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The Pharmacognostic Standards, Antioxidant and Antidiabetic Activities, and Hepatic Safety Profile of An Indonesian Antidiabetic Polyherbal Formulation

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ABSTRACT

This study evaluated the physicochemical characters, antioxidant activities, total phenolic content, glucose uptake stimulatory activities, and the hepatic safety of a polyherbal formulation containing seven plant components used by *Klinik Wisata Kesehatan Jamu* Kalibakung, Tegal, Indonesia, to treat diabetic patients. The selected physicochemical parameters of the formulation were characterized according to the WHO quality control methods for herbal materials. Furthermore, the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH RSA), ferric reducing antioxidant power (FRAP), and total phenolic content (TPC) were evaluated in accordance with the standard method. Also, the cytotoxic effects of formulation on the skeletal muscle L6 and hepatic HepG2 cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The glucose uptake stimulatory activity of the formulation in L6 was evaluated using the indirect glucose oxidase method. Meanwhile, the physicochemical properties were specified as follows: foreign matters (1.32±0.05%), loss on drying (11.50±0.07%), total ash (5.68±0.07%), acid-insoluble ash (0.94±0.04%), water-soluble extractable (18.22±0.60%), and ethanol-soluble extractable (16.90±0.77%). The results showed ethanol extract had superior DPPH RSA (960.70±2.58 mM Trolox equivalent (TE)/ g dry weight (DW)), FRAP (1112.69±8.39 mM TE/g DW), and TPC (1768.40±32.40 mg gallic acid equivalent (GAE)/g DW) over its water counterpart. The respective IC_{50} values of water extract on L6 and HepG2 were 209.10±5.45 and 225.17±11.00 µg/mL, while those of ethanol extract were 94.80±1.56 and 40.24±3.53 µg/mL. Compared to the control, the water and ethanol extracts increased glucose uptake by 23.88±5.21 and 55.58±1.24% respectively. This study characterized the physicochemical parameters for an antidiabetic polyherbal formulation with excellent antioxidant and glucose uptake stimulatory activities. Also, its current use as a decoction was safe for the skeletal muscle and hepatic cells.

Keywords: Polyherbal formulation, Pharmacognostic specification, Antioxidant activity, Glucose uptake, Hepatotoxic effect.

INTRODUCTION

Diabetes is one of the three leading causes of non-communicable deaths in Indonesia, with a prevalence in 10.9% adults (Indonesian MoH, 2018). Interestingly, herbal medicines are frequently used by diabetic patients who take them concurrently or alternately with conventional oral antidiabetic agents (Ligita *et al*., 2019). *Jamu pahitan* is a well-known polyherbal formulation used for the traditional management of diabetes with the king of bitter (*Andrographis paniculata* (Burm. f.) Nees) as the main plant component. Several versions of the formulation are included in the science-based *jamu* development program (*program saintifikasi jamu*) and show a good decreasing effect on blood glucose levels in diabetic patients (Rahayu *et al*., 2016; Wijayanti *et al*., 2016). The formulation currently used in *Klinik Wisata Kesehatan Jamu* Kalibakung, Tegal, Indonesia, consists of seven plant components (Table I).

As in general herbal medicines, a polyherbal formulation should be of good quality to maintain therapeutic efficacy and safety. The standardization process is essential to guarantee the quality of a formulation, in which the standards or inherent characteristics, constant parameters, absolute qualitative and quantitative values are specified (Das *et al*., 2019; Mukhi *et al*., 2016). Because there is no standard for the quality of *Jamu pahitan* to date, the values of selected physicochemical parameters of the Tegal-originated formulation were characterized in this study.

Oxidative stress is one of the underlying contributors to the development of chronic complications in diabetes. The reactive oxygen species (ROS) persistently increase while the cellular antioxidant activity significantly decreases under hyperglycemia. These conditions can lead to an oxidative stress that eventually causes endothelial dysfunction, insulin resistance, and negative changes in pancreatic β cell functions (Bandeira *et al*., 2013). This study investigated the formulation's antioxidant properties by using DPPH RSA and FRAP assays. The antioxidant activity can be attributed to several compounds present in the extract, particularly phenolic ones. Therefore, the TPC in the extracts was also analyzed.

Being included in the science-based *jamu* development program enables the efficacy and safety profile evaluation of the formulation on humans. However, it is impossible to understand all the exact mechanisms of pharmacological actions through the studies in the program only, hence, additional *in-vitro* evaluations are needed. The hyperglycemia in diabetes resulted from eight pathophysiological processes, with the decreased glucose uptake in peripheral tissues as one of the most important contributing factors (DeFronzo, 2009). The *in-vitro* model of glucose uptake stimulatory activity of the formulation extracts was evaluated on the skeletal muscle L6 cell lines in this study.

