 8.0
Baeckea frutescens L.	BF1314.023	Jungrahab	Myrtaceae	Bark	Dalimartha (2000)	68.6 ± 15.8
Blumea balsamifera (L.) DC.	BB1314.007	Sembung	Asteraceae	Leaves	Elfahmi et al. (2014)	72.2 ± 8.7
Brucea javanica (L.) Merr.	BJ1314.031	Buah Makasar	Simaroubaceae	Seeds	Noorshahida et al. (2009)	37.4 ± 8.2
Caesalpinia sappan L.	CS1314.17	Kayu secang	Fabaceae	Wood	Tong et al. (2013)	161.1 ± 19.9
Carica papaya L.	CP1314.049	Pepaya	Caricaceae	Leaves	Juárez-Rojop et al. (2012)	91.1 ± 9.3
Centella asiatica (L.) Urb.	CA1314.041	Pegagan	Apiaceae	Whole plant	Elfahmi et al. (2014)	65.9 ± 3.6
Cinnamomum burmanni (Nees & T.Nees) Blume	CB1314.065	Kayu manis	Lauraceae	Bark	Hariana (2006)	98.0 ± 15.8
Cinnamomum cassia (L.) J.Presl	CC1314.015	Kembang lawang	Lauraceae	Fruits	Hariana (2006)	70.8 ± 11.7
Clerodendrum serratum (L.) Moon	CS1314.013	Senggugu	Verbenaceae	Roots	Hariana (2006)	21.8 ± 2.7
Coriandrum sativum L.	CS1314.068	Ketumbar	Apiaceae	Seeds	Rajeshwari et al. (2011)	64.9 ± 26.7
Curcuma aeruginosa Roxb.	CA1314.039	Temu ireng	Zingiberaceae	Rhizomes	Hariana (2006)	63.6 ± 14.6
Curcuma heyneana Valeton & Zijp	CH1314.038	Temu giring	Zingiberaceae	Rhizome	Hariana (2006)	79.6 ± 15.1
Curcuma zanthorrhiza Roxb.	CX1314.040	Temulawak	Zingiberaceae	Rhizome	Elfahmi et al. (2014)	55.8 ± 0.1
Curcuma zedoaria (Christm.) Roscoe	CZ1213.001	Temu putih	Zingiberaceae	Rhizome	Hariana (2006)	95.0 ± 15.2
Cymbopogon citratus (DC.) Stapf	CC1314.053	Sere minyak	Poaceae	Leaves	Hariana (2006)	105.5 ± 27.9
Elephantopus scaber L.	E1113.001	Tapak liman	Asteraceae	Leaves	Hariana (2006)	101.0 ± 32.9
Eleutherine americana (Aubl.) Merr. ex K.Heyne	EA1314.055	Bawang sabrang	Iridaceae	Bulb	Febrinda et al. (2014)	130.9 ± 9.3

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Eugenia aromatica (L.) Baill.	EA1314.050	Cengkeh	Myrtaceae	Leaves	Hariana (2006)	119.1 ± 8.5
Eugenia polyantha Barb. Rodr.	EP1314.056	Daun salam	Myrthaceae	Leaves	Dalimartha (2000)	102.9 ± 21.2
Eurycoma longifolia Jack	EL1314.057	Pasak bumi	Simaroubaceae	Roots	Husen et al. (2004)	223.1 ± 15.7
Foeniculum vulgare Mill.	FW1314.003	Adas-Jogja	Apiaceae	Seeds	Hariana (2006)	12.9 ± 5.3
Guazuma ulmifolia Lam.	GU1314.033	Jati Belanda	Sterculiaceae	Leaves	Elfahmi et al. (2014)	68.2 ± 14.1
Gynura procumbens (Lour.) Merr.	GP1314.058	Sambung nyawa	Asteraceae	Leaves	Wijayakusuma (2006)	64.4 ± 7.2
Helicteres isora L.	HI1314.063	Kayu ulet	Sterculiaceae	Fruits	Hariana (2006)	110.8 ± 6.3
Kaempferia angustifolia Roscoe	KA1314.037	Kunci pepet	Zingiberaceae	Rhizome	Elfahmi et al. (2014)	22.9 ± 0.2
Kaempferia galanga L.	KG1314.059	Kencur	Zingiberaceae	Rhizome	Elfahmi et al. (2014)	89.0 ± 13.6
Leucaena leucocephala (Lam.) de Wit	LL1314.075	Lamtoro	Fabaceae	Fruit	Wijayakusuma (2006)	59.7 ± 19.2
Leucas lavandulifolia Sm.	LL1314.014	Leng-lengan	Labiatae	Whole plant	Hariana (2006)	35.0 ± 6.4
Melaleuca leucadendra (L.) L.	ML1314.024	Merica bolong	Myrtaceae	Fruits	Elfahmi et al. (2014)	46.7 ± 6.7
Melastoma polyanthum Burm. f.	MP1314.019	Senggani	Melastomataceae	Roots	Hariana (2006)	56.5 ± 2.1
Mimosa pigra L.	MP1314.018	Putri malu	Fabaceae	Whole plant	Dalimartha (2000)	42.5 ± 6.8
Momordica charantia L.	MC1314.043	Pare	Cucurbitaceae	Fruits	Wijayakusuma (2006)	79.0 ± 14.6
Morinda citrifolia L.	MC1314.029	Mengkudu	Rubiaceae	Fruits	Wijayakusuma (2006)	53.8 ± 6.9
Moringa oleifera Lam.	MO1314.071	Kelor	Moringaceae	Leaves	Yassa and Tohamy (2014)	128.0 ± 1.7
Myristica fragrans Houtt.	MY1013.001	Pala	Myristicaceae	Fruits	Hariana (2006)	106.1 ± 2.5
Nigella sativa L.	NS1314.028	Jintan hitam	Ranunculaceae	Seeds	Dalimartha (2000)	26.3 ± 5.5
Ocimum gratissimum L.	OG1314.067	Selasih	Lamiaceae	Seeds	Elfahmi et al. (2014)	92.5 ± 24.0
Orthosiphon aristatus (Blume) Miq.	OA1314.061	Kumis kucing	Lamiaceae	Whole plant	Elfahmi et al. (2014)	51.5 ± 2.9
Phaleria macrocarpa (Scheff.) Boerl.	PM1314.034	Mahkota dewa	Thymelaeaceae	Fruits	Wijayakusuma (2006)	86.7 ± 8.7
Phyllanthus urinaria L.	PU1314.012	Meniran	Phyllanthaceae	Whole plant	Dalimartha (2000)	105.5 ± 21.9
Physalis angulata L.	PA1314.32	Ciplukan	Solanaceae	Whole plant	Wijayakusuma (2006)	99.9 ± 32.6
Pimpinella anisum L.	PA1314.045	Adas manis	Apiaceae	Seeds	Rajeshwari et al. (2011)	280.6 ± 0.6
Piper betle L.	PB1314.062	Sirih	Piperaceae	Leaves	Arambewela et al. (2005)	159.6 ± 17.9
Piper crocatum Ruiz & Pav.	PC1314.066	Sirih merah	Piperaceae	Leaves	Safithri and Fahma (2008)	188.5 ± 9.2