A long-term intake of oral hypoglycemic medications is required to manage diabetes (Chawla *et al*., 2020), which is similarly applied for herbal medicines. The doctors at *Klinik Wisata Kesehatan Jamu* Kalibakung prescribe the formulation for four weeks, and the treatment may proceed as needed. However, the prolonged use of herbal medicines is associated with herb-induced liver injury (HILI) (Lin *et al*., 2019). Therefore, this study evaluated the formulation's safety to the liver by utilizing hepatic HepG2 cell lines as the model to predict the hepatotoxic effects. Since the taste of decoction of this formulation is inappreciably bitter, both water and ethanol extracts were assessed to find a more convenient preparation with a better efficacy and safety profile.

MATERIAL AND METHODS

Sample

The crude drugs of the antidiabetic polyherbal formulation were purchased from *Klinik Wisata Kesehatan Jamu* Kalibakung, Tegal, Indonesia, which is a clinic under the science-based *jamu* development program network. The formulation was a mixture of seven plant components crude drugs (Table I) and was packed in a plastic bag per daily dose.

The physicochemical character determination

The shape, color, odor, and taste of the crude drugs were organoleptically observed. Foreign matter, loss on drying, total ash, acid-insoluble ash, water-soluble extractable, and ethanol-soluble extractable of the powdered crude drugs were determined according to the WHO method (WHO, 2011).

Preparation of extracts

The powdered crude drugs were boiled in the water at a ratio of 1:100 (w/v) for an hour. The filtrate was further evaporated over the water bath to dryness to obtain the water extract (Habibie *et al*., 2017).

Table I. The composition of the antidiabetic polyherbal formulation

Plant name	Scientific name	Family	Part used	Weight (g)
King of bitter, *sambiloto*	*Andrographis paniculata* (Burm.f.) Nees	Acanthaceae	Aerial parts	5
Java tea, *kumis kucing*	*Orthosiphon aristatus* (Blume) Miq.	Lamiaceae	Aerial parts	3
Indonesian bay leaf, *salam*	*Syzygium polyanthum* (Wight) Walp.	Myrtaceae	Leaves	5
Indonesian cinnamon, *kayu manis jangan*	*Cinnamomum verum* J.Presl	Lauraceae	Barks	7
Javanese turmeric, *temulawak*,	*Curcuma zanthorrhiza* Roxb.	Zingiberaceae	Rhizomes	15
Turmeric, *kunyit*	*Curcuma longa* L.	Zingiberaceae	Rhizomes	10
Seed-under-leaf, *meniran*	*Phyllanthus niruri* L.	Phyllantaceae	Aerial parts	5

Additionally, the powdered crude drugs were extracted under ultra-sonification in the absolute ethanol with a ratio of 1:100 (w/v) for an hour. After filtration, the filtrate was evaporated to dryness in a rotary evaporator to obtain the ethanol extract (Chewchinda *et al*., 2018).

The *in-vitro* antioxidant activities and total phenolic content analysis

The DPPH RSA assay was carried out by mixing the extracts, in a concentration of 10 mg/mL in their respective solvent, with a 0.025 mg/mL DPPH methanolic solution at a ratio of 1:10 (v/v). Furthermore, the absorbance of the mixture was read at a wavelength of 517 nm using a UV – visible spectrophotometer (Thermo Scientific, USA) after storing at room temperature and protected from light for 30 mins. The DPPH RSA was presented as mM Trolox equivalent (TE)/g dry weight (DW) crude drugs (Thaipong *et al*., 2006).

The FRAP was determined by mixing extracts, in a concentration of 10 mg/mL in their respective solvent, with FRAP reagent in a ratio of 1:19 (v/v). The absorbance of the mixture was read at 594 nm after storing at room temperature for 30 mins. Additionally, the FRAP was reported as mM TE/g DW crude drugs (Frond *et al*., 2019).

TPC was determined by mixing the extracts, in a concentration of 10 mg/mL in their respective solvent, with water and a Folin-Ciocalteau reagent (MilliporeSigma) in a ratio of 1:79:5 (v/v/v). Fifteen parts of saturated sodium carbonate were further added to each mixture after 5 mins. The absorbance of the final mix was read at 764 nm after standing at room temperature for two hours. Subsequently, the TPC was reported as mg Gallic acid equivalents (GAE)/g DW crude drugs (Tuekaew *et al*., 2014).

Cell lines

The cell lines used in this study included L6 skeletal muscle cells (CRL-1458™, passage number of 27-33) and HepG2 hepatic cells (HB-8065™, passage number of 33-35) that were purchased from ATCC (US). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) respectively. All maintaining media were supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P-S). An incubator (Thermo Scientific, US) was used to examine all the cell incubations at a temperature of 37°C and under 5% CO_2. In addition, all cell culture supplies were purchased from Thermo Fisher Scientific (US).

The *in-vitro* glucose uptake assay

The MTT reduction assay was used to evaluate the cytotoxic effects of the formulation extracts on L6 as previously reported (Inthongkaew *et al*., 2017). At a density of $2x10^4$ cells/well, the cells were cultured in DMEM enriched with 10% FBS and 1% P-S and incubated for 24 hours at a working volume of 100 µl. They were further incubated in DMEM containing ethanol and water extracts of the formulation at concentrations of 25, 50, 100, and 200 µg/mL for 24 hours. Furthermore, the DMEM containing 0.5% DMSO was used as the control. MTT Assay Kit (Abcam, UK) was used to evaluate the viability of L6 treated with the extracts by following the manufacturer's protocol. The absorbance was read in a microplate reader (BMG LABTECH, Germany) at a wavelength of 590 nm. In addition, the concentrations exerting 50% of cell viability (IC_{50}) of each extract were mathematically calculated from the concentration-viability curve accordingly.

Figure 1. The macroscopic observation (A) and the physicochemical properties values (B) of the crude drugs of the antidiabetic polyherbal formulation from Tegal

For the evaluation of glucose uptake, L6 myoblasts were seeded at a density of 2×10^4 cells/well in DMEM supplemented with 10% FBS and 1% P-S in a working volume of 500 µl. The cells were further maintained in DMEM with 2% FBS and 1% P-S as the differentiate media after reaching 90% confluence and were allowed to differentiate into myotubes for at least seven days, with media changing every other day. The myotubes were incubated in differentiated media containing water and ethanol extracts of the formulation at a concentration of 20 µg/mL for 24 hours. Insulin (at 100 nM; Eli Lilly, US) and differentiate media (with 0.5% DMSO) functioned as the positive and control group respectively. The media was collected, and the volume was corrected to 500 µl with sterile water. Glucose Assay Kit (Abcam, UK) was used to analyze the glucose level in the media following the manufacturer's protocol. Also, the absorbance was measured at a wavelength of 570 nm. The glucose uptake in cells treated with each extract concentration was calculated by subtracting the final glucose level from their initial counterparts. Subsequently, the glucose uptake stimulatory activity of each sample was relatively calculated as the percentage of the control group (Inthongkaew *et al*., 2017).

The hepatotoxic effect evaluation

The MTT reduction assay to evaluate the hepatotoxic effects of the formulation extracts on HepG2 cells was conducted in the same manner, as L6 cells using a similar cell density of $2x10^4$ cells/well. In this assay, DMEM/F-12 was used instead of DMEM as the media. The tested concentrations were 6.25, 12.5, 25, 50, and 100 µg/mL (Siddiqui *et al*., 2019).

Data analysis

Data were presented as the mean value ± standard error of the mean (SEM). One-way ANOVA and Duncan test were used to compare the cell viability between concentrations of each extract on the respective cell line. Also, the independent-samples t-test was used to compare DPPH RSA, FRAP, TPC, and IC_{50} on the respective cell line of each extract. The correlation between TPC - antioxidant activities and TPC - IC_{50} on each cell line of extracts was calculated by the Pearson correlation test. The difference was considered significant at a p-value <0.05, and IBM SPSS Statistics v.13 (IBM, USA) was used to conduct all statistical analyses.

RESULT AND DISCUSSION

The presence of crude drugs of Javanese turmeric, Java tea, turmeric, as well as Indonesian cinnamon and bay leaves was readily observed in the mixture (Figure 1). The bay leaves were elliptical laminae brown, had a pleasant odor, and slightly bitter with astringent effects. Meanwhile, Javanese turmeric had thin but hard fragments of primary rhizomes in lanceolate shaped, external light brown color and somewhat yellowish cortex, with a distinctive aromatic solid odor and slightly bitter taste. The turmeric appeared as thin fragments of rhizomes with internal brown color and specific internal orange color as well as a fragrant, pungent aroma, overwhelmingly earthy and bitter taste. Also, the Java tea was entire and broken, dark green, ovate-lanceolate leaves with a slightly aromatic odor, somewhat bitter, salty, and had an astringent taste. The Indonesian cinnamon appeared as barks in brown color, delicate, fragrant, woody, with a spicy aroma and arm sweet flavor.