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Piper nigrum L.	PN1314.027	Lada hitam	Piperaceae	Fruits	Onyesife et al. (2014a)	212.3 ± 1.2
Plantago major L.	PM1314.072	Daun sendok	Plantaginaceae	Leaves	Wijayakusuma (2006)	106.1 ± 3.5
Ruellia tuberosa L.	RT 1314.001	Ceplikan	Acanthaceae	Fruits	Dalimartha (2000)	57.5 ± 26.0
Swietenia mahagoni (L.) Jacq.	SM1314.020	Mahoni	Meliaceae	Seeds	Elfahmi et al. (2014)	45.5 ± 6.5
Syzygium aromaticum (L.) Merr. & L.M.Perry	SA1314.022	Cengkeh	Myrtaceae	Fruits	Elfahmi et al. (2014)	225.3 ± 0.7
Tamarindus indica L.	TI1314.047	Asam Jawa	Fabaceae	Fruits	Hariana (2006)	13.4 ± 7.5
Terminalia bellirica (Gaertn.) Roxb.	TB1314.009	Joho	Combretaceae	Fruits	Sabu and Kuttan (2002)	276.6 ± 6.4
Tinospora crispa (L.) Hook. f. & Thomson	TC1314.021	Brotowali	Menispermaceae	Bark	Elfahmi et al. (2014)	213.9 ± 5.1
Typhonium flagelliforme (Lodd.) Blume	TF1314.005	Keladi tikus	Araceae	Rhizome	Hariana (2006)	29.3 ± 9.9
Rosiglitazone 1 μM						178.3 ± 10.22

Remarks: Concentration of tested plant extracts: 50 μg/mL. IiGUE, insulin-induced glucose uptake enhancement. *Percentage of values, relative to control. Data are expressed as the mean ± SD (n = 3) and are representative of at least three independent experiments.

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2.3. Plant extraction

The dried parts of the plants were reduced to coarse powder and 5 g per plant of the powder were macerated at ambient temperature with 50% (v/v) aq. methanol for 24 h. Each supernatant was filtered and evaporated by a rotary evaporator (EYELA, Tokyo, Japan) under vacuum at 30°C. Most of the plant extracts generated 10-20% (w/w) yield. Each dried extract was solubilized in 50% (v/v) aq. DMSO to reach tested concentrations.

2.4. Cell culture

The murine 3T3-L1 pre-adipocytes (cell no. JCRB9014) were supplied by the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were seeded into 48-well multiplates (Greiner Bio-one, Tokyo, Japan) and cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin at 37°C in a humidified atmosphere of 10% CO_2. To induce adipocyte differentiation, a day after reaching confluence, the cells were cultured in DMEM supplemented with 10% (v/v) FBS, antibiotics, 0.5 mM IBMX, 0.25 μM DEX, and 5 μg/mL insulin. Two days after the induction, culture medium was replaced with DMEM containing 10% (v/v) FBS, antibiotics, and 5 μg/mL insulin for 4 days to enhance the differentiation. On the sixth day after differentiation induction, medium was refreshed with DMEM containing 10% (v/v) FBS and antibiotics.

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2.5. Insulin-induced glucose uptake-enhancing assay

Based on the results of a preliminary study using the optimum concentration, 50 µg/mL of each plant extract was added to mature adipocytes on day 8, while 1 µM rosiglitazone (a PPARγ ligand) was used as the reference compound. Insulin stimulation and glucose uptake were conducted on day 12, subsequently followed by the enzymatic fluorescence assay. Insulin-induced glucose uptake enhancing assay was performed by following the protocol of Kato and Kawabata (2010) with minor modifications. Figure 1 illustrates the administration of samples and procedure for glucose uptake.

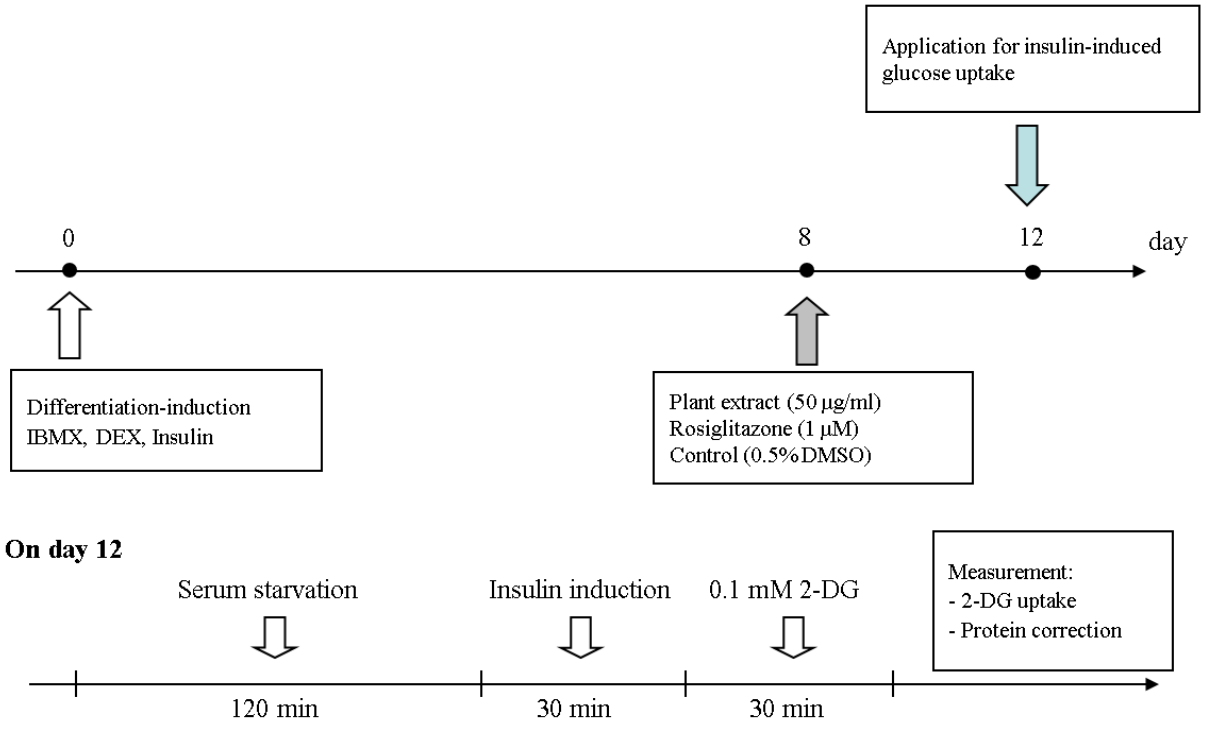


Figure 1. Scheme of the experimental procedure for insulin-induced glucose uptake in 3T3-L1 cells.

For serum starvation, the cells were rinsed twice and incubated in a serum-free DMEM medium for 2 h. After being washed a couple of times, the cells were incubated with or without 100 nM insulin in KRPH buffer for 30 min to perform insulin stimulation. KRPH buffer contained 20 mM HEPES, 136 mM NaCl, 4.7 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$ at pH 7.4. The cells were then incubated with 0.1 mM 2-DG in KRPH buffer for another 30 min. To remove the excess of glucose and halt its uptake, the cells were rinsed with cold KRPH buffer three times. Cytolysis was performed by adding 50 µL of 0.1 M NaOH to each well. Cell lysate was frozen and then heated to 85°C for 45 min. The lysate was neutralized by adding 0.1 M HCl, and TEA buffer (50 mM TEA, 50 mM KCl, 0.5 mM $MgCl_2$, pH 8.1) was subsequently added. The uptake of 2-DG was subsequently measured using an enzymatic fluorescence assay as previously described by Yamamoto et al. (2006).

The enzymatic fluorescence assay cocktail contained 50 µM resazurin sodium salt, 1.3 mM ATP, 20 µM NADP+, 12 U/mL hexokinase, 32 U/mL G6PDH, and 4 U/mL diaphorase in TEA buffer. For the enzymatic reaction, 50 µL of the cell lysate was mixed with 50 µL of the assay cocktail and subsequently incubated at 37ºC for 60 min. Fluorescence excitation and emission were measured at wavelengths of 530 nm and 590 nm, respectively, using a Synergy™ MX microplate reader (Bio-tech Instruments Inc., Winooski, USA). The amount of protein in each well was determined using the Quickstart protein assay, with BSA as the standard protein. Glucose uptake values were then normalized to the protein value of the cell lysate. The insulin-induced glucose uptake was calculated by subtracting the uptake of non insulin-stimulated glucose from that of insulin-stimulated uptake. The glucose uptake level of control cells was set as 100%, while the glucose uptake level of treated cells was calculated relative to the control

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value. If the value is higher than 100%, this indicates an enhancement of glucose uptake after insulin induction and *vice versa*.

2.6. Lipid accumulation assay

For lipid accumulation studies, plant extracts were added to 3T3-L1 cells on day 4 after differentiation induction. The Oil Red O staining assay was performed on day 9, when the 3T3-L1 adipocytes reached maturity, to detect the accumulated lipid droplets. An Oil Red O stock was prepared as previously described by Kuri-Harcuch and Green (1978). First, the cells were rinsed twice with PBS and fixed with 10% (v/v) formalin/PBS for 1 h at ambient temperature. The cells were then washed twice with PBS and stained with the Oil Red O solution (0.36% Oil Red O in 60% (v/v) isopropyl alcohol) for another 30 min. To remove the excess of the Oil Red O solution, the cells were washed three times with PBS. For visualization, the stained lipid droplets were photographed using the Olympus CKX41 microscope (Olympus, Tokyo, Japan). For quantitative analysis, the Oil Red O staining was dissolved with isopropyl alcohol and the optical density (OD) was measured at a wavelength of 520 nm using a Synergy™ MX microplate reader (Tokyo, Japan). The accumulated lipids of control cells were set as 100%, and the lipid contents from the treated cells were calculated relative to the non-treatment value.

2.7. Cytotoxicity assay

The cell viability was tested using the cell counting kit solution (CCK-8) from Dojindo Molecular Laboratories Inc. (Kumamoto, Japan). The solution contained the highly water-

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soluble tetrazolium salt (Ishiyama et al., 1997). The 3T3-L1 pre-adipocytes were plated in 96-well plates (5×10^3 cells per well) and incubated for 24 h. The cells were then incubated with plant extracts for 6, 12, 24, and 48 h at 37°C with 10% CO_2. DMSO (0.5 %) and 1% Triton X-100 served as the negative control and cytotoxic reference, respectively. After treatment, 10 µL of the CCK-8 solution was added to each well, and the 96-well plate was incubated at 37°C for an additional 3 h. The optical density (OD) was measured at a wavelength of 450 nm using the same Synergy™ MX microplate reader (Tokyo, Japan). The cell viability (% of control) was calculated by the following formula:

$$[OD]_{test}/[OD]_{control} \times 100 \quad (1)$$

where $[OD]_{test}$ is the absorbance of the tested extracts, while $[OD]_{control}$ represents the absorbance of the negative control.

2.8. Statistical analysis

The experiments were undertaken at least in triplicate under identical conditions, and the mean value ± standard deviation (SD) of the representative data is presented. Statistical differences were determined using the one-way analysis of variance (ANOVA) followed by the Dunnett's *post hoc* test. P values less than 0.01 were considered significantly different.

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3. Results and Discussion

3.1. Screening of plant extracts for insulin-induced glucose uptake-enhancing activity

Fifty-nine plant extracts were screened for their ability to enhance glucose uptake upon the induction of insulin against fully differentiated 3T3-L1 cells, and their activities are presented in Table 1. Using 3T3-L1 adipose model cells, the insulin-induced glucose uptake-enhancing assay was performed to determine glucose consumption after treatment with the plant extracts. The activity represents the plants' potential to reduce insulin resistance in body tissues, such as adipocytes, resulting in hypoglycemic effects.

Figure 2 depicts the 13 plant extracts that demonstrated insulin-induced glucose uptake-enhancing activity at the concentration of 50 μg/mL, including *Andrographis paniculata* (Burm.f.) Nees (whole plant) (AP), the *Areca catechu* L. (fruits) (AC), the *Caesalpinia sappan* L. (wood) (CS), *Eleutherine americana* (Aubl.) Merr. ex K.Heyne (bulb part) (EA), *Eurycoma longifolia* Jack (root) (EL), *Moringa oleifera* Lam. (leaves) (MO), *Piper betle* L. (leaves) (PB), *Piper crocatum* Ruiz & Pav. (leaves) (PC), *Piper nigrum* L. (fruits) (PN), *Syzygium aromaticum* (L.) Merr. & L.M.Perry (fruits) (SA), *Terminalia bellirica* (Gaertn.) Roxb. (fruits) (TB), *Tinospora crispa* (L.) Hook. f. & Thomson (bark) (TC), and *Pimpinella anisum* L (seeds) (PA). Those plant extracts showed percentages of insulin-induced glucose uptake activity ranging from 126% to 281%, while rosiglitazone displayed 178% relative to control. Among those plants, MA, AP, EA, AC, PB, and CS were less active than rosiglitazone, while PC, PN, TC, EL, SA, TB, and PA exerted a stronger activity than rosiglitazone.

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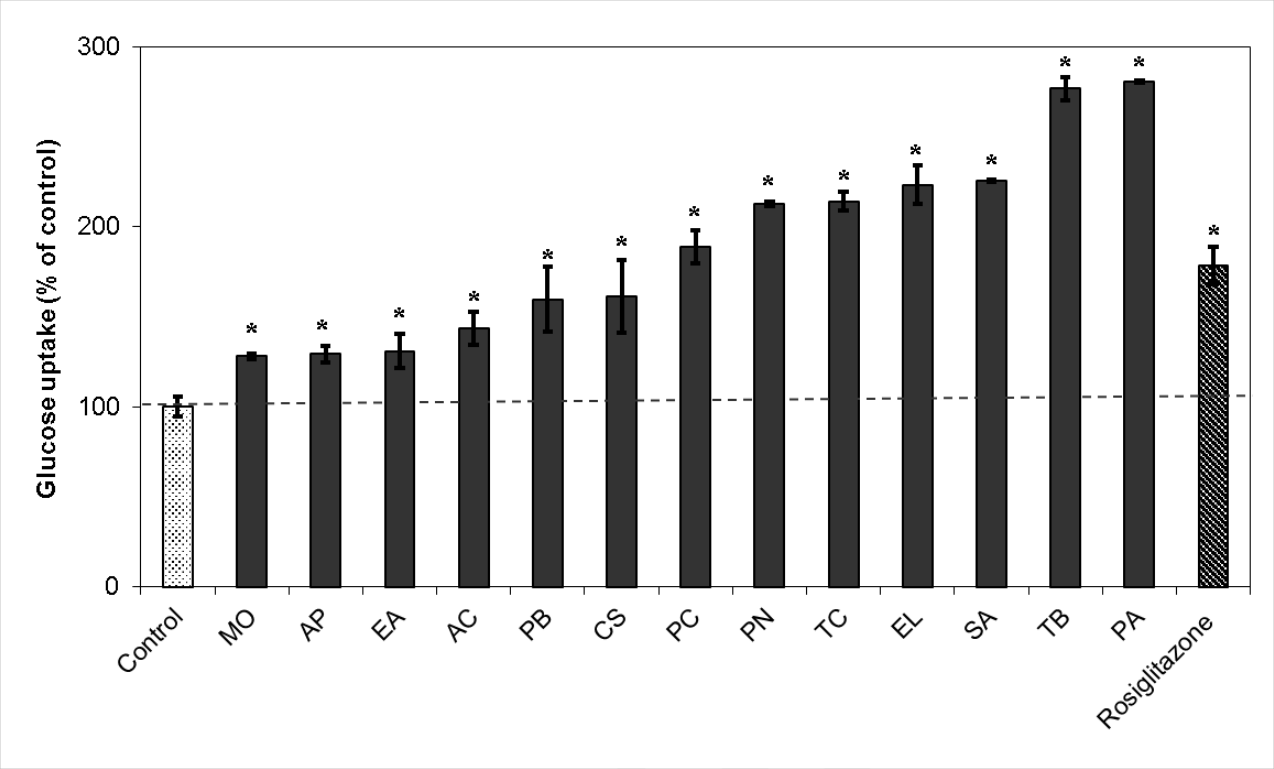