King of bitter was supposed to be dark green lanceolate leaves with quadrangular stems, have a slightly specific odor, and extremely bitter to taste. The seed-under-leaf should appear as small roundish leaves and stalks in brownish-green with aromatic odor and bitter taste. However, both were not visibly distinguishable from the tiny fragments of other leafy crude drugs.

This study evaluated the crude drugs' physicochemical properties of the antidiabetic polyherbal formulation currently used in the science-based *jamu* development program for preliminary characterization of its quality (Figure 1). The quality of crude drugs can be defined by three aspects, namely identity, purity, and content (Alamgir, 2017). Furthermore, the entire plant components of the formulation are included in the Indonesian Herbal Pharmacopeia (IHP) 2017. Therefore, the obtained values of all formulation parameters were compared with the range of their respective standard for the individual components (Indonesian MoH, 2017). Although IHP 2017 does not require foreign matter specifications, it is an important parameter to define a crude drug's identity and purity aspects. The American Herbal Products Association (AHPA) set the limit for the foreign matter for at least 2-5%, depending on tested crude drugs' nature (AHPA, 2017). Hence, the crude drugs of the formulation met the criteria set by AHPA.

The loss on drying, total ash, and acid-insoluble ash specified the purity aspects of the drugs. The loss on drying is correlated with the high moisture in the drugs, which is associated with bioactive decomposition and microbial growth risks (Agarwal *et al*., 2014). The acceptable loss on drying for the crude drugs of all plant components in the formulation was not more than 10% (Indonesian MoH, 2017). Therefore, the loss on drying was higher than the range of the plant components. The moisture in the drugs might have originated from the incomplete drying process or absorbed from the air during transport and storage. The latter was likely the cause of the high moisture as it was prepared and packed in Tegal, Indonesia before transporting to Bangkok, Thailand. The ash contents represent the inorganic impurities in the drugs, which might occur naturally in the plant or acquired from the environment during production (Tauheed *et al*., 2017). The total ash for the crude drugs of the plant components specified in IHP 2017 ranged from 2.5% (Indonesian bay leaf) to 10.2% (king of bitter). Hence, the total ash of the formulation was within the range of its plant components. Similar trend was observed in the acid-insoluble ash, where the range of standard for the plant components was not more than 0.3% (Indonesian cinnamon) - 3.4% (Java tea).

The ethanol- and water-soluble extractable specified the content aspect of crude drug's quality, which can be mainly utilized when the chemical or biological content evaluation is unavailable (Alamgir, 2017). The water-soluble extractable of the formulation was within the range of its plant components' standard, with at least 4.0% (Indonesian cinnamon) – 20.3% (turmeric). Meanwhile, the ethanol-soluble extractable of the plant components specified in IHP 2107 ranged from 3.6% (Javanese turmeric) to 19.7% (Indonesian bay leaf). Therefore, the same pattern was observed in both parameters. The higher value of the water-soluble extractable than the ethanol's indicated that most bioactive compounds in the formulation had higher polarity.

Klinik Wisata Kesehatan Jamu Kalibakung prescribed 50 g of the formulation to be extracted in water by decoction method. The obtained extract was divided into three doses and orally taken for a day. The water extract in this study represented the actual use of the formulation, while ethanol extract was evaluated to determine whether it exerts a better efficacy and safety profile. Furthermore, the water extract generated a much higher extraction yield (34.24%) than its ethanol counterpart (11.25%). The yield of extraction for herbal materials was widely varied, with solvent and method of extraction as the defining factors. The higher yield of water extract represented the presence of a high fraction of polar compounds in the formulation. This was also observed in a Sri Lankan ayurvedic polyherbal formulation indicated for treating inflammation and a polyherbal nutritional supplement for horses (Cecchini *et al*., 2014; Wakkumbura *et al*., 2020). The decoction method used for preparing water extract generated a higher extraction yield than its ethanol counterpart prepared by an ultrasonic-assisted method, this is similar to a report on ironwort crude drugs (Celep *et al*., 2019).

The ethanol extract showed a statistically better DPPH RSA and FRAP than water (Table 2). Similar to this study, the superior DPPH RSA and FRAP of the ultrasonic-assisted extraction-derived extracts over their decoction-originated counterparts were reported in beleric and horsemint respectively (Dharmaratne *et al*., 2018; Patonay *et al*., 2019). A better DPPH RSA of ethanol

extract than water was observed in a Sri Lankan antiinflammatory polyherbal formulation. The same trend of FRAP was reported in a traditional Thai tonifying formulation (Wakkumbura *et al*., 2020; Wetchakul *et al*., 2019).