Figure 2. Thirteen Indonesian plant extracts that enhance insulin-induced glucose uptake. Values are normalized to the amount of protein from each well. Data are expressed as the mean ± SD, (n = 5 for AC, PB, CS; n = 3 for the others) and are representative of at least three independent experiments ($^{*}p < 0.01$ versus control).

Those 13 Indonesian medicinal plants have been used traditionally for the treatment of diabetes symptoms. *Andrographis paniculata* (Burm.f.) Nees, *Tinospora crispa* (L.) Hook. f. & Thomson, and *Syzygium aromaticum* (L.) Merr. & L.M.Perry are constituents of anti-diabetic herbal products currently available in the Indonesian market (Elfahmi et al., 2014). The results of this study indicate their efficacy in the treatment of diabetes via the enhancement of insulin sensitivity. Moreover, a number of previous studies have reported the anti-hyperglycemic effects of *Areca catechu* L. (Ghate et al., 2014), *Caesalpinia sappan* L. (Tong et al, 2013), *Eleutherine americana* (Aubl.) Merr. ex K.Heyne (Ieyama et al., 2011), *Moringa oleifera* Lam. (Yassa and Tohamy, 2014), *Piper betle* L. (Arambewela et al., 2005), *Piper crocatum* Ruiz & Pav. (Safithri

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and Fahma, 2008), *Terminalia bellirica* (Gaertn.) Roxb. (Kasabri et al., 2010), and *Pimpinella anisum* L. (Rajeshwari et al., 2011) through various mechanisms of actions.

3.2. Lipid suppression from glucose uptake-enhancing plant extracts

Overproduction of lipids in adipose tissue is considered a sign of dyslipidemia, which could lead to obesity and exacerbate T2DM conditions. For this reason, studies on the regulation of adipogenesis inform the medical community of the best method for treating obesity-associated diabetes. The 13 plant extracts that exerted insulin-induced glucose uptake-enhancing activity were subjected to a lipid accumulation assay to examine their biological effects on the lipid production of adipocytes. Various concentrations of the extracts were added on day 4 after the induction of differentiation, to investigate their effects on intracellular lipid formation (lipogenesis).

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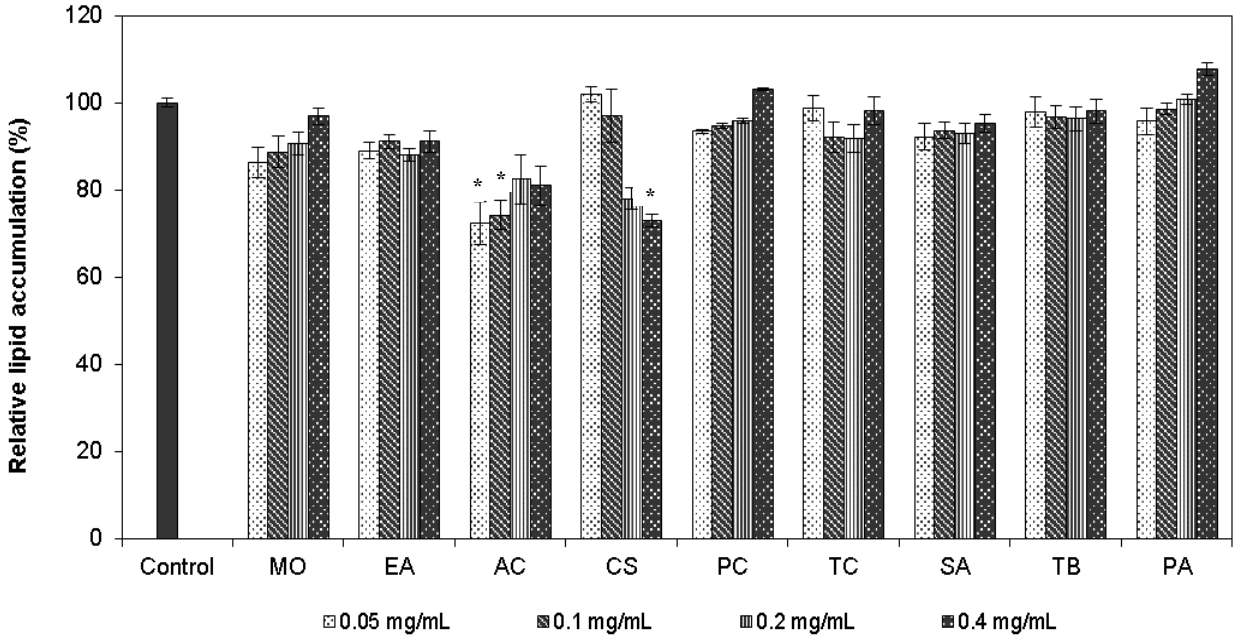


Figure 3. Lipid accumulation in 3T3-L1 adipocytes after treatment with *Moringa oleifera* Lam. (MO), *Eleutherine americana* (Aubl.) Merr. ex K.Heyne (EA), *Areca catechu* L. (AC), *Caesalpinia sappan* L. (CS), *Piper crocatum* Ruiz & Pav. (PC), *Tinospora crispa* (L.) Hook. f. & Thomson (TC), *Syzygium aromaticum* (L.) Merr. & L.M.Perry (SA), *Terminalia bellirica* (Gaertn.) Roxb. (TB), and *Pimpinella anisum* L. (PA). Data are expressed as the mean ± SD (n = 3) and are representative of three independent experiments ($^{*}p < 0.01$ versus control).

Figure 3 shows that 9 out of the 13 glucose uptake-enhancing plants did not demonstrate reduced lipid accumulation in 3T3-L1 adipocytes at concentrations of 0.05, 0.1, 0.2, and 0.4 mg/mL, including *Moringa oleifera* Lam. (leaves), *Eleutherine americana* (Aubl.) Merr. ex K.Heyne (bulb), *Areca catechu* L. (fruits), *Caesalpinia sappan* L. (wood), *Piper crocatum* Ruiz & Pav. (leaves), *Tinospora crispa* (L.) Hook. f. & Thomson (bark), *Syzygium aromaticum* (L.) Merr. & L.M.Perry (fruits), *Terminalia bellirica* (Gaertn.) Roxb. (fruits), and *Pimpinella anisum* L. (seeds).

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That being said, the extracts of *Andrographis paniculata* (Burm.f.) Nees (whole plant), *Eurycoma longifolia* Jack (root), *Piper betle* L. (leaves), and *Piper nigrum* L. (fruits) were selected from the results of the Oil Red O staining, as they exert lipid-lowering activities.

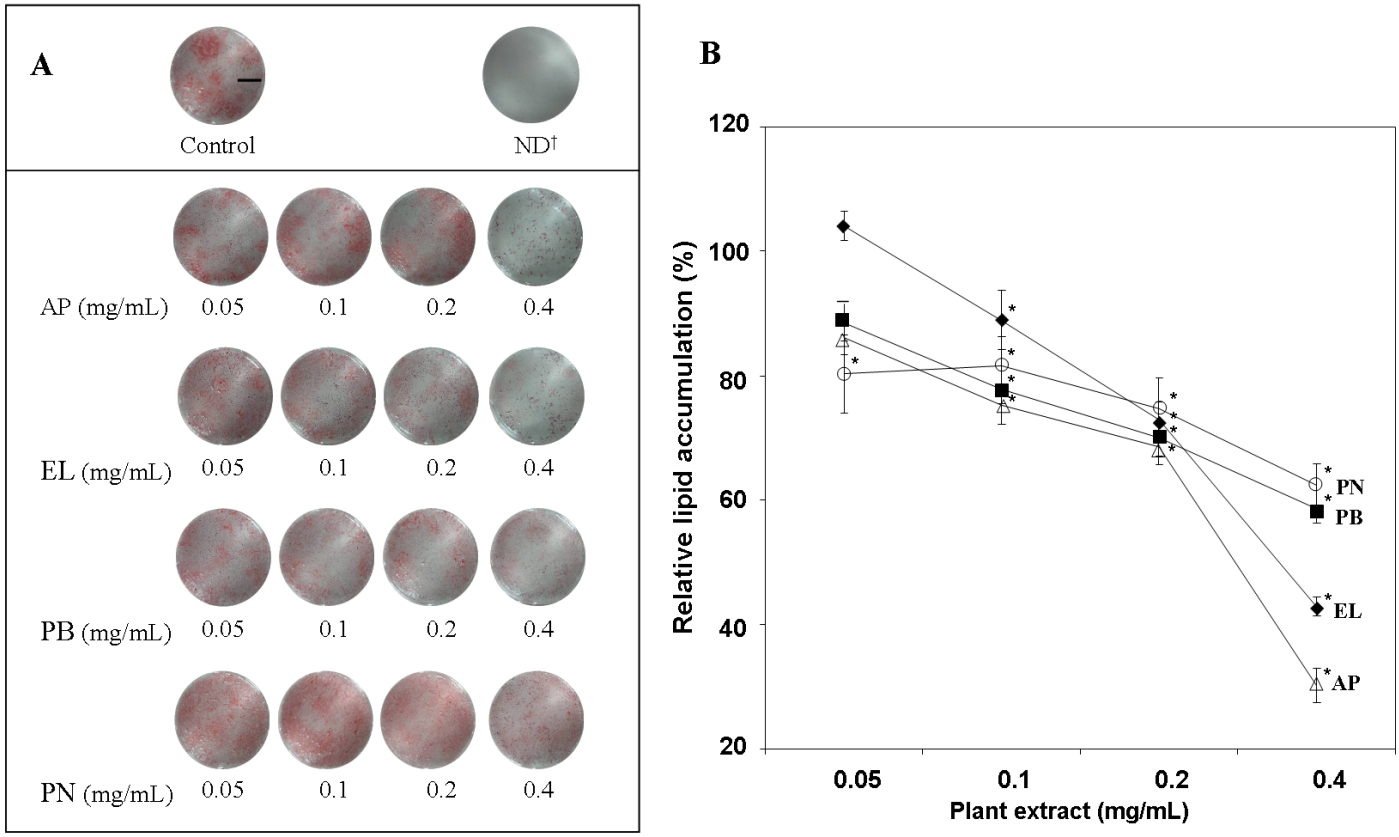


Figure 4. Lipid-suppressing activity of *Andrographis paniculata* (Burm.f.) Nees (AP), *Eurycoma longifolia* Jack (EL), *Piper betle* L. (PB), and *Piper nigrum* L. (PN) on lipogenesis in 3T3-L1 adipocytes. (A) Photograph of Oil Red O-stained lipid droplets. Scale bar corresponds to 3 mm. ND, non-differentiation. (B) Dissolved Oil Red O absorbance was measured at 520 nm. Data are expressed as the mean ± SD (n = 3) and are representative of three independent experiments ($^{*}p < 0.01$ versus control).

Figure 4A presents the Oil Red O-stained lipid droplets of the 3T3-L1 adipocytes after incubation with 4 active plant extracts at varying concentrations. With the increasing concentration of the plant extracts, the intensity of the red color observed was reduced, thus

implying the suppression of lipid droplets. Measurement from the spectrophotometer confirmed that treatment in 3T3-L1 adipocytes with those 4 plant extracts substantially reduced the accumulation of intracellular lipids in a concentration-dependent manner (Figure 4B).

3.3. Cytotoxicity of Andrographis paniculata (Burm.f.) Nees (whole plant)*, Eurycoma longifolia* Jack (root)*, Piper betle* L. (leaves)*,* and *Piper nigrum* L. (fruits) extracts

A viability assay was performed to examine the concentrations at which *Andrographis paniculata* (Burm.f.) Nees, *Eurycoma longifolia* Jack, *Piper betle* L., and *Piper nigrum* L. extracts are cytotoxic in the same 3T3-L1 pre-adipocytes. It is important to examine cytotoxicity when observing potential lipid-lowering activity, in order to distinguish between selectivity of action and cell death. A preliminary study on different incubation times from 6, 12, and 24 to 48 h was undertaken to determine the culture period. Optimum values of cell viability following exposure to samples were obtained from 24 h of incubation. Therefore, subsequent experiments were performed based on 24 h of incubation.

The 3T3-L1 pre-adipocytes were treated with the same concentrations used in the Oil Red O assay (0.05, 0.1, 0.2, and 0.4 mg/mL) for 24 h. As presented in Figure 5A and 5B, *Andrographis paniculata* (Burm.f.) Nees (whole plant) and *Piper betle* L. (leaves) caused a significant decrease in the survival rates ($p < 0.01$ versus control). A reduction in intracellular lipid by those 2 plants was not attributed to the decreasing size and number of lipid droplets. Instead, lipid accumulation levels were mainly reduced because of 3T3-L1 pre-adipocytes cell death. Therefore, the cytotoxicity of *Andrographis paniculata* (Burm.f.) Nees (whole plant) and *Piper betle* L. (leaves) suggests a possible drawback to their development as safe anti-hyperlipidemic agents.

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Figure 5C indicated that the extract of the *Eurycoma longifolia* Jack (root) did not cause cells' death at all tested concentrations (0.05, 0.1, 0.2, and 0.4 mg/mL). Figure 5D shows that treatment of low concentrations of *Piper nigrum* L. (fruits) (0.05, 0.1, and 0.2 mg/mL) caused no decrease in cell viability, although the highest concentration of the extract (0.4 mg/mL) was cytotoxic against 3T3-L1 pre-adipocytes. Consequently, these cell viability assay results confirm that the lipid-lowering activity of *Eurycoma longifolia* Jack (root) and *Piper nigrum* L. (fruits) was not due to cytotoxicity in the 3T3-L1 cells, but in fact both plants decreased the amount of the intracellular lipids.

Given their beneficial effects on glucose uptake, intracellular lipid content, and cell viability, *Eurycoma longifolia* Jack (root) and *Piper nigrum* L. (fruits) are promising sources of anti-diabetic and anti-obesity compounds. The enhancement of glucose uptake after insulin induction by those 2 plant extracts may be mediated by PPARγ modulation, as the receptor is located abundantly in adipose cells (Chen et al., 2013: Richard et al., 2014). Following PPARγ activation, insulin sensitivity increased, leading to hypoglycemic effects. Moreover, the lipid-lowering activity of both plants is speculated to be caused by the inhibition of the transcriptional regulation of lipid synthesis and/or stimulation of lipolysis in 3T3-L1 adipocytes (Kim et al., 2014; Oppi-Williams et al., 2013).