The available data suggested that all plant components of the formulation were responsible for the antioxidant capacity except king of bitter and Javanese turmeric. The IC_{50} value of the DPPH RSA of Indonesian cinnamon water extracts and the seed-under-leaf phenolic compound-enrich extract was 3.45- 20.53 and 6.40 µg/mL respectively. On the other hand, the Indonesian bay leaf ethanol extract scavenged DPPH by half at 437.89 ppm and showed ferric reduction capacity equivalent to 1 mM Fe_2SO_4 at 684.00 ppm. Furthermore, turmeric water and ethanol extracts showed respective DPPH RSA and FRAP of 5.31 and 1.08 µg/mL as well as 646.67 and 3475.36 µM Fe^{2+}/100 g sample (Ervina *et al*., 2019; Hartanti *et al*., 2020; Navarro *et al*., 2017; Tanvir *et al*., 2017). A Malaysian study reported that Indonesian cinnamon (IC_{50} = 11.03 µg/mL) showed the highest DPPH RSA, subsequently followed by bay leaf (IC_{50} = 15.48 µg/mL) and Java tea (IC_{50} = 53.51 µg/mL), while king of bitter (IC_{50} = 143.7 µg/mL) and Javanese turmeric (IC_{50} = 326.3 µg/mL) weakly scavenged DPPH (Ismail *et al*., 2017). Therefore, the high DPPH RSA and FRAP of the formulation can be attributable to the plant components with their antioxidant property. Also, the presence of more bioactive compounds from each plant component in a polyherbal formulation enables the polyvalence effect, which can increase the overall therapeutic effect or lessen the toxicity compared to the single herbal preparation (Houghton, 2009).

Table II. The DPPH RSA, FRAP, and TPC of the water and ethanol extracts of the formulation and their correlation

Extracts	Water extract	Ethanol extract
TPC (mg GAE/g DW)	85.08±1.85	1768.40±32.40*
DPPH (mM TE/g DW)	263.60±5.23	960.70±2.58*
FRAP (mM TE/g DW)	121.17±2.25	1112.69±8.39*

The asterisk (*) indicated the statistically higher value between water and ethanol extracts in a given parameter at p-value < 0.05 (n=3).

The ethanol extract formulation obtained from ultrasonic-assisted extraction contained a higher level of phenolic compounds than its water counterpart (Table II). The extracted phenolic compounds of a herbal material depended on the time, temperature, and solvent of extraction (Casagrande *et al*., 2018). This result is similar to a report on the effect of the extraction method on the TPC of Asiatic pennywort and studies on the polyherbal formulation in Thailand and Sri Lanka (Wakkumbura *et al*., 2020; Wetchakul *et al*., 2019; Zainal *et al*., 2019).

Indonesian cinnamon, seed-under-leaf, and turmeric were likely responsible for the high level of TPC in the formulation. The TPC in Indonesian cinnamon water extracts varied between 105.71-259.08 mg GAE/g sample, while that of seed-under-leaf ethanol extract was 328.80 mg GAE/g sample (Ervina *et al*., 2019; Navarro *et al*., 2017). Turmeric water and ethanol extracts of different varieties from Srilanka contained a high level of phenolic compounds with TPC of 45.2-76.8 and 661.5-160.7 mg GAE/g sample (Tanvir *et al*., 2017). On the other hand, a low level of phenolic compounds was detected in Indonesian bay leaf, Java tea, king of bitter, and Javanese turmeric, with TPC of 2.19, 0,95, 0,21, and 0.20 mg GAE/g sample respectively (Ismail *et al*., 2017).

Both water and ethanol extracts of the formulation showed a significant proliferation stimulatory activity on L6 at 25 µg/mL. Therefore, the formulation extract stimulated regeneration of the muscle cells at a lower concentration. This phenomenon can be beneficial in the treatment of diabetes, as the more skeletal muscle cells available will utilize more glucose in the blood, and consequently reduce the blood glucose level as previously reported in the adipose cells (Smith & Kahn, 2016). The water extracts exerted toxic effects at 200 µg/mL, when its ethanol counterpart was already toxic at 50 µg/mL (Figure 2). Hence, the IC_{50} of water extract was significantly higher than the ethanol's, with the respective value of 209.10±5.45 and 94.80±1.56 µg/mL. The data on the cytotoxic effect of plant components for the formulation on L6 was limited. Deoxyandrographolide isolated from king of bitter at 20 mM did not cause any toxic effects on the cells (Arha *et al*., 2015). Conversely, cinnamon ethanolic-water and turmeric ethyl acetate extracts were toxic at a concentration higher than 100 µg/mL (Kadan *et al*., 2013; Prathapan *et al*., 2012).