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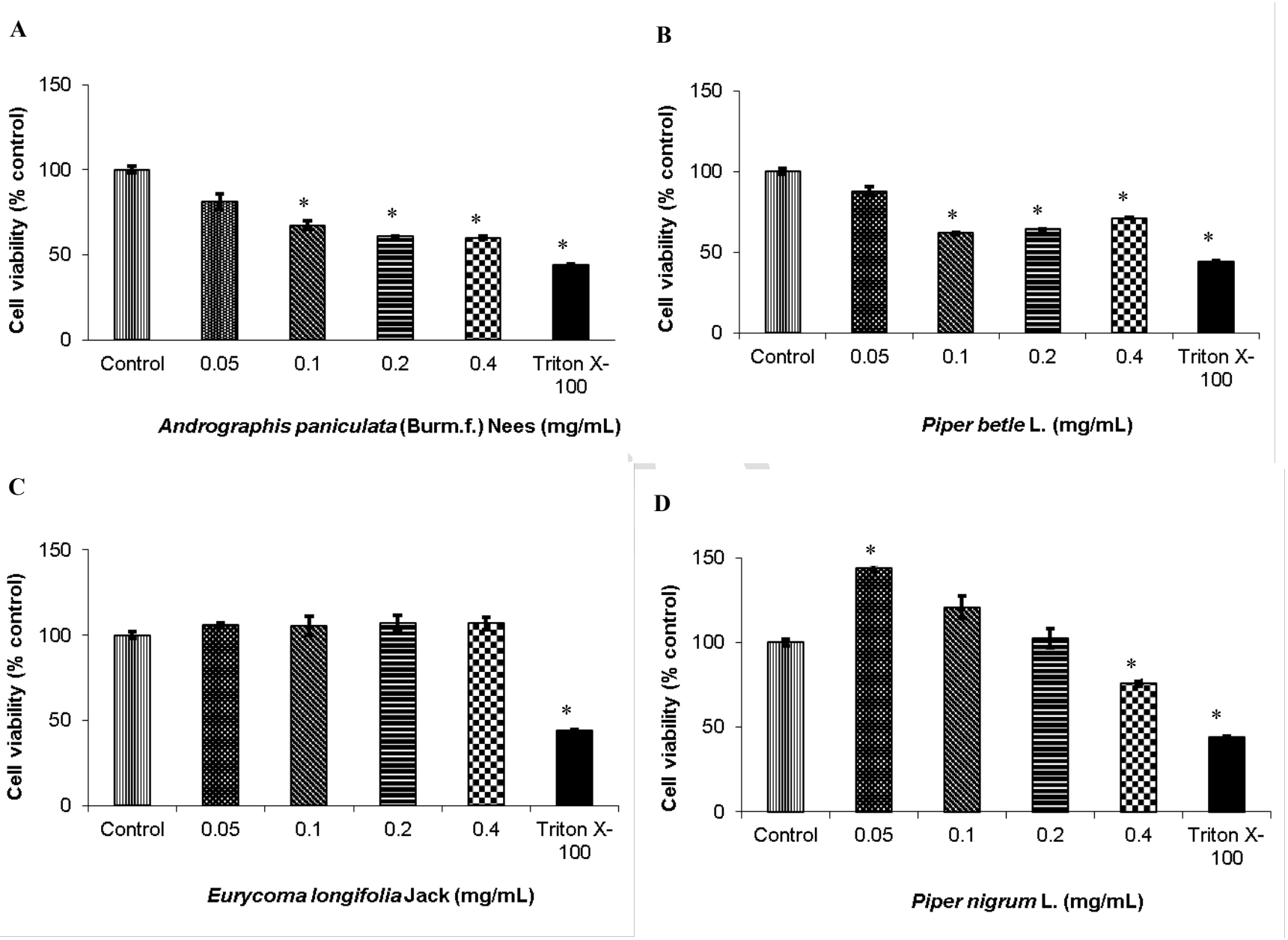


Figure 5. Cell viability of 3T3-L1 pre-adipocytes upon treatment with *Andrographis paniculata* (Burm.f.) Nees (whole plant) (A), *Piper*

betle L. (leaves) (B), *Eurycoma longifolia* Jack (root) (C), and *Piper nigrum* L. (fruits) (D). All data are expressed as the mean ± SD ($n = 3$) and are representative of three independent experiments ($^{*}p < 0.01$ versus control).

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Roots of *Eurycoma longifolia* Jack have been used empirically for erectile dysfunction (ED), one of the complications cause by diabetes. An association between diabetes and ED development has been well-documented, with more than half of diabetic men having experienced ED that led to impotency (Thorve et al., 2011). Moreover, scientific studies have reported the use of *Eurycoma longifolia* Jack for the treatment of sexual dysfunction either *in vivo* in animal models or *in vitro* in human spermatozoa (Ang et al., 2003; Erasmus et al., 2012; Solomon et al., 2014). In this study, the anti-diabetic effect of the same *Eurycoma longifolia* Jack (root) demonstrated enhanced insulin sensitivity in adipocytes. This finding supports the previous study undertaken by Husen et al. (2004), who demonstrated hypoglycemic effects in streptozotocin-induced diabetic rats. In spite of limited scientific research on this particular bioactivity, previous *in vivo* and current *in vitro* works have encouraged further research on its anti-diabetic properties.

A number of studies have also demonstrated the cytotoxic effects of *Eurycoma longifolia* Jack against a wide range of human cancer cell lines, such as human lung, breast, and colon cancer cell lines (Jiwajinda et al., 2002; Kardono et al., 1991; Kuo et al., 2004). However, Nurhanan et al. (2005) reported that this plant displayed no significant cytotoxic effects in the MDBK (kidney) normal cell line, thus indicating a selective cytotoxicity. In the same way, the present study does not demonstrate the death of the 3T3-L1 adipose cells upon treatment. Taking the cytotoxicity experiment into account, the results of the present study confirm the lipid-lowering activity of the *Eurycoma longifolia* Jack (root) extract.

So far, no scientific studies have reported the anti-hyperlipidemic activity of this species. Hence, this is the first work reporting the lipid-suppressing activity of *Eurycoma longifolia* Jack (root) in 3T3-L1 adipocytes. Collectively, the glucose uptake, lipid accumulation, and

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cytotoxicity results demonstrate the potential of *Eurycoma longifolia* Jack (root) as an anti-diabetic agent for the regulation of glucose and lipid metabolism.

In addition to its use as a spice in Indonesian cooking, Indonesian herbal industries include *Piper nigrum* L. (fruits) in the formulation of their herbal products for immunostimulation and the elimination of fatigue along with *Eurycoma longifolia* Jack (root) and other medicinal plants (Elfahmi et al., 2014). The current study highlights the activity of *Piper nigrum* L. (fruits) extract as a potent anti-diabetic activity via its beneficial effect on insulin sensitivity. This *in vitro* work supports the hypoglycemic effects of the plant leaves' ethanolic extract in alloxan-induced diabetic rats (Onyesife et al., 2014a).

In the anti-obesity study, the results demonstrate the lipid-lowering activity of the *Piper nigrum* L. (fruits) extract in a concentration-dependent manner. In agreement with this finding, piperine, a major bioactive molecule isolated from this plant, was found to be able to lower body weight and hepatic lipid levels dose-dependently in high fat-diet mice (Choi et al., 2013). Furthermore, Jwa et al. (2012) and Vijayakumar and Nalini (2006) suggested that piperine improved insulin signaling through thyrogenic activation to modulate apolipoprotein and LXR-α mediated lipogenesis in hyperlipidemic experimental animals.