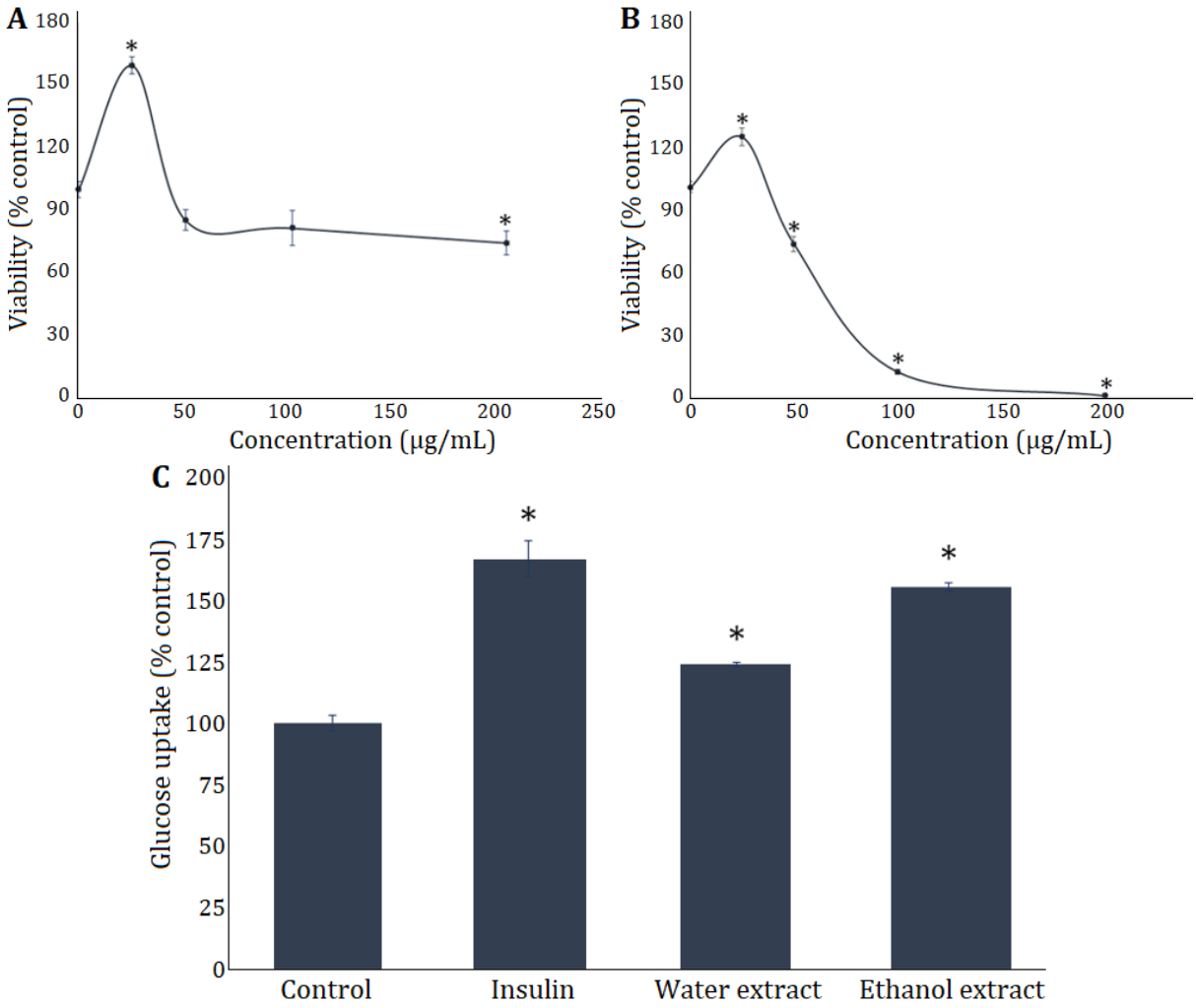

Figure 2. The cytotoxic effect and glucose uptake stimulatory activity of the formulation extract on L6 cells showed the viability of the cells treated by the water (A) and ethanol (B) extracts of the formulation as well as the glucose uptake stimulation (C). The asterisk (*) indicated a statistically different value on each point/bar compared to the control. All statistical evaluations were conducted at p-value <0.05 (n = 3).