In their toxicology study, Onyesife et al. (2014b) reported that *Piper nigrum* L. (leaves) extracts of up to 5 g/kg body weight showed no toxicity to the liver and kidney of rats. With respect to the *in vivo* results, this *in vitro* study in 3T3-L1 cells demonstrated that the fruits of *Piper nigrum* L. did not cause cytotoxicity at low concentrations. Cytotoxicity at the highest concentration (0.4 mg/mL), however, suggests that the toxic compounds of the *Piper nigrum* L. (fruits) extract may exert their activities only at high concentrations. Having taken into account the enhancement of insulin sensitivity, the suppression of lipid production, and cytotoxicity, this

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work may facilitate the development of the *Piper nigrum* L. (fruits) extract for the treatment of diabetes and obesity.

4. Conclusions

This study has demonstrated that out of 59 Indonesian medicinal plants, both *Eurycoma longifolia* Jack (root) and *Piper nigrum* L. (fruits) are capable of modulating glucose and lipid metabolism. The two plant extracts doubled the amount of glucose taken up into adipocytes after insulin induction and suppressed lipid production in a concentration-dependent manner in the same cells.

These findings suggest that both of the plants possess unique mechanisms to increase glucose uptake after the induction of insulin and suppress lipid production in 3T3-L1 adipocytes simultaneously. These findings justify the use of *Eurycoma longifolia* Jack and *Piper nigrum* L. in obesity-associated T2DM. Further studies of both plants are being conducted to investigate their cellular mechanisms as well as the isolation of their bioactive constituents.

Conflict of Interests

The authors declare that there are no conflicts of interests.

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References

Ang, H.H., Ngai, T.H., Tan, T.H., 2003. Effects of *Eurycoma longifolia* Jack on sexual qualities in middle aged male rats. Phytomedicine 10, 590–593. doi:10.1078/094471103322331881

Arambewela, L.S.R., Arawwawala, L.D.A.M., Ratnasooriya, W.D., 2005. Antidiabetic activities of aqueous and ethanolic extracts of *Piper betle* leaves in rats. Journal of Ethnopharmacology 102, 239–245. doi:10.1016/j.jep.2005.06.016

Atangwho, I.J., Ebonga, P.E., Eyonga, E.U., Asmawib, M.Z., Ahmad, M., 2012. Synergistic antidiabetic activity of *Vernonia amygdalina* and *Azadirachta indica*: Biochemical effects and possible mechanism. Journal of Ethnopharmacology 141, 878– 887. doi:10.1016/j.jep.2012.03.041

Chen, L., Li, Q.Y., Shi, X.J., Mao, S.L., Du, Y.L., 2013. 6-Hydroxydaidzein enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells. Journal of Agricultural and Food Chemistry 61, 10714–10719. doi:10.1021/jf402694m

Choi, S., Choi, Y., Choi, Y., Kim, S., Jang, J., Park, T., 2013. Piperine reverses high fat diet-induced hepatic steatosis and insulin resistance in mice. Food Chemistry 141, 3627–3635. doi:10.1016/j.foodchem.2013.06.028

Choi, S.S., Cha, B.Y., Lee, Y.S., Yonezawa, T., Teruya, T., Nagai, K., Woo, J.T., 2009. Magnolol enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells. Life Sciences 84, 908–914. doi:10.1016/j.lfs.2009.04.001

Dalimartha, S., 2000. Atlas tumbuhan obat Indonesia,second ed. Trubus Agriwidya, Jakarta. (in Indonesian)

Elfahmi, Woerdenbag, H.J., Kayser, O., 2014. Jamu: Indonesian traditional herbal medicine towards rational phytopharmacological use. Journal of Herbal Medicine 4, 51–73. doi:10.1016/j.hermed.2014.01.002

Pre-print

Erasmus, N., Solomon, M.C., Fortuin, K.A., Henkel, R.R., 2012. Effect of *Eurycoma longifolia* Jack (tongkat ali) extract on human spermatozoa *in vitro*. Andrologia 44, 308–314. doi: 10.1111/j.1439-0272.2012.01282.x.

Febrinda, A. E., Yuliana, N. D., Ridwan, E., Wresdiyati, T., Astawan, M., 2014. Hyperglycemic control and diabetes complication preventive activities of bawang dayak (*Eleutherine palmifolia* L. Merr.) bulbs extracts in alloxan-diabetic rats. International Food Research Journal 21(4), 1405–1411.

Ghate, R., Patil, V.P., Hugar, S., Matha, N.H., Kalyane, N.V., 2014. Antihyperglycemic activity of *Areca catechu* flowers. Asian Pacific Journal Tropical Disease 4 (Suppl 1), S148–S152. doi:10.1016/S2222-1808(14)60430-8

Han, K.L., Ung, H.J., Ohn, H.S., Wang, J.H., 2006. Ginsenoside 20(S)-Protopanaxatriol (PPT) activates peroxisome proliferator-activated receptor γ (PPARγ) in 3T3-L1 Adipocytes. Biological and Pharmaceutical Bulletin 29(1), 110–113. doi.org/10.1248/bpb.29.110

Hariana, A., 2006. Tumbuhan obat dan khasiatnya, second ed. Penebar Swadaya, Jakarta. (in Indonesian)

Husen, R., Pihie, A.H.L., Nallappan, M., 2004. Screening for antihyperglycaemic activity in several local herbs of Malaysia. Journal of Ethnopharmacology 95, 205–208. doi:10.1016/j.jep.2004.07.004

International Diabetes Federation, 2014. IDF Diabetes Atlas, sixth edition 2014 update. Brussels, Belgium.

Ieyama, T., Gunawan-Puteri, M.D., Kawabata, J., 2011. α-glucosidase inhibitors from the bulb of *Eleutherine americana*. Food Biochemistry 128, 308–311. doi:10.1016/j.foodchem.2011.03.021

Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y., Ueno, K., 1997. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta 44, 1299–1305. doi:10.1016/S0039-9140(97)00017-9

Jiwajinda, S., Santisopasri, V., Murakami, A., Kawanaka, M., Sugiyama, H., Gasquet, M., Elias, R., Balansard, G., Ohigashi, H., 2002. *In vitro* anti-tumor promoting and anti-parasitic activities of the quassinoids from E*urycoma longifolia*, a medicinal plant in Southeast Asia. Journal of Ethnopharmacology 82, 55–58. doi:10.1016/S0378-8741(02)00160-5

Juárez-Rojop, I.E., Díaz-Zagoya, J.C., Ble-Castillo, J.L., Miranda-Osorio, P.H., Castell-Rodríguez, A.E., Tovilla-Zárate, C.A., Rodríguez-Hernández, A., Aguilar-Mariscal, H., Ramón-Frías, T., Bermúdez-Ocaña, D.Y., 2012. Hypoglycemic effect of *Carica papaya* leaves in streptozotocin-induced diabetic rats. BMC Complementary and Alternative Medicine 12, 236. doi:10.1186/1472-6882-12-236

Pre-print

Jwa, H., Choi Y, Park, U.H., Um S.J., Yoon S.K., P.T., 2012. Piperine, an LXRα antagonist, protects against hepatic steatosis and improves insulin signaling in mice fed a high-fat diet. Biochemical Pharmacology 84(11), 1501–1510. doi: 10.1016/j.bcp.2012.09.009

Kardono, L.B., Angerhofer, C.K., Tsauri, S., Padmawinata, K., Pezzuto, J.M., Kinghorn, A.D., 1991. Cytotoxic and antimalarial constituents of the roots of *Eurycoma longifolia*. Journal of Natural Products 54(5), 1360–1367.