The glucose uptake stimulatory activity of the formulation was evaluated in the skeletal muscle cells that play a crucial role in postprandial glucose control and are one of the main target sites for insulin action (DeFronzo & Tripathy, 2009). This analysis was carried out at the safe concentration (20 µg/mL) of both extracts. The assay was conducted on the L6 myotubes for a minimum of 7-day culture in the differentiate media with 2% FBS. Compared to the control, insulin increased glucose uptake in the cells by 66.92±0.23%, while the water and ethanol extract increased by 23.88±5.21 and 55.58±1.24% respectively. Compared to insulin, the respective efficacy of the extracts was 74.21 and 93.21%. The ethanol extract significantly stimulated glucose uptake to the cells better than its water counterpart, with the statistically comparable efficacy to the insulin (Figure 2). The stimulatory activity of the formulation can be attributable to the king of bitter, Java tea, and turmeric. Deoxyandrographolide isolated from king of bitter enhanced glucose uptake in L6 with the presence of insulin. Furthermore, andrographolide and 50% methanol extract of the plant component showed glucose uptake stimulatory activity in C2C12 myoblast cells and 3T3-L1 adipocytes respectively (Arha *et al.*, 2015; Hsu *et al.*, 2004; Lahrita *et al.*, 2015).

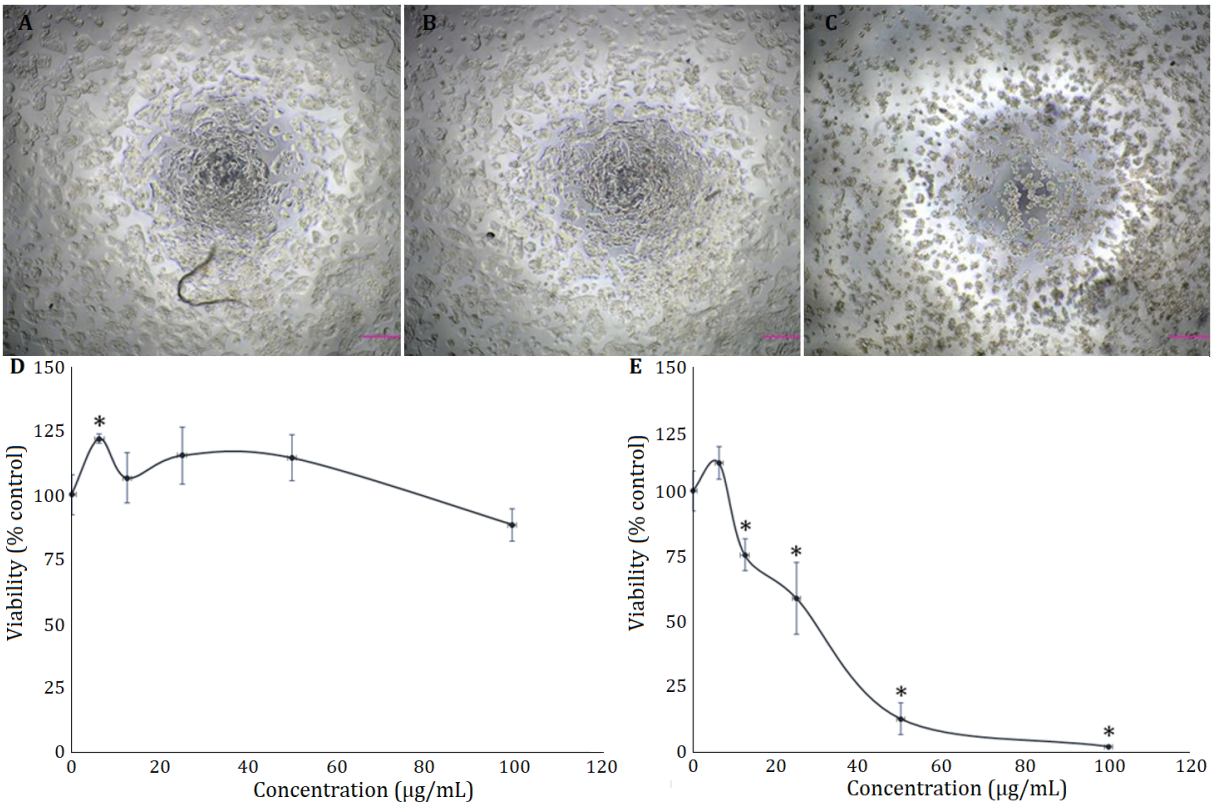

Figure 3. The profile of potential hepatotoxic effects of the formulation on HepG2. The comparison of cells in control (A) and those treated with 50 µg/mL of water (B) and ethanol (C) extracts of the formulation, the scale bar is 500 µm, as well as the viability profile of HepG2 treated by the water (D) and ethanol (E) extracts. The asterisk (*) indicated the statistically different viability compared to the control, evaluated at p-value <0.05 (n = 3).