Kasabri, V., Flatt, P.R., Abdel-Wahab, Y.H.A., 2010. *Terminalia bellirica* stimulates the secretion and action of insulin and inhibits starch digestion and protein glycation *in vitro*. British Journal of Nutrition 103, 212–217. doi: 10.1017/S0007114509991577

Kato, E., Kawabata, J., 2010. Glucose uptake enhancing activity of puerarin and the role of C-glucoside suggested from activity of related compounds. Bioorganic & Medicinal Chemistry Letters 20, 4333–4336. doi: 10.1016/j.bmcl.2010.06.077

Kim, S.O., Sakchaisri, K., Asami, Y., Ryoo, I.J., Choo, S.J., Yoo, I.D., Nak-Kyun Soung, N.K., Kim,Y.S., Jang, J.H., Kim, B.Y., Ahn, J.S., 2014. Illudins C2 and C3 stimulate lipolysis in 3T3-L1 adipocytes and suppress adipogenesis in 3T3-L1 preadipocytes. Journal of Natural Products 77, 744−750. doi: 10.1021/np400520a

Kuo, P.C., Damu, A.G., Lee, K.H., Wu, T.S., 2004. Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*. Bioorganic & Medicinal Chemistry 12, 537–544. doi:10.1016/j.bmc.2003.11.017

Kuri-Harcuch, W., Green, H., 1978. Adipose conversion of 3T3 cells depends on a serum factor. Proceedings of the National Academy of Sciences of the United States of America 75(12), 6107–6109.

Mayerson, A.B., Hundal, R.S., Dufour, S., Lebon, V., Befroy, D., Cline, G.W., Enocksson, S., Inzucchi, S.E., Shulman, G.I., Petersen, K.F., 2002. The Effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. Diabetes 51, 797–802. doi:10.2337/diabetes.51.3.797

NoorShahida, A., Wong, T.W., Choo, C.Y., 2009. Hypoglycemic effect of quassinoids from *Brucea javanica* (L.) Merr (Simaroubaceae) seeds. Journal of Ethnopharmacology, 124, 586–591. doi:10.1016/j.jep.2009.04.058

Nurhanan, M.Y., Azimahtol Hawariah, L.P., Mohd Ilham, A., Mohd Shukri, M.A., 2005. Cytotoxic effects of the root extracts of *Eurycoma longifolia* Jack. Phytotherapy Research 19, 994–996. doi:10.1002/ptr.1759

Onyesife, C.O., Victor, N.O., Emeka, G.A., 2014a. Hypoglycemic potentials of ethanol leaves extract of black pepper (*Piper nigrum*) on alloxan-induced diabetic rats. Annals of Biological Research 5(6), 26–31.

Pre-print

Onyesife, C.O., Victor, N.O., Emeka, G.A., 2014b. Toxicological assessments of *Piper nigrum* on alloxan induced diabetic rats. American-Eurasian Journal of Toxicological Sciences 6(2), 30–34. doi: 10.5829/idosi.aejts.2014.6.2.8490

Oppi-Williams, C., Suagee, J.K., Corl, B.A., 2013. Regulation of lipid synthesis by liver X receptor α and sterol regulatory element-binding protein 1 in mammary epithelial cells. Journal of Dairy Science 96,112–121. doi: 10.3168/jds.2011-5276

Pushparaj, P., Tan, C.H., Tan, B.K.H., 2000. Effects of *Averrhoa bilimbi* leaf extract on blood glucose and lipids in streptozotocin-diabetic rats. Journal of Ethnopharmacology 72, 69–76. doi:10.1016/S0378-8741(00)00200-2

Rajeshwari, U., Shobha, I., Andallu, B., 2011. Comparison of aniseeds and coriander seeds for antidiabetic, hypolipidemic and antioxidant activities. Spatula DD 1(1), 9–16. doi:10.5455/spatula.20110106123144

Richard, A.J., Burris, T.P., Sanchez-Infantes, D., Wang, Y.J., Ribnicky, D.M., Stephens, J.M., 2014. *Artemisia* extracts activate PPARγ, promote adipogenesis, and enhance insulin sensitivity in adipose tissue of obese mice. Nutrition 30, S31–S36. doi:10.1016/j.nut.2014.02.013

Sabu, M.C., Kuttan, R., 2002. Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. Journal of Ethnopharmacology 81, 155–160. doi:10.1016/S0378-8741(02)00034-X

Safithri, M., Fahma, F., 2008. Potency of *Piper crocatum* decoction as an antihiperglycemia in rat strain Sprague dawley. Hayati Journal of Biosciences 15(1), 45-48.

Solomon, M.C., Erasmus, N., Henkel, R.R., 2014. *In vivo* effects of *Eurycoma longifolia* Jack (tongkat ali) extract on reproductive functions in the rat. Andrologia 46, 339–348. doi: 10.1111/and.12082

Thorve, V.S., Kshirsagara, A.D., Vyawaharea, N.S., Joshib, V.S., Ingalea, K.G., Mohitea, R.J., 2011. Diabetes-induced erectile dysfunction: epidemiology, pathophysiology and management. Journal of Diabetes and Its Complications 25, 129–136. doi:10.1016/j.jdiacomp.2010.03.003

Tong, X.Z., Zhu, H., Shi, Y., Xu, H.T., Wang, B., Zhao, J.H., 2013. An LC/MS/MS method for simultaneous quantitation of two homoisoflavones: Protosappanin B and brazilin with hypoglycemic activity in rat plasma and its application to a comparative pharmacokinetic study in normal and streptozotocin-treated rats. Journal of Ethnopharmacology 148, 682–690. doi:10.1016/j.jep.2013.05.029

Vijayakumar, R.S., Nalini, N., 2006. Piperine, an active principle from *Piper nigrum*, modulates hormonal and apo lipoprotein profiles in hyperlipidemic rats. Journal of Basic Clinical Physiology and Pharmacology 17(2), 71–86. doi: 10.1515/JBCPP.2006.17.2.71

Pre-print

Virtanen, K.A., Hallsten, K., Parkkola, R., Janatuinen, T., Lonnqvist, F., Viljanen, T., Ronnemaa, T., Knuuti, J., Huupponen, R., Lonnroth, P., Nuutila, P., 2003. Differential effects of rosiglitazone and metformin on adipose tissue distribution and glucose Uptake in type 2 diabetic subjects. Diabetes 52, 283–290. doi:10.2337/diabetes.52.2.283

Wijayakusuma, H., 2006. Bebas Diabetes Mellitus ala Hembing, third ed. Puspa Swara, Jakarta. (in Indonesian)

Yamamoto, N., Sato, T., Kawasaki, K., Murosaki, S., Yamamoto, Y., 2006. A nonradioisotope, enzymatic assay for 2-deoxyglucose uptake in L6 skeletal muscle cells cultured in a 96-well microplate. Analytical Biochemistry 351, 139–45. doi:10.1016/j.ab.2005.12.011

Yang, Y., Shang, W., Zhou, L., Jiang, B., Jin, H., Chen, M., 2007. Emodin with PPARγ ligand-binding activity promotes adipocyte differentiation and increases glucose uptake in 3T3-Ll cells. Biochemical and Biophysical Research Communications 353, 225–230. doi:10.1016/j.bbrc.2006.11.134

Yassa, H.D., Tohamy, A.F., 2014. Extract of *Moringa oleifera* leaves ameliorates streptozotocin-induced Diabetes mellitus in adult rats. Acta Histochemica 116, 844–854. doi:10.1016/j.acthis.2014.02.002

Pre-print