Conversely, Java tea enhanced glucose uptake in INS-1 cells by the mechanism of phosphatidylinositol 3-kinase activation (Lee *et al*., 2015). Subsequently, turmeric can contribute to the glucose uptake stimulatory of the formulation as the ethyl acetate extract and curcumin enhance glucose uptake in L6 cells (Kim *et al*., 2010; Na *et al*., 2011). The enhancement of glucose uptake in a skeletal muscle cell line was reported as the antidiabetic mechanism of other polyherbal formulations, particularly those developed from Ayurvedic original recipes (Perumal *et al*., 2022; Telapolu *et al*., 2018).

The cytotoxic activity of the formulation was evaluated on HepG2, a cell line known for its suitability to differentiate the hepatotoxicants from hepato-safe agents (Van-den-Hof *et al*., 2014). A similar growth profile of HepG2 was observed in the control and those treated with the formulation water extract at 50 µg/mL. On the other hand, sparser growth and somehow fragmented cells were observed in those treated with the ethanol extracts at the same concentration. The water extracts stimulated cell proliferation at 6.25 µg/mL, and all the tested concentrations were safe. However, the ethanol extract was already toxic at 12.5 µg/mL. The IC_{50} of the water extract was higher than the ethanol's, with 218.25±14.03 and 40.24±3.53 µg/mL (Figure 3).

The relatively nontoxic effects of water extract suggested that its recommendation as a decoction is safe for the hepatic tissue. This can be attributed to the safety of the plant constituents. The IC_{50} value for king of bitter and Java tea water extracts on HepG2 was >250 µg/mL (Chassagne *et al*., 2018). However, the water extract of seed-under-leaf induced apoptosis on the HepG2 cells (Júnior *et al*., 2012). The higher toxic effects of the ethanol extract over its water counterparts were also consistent with the previous studies.

Table III. Correlation between TPC, DPPH RSA, FRAP, and cytotoxicity of the formulation extracts

Extract	R-value between TPC and			
	DPPH RSA	**FRAP**	**IC_{50} on L6**	**IC_{50} on HepG2**
Water extract	0.936	0.885	0.999**	0.999**
Ethanol extract	0.935	0.919	0.999**	0.999**

R = Pearson's correlation coefficient. The double asterisks (**) indicated significant correlation at $p < 0.01$ ($n = 3$)

The ethanol extracts of the king of bitter and Java tea with some of their fractions were toxic to HepG2 (Chassagne *et al*., 2018). The highly toxic effects of ethanol extract can also be attributed to turmeric. Curcumin is better extracted in ethanol and resulted in the IC_{50} value of 6.38 µg/mL on the same cells (Yang *et al*., 2020).

Strong positive correlations were observed between TPC and DPPH RSA, TPC and FRAP, TPC and IC_{50} on L6, as well as TPC and IC_{50} on HepG2 in water and ethanol extracts. Furthermore, there was a significant correlation between TPC and IC_{50} on both cells (Table 3). Therefore, the phenolic compounds were likely responsible for the free radical scavenging and reduction capacities of the formulation, as well as the amelioration in toxic effects on both cell lines. DPPH assay quantified the tested compounds' ability to transfer hydrogen atoms and single electrons to scavenge the free radical. In contrast, the FRAP assay quantified the single electron transfer during ferric reduction to ferrous. The structure of phenolic compounds, such as the aromatic ring and the double bonds, likely facilitated both mechanisms (Craft *et al*., 2012; Pisoschi *et al*., 2016; Santos-Sánchez *et al*., 2019). The same trend was shown in the extracts for all plant components of the formulation as king of bitter, Javanese turmeric, Java tea, turmeric, Indonesian cinnamon, Indonesian bay leaf, and seed-under-leaf demonstrated strong positive correlations between TPC and their antioxidant activities (Ismail *et al*., 2017; Sepahpour *et al*., 2018; Wijewardhana *et al*., 2019; Yahaya *et al*., 2019). Similarly, the strongly correlated TPC in the polyherbal formulations with their antioxidant capacities was reported in Thai recipe for nausea, vomiting, dizziness, and fainting in elderly as well as immunomodulatory supplement for horses (Cecchini *et al*., 2014; Tuekaew *et al*., 2014).

The presence of more phenolic compounds in the extract correlated with the lower toxic effects on L6 and HepG2. The negative regulation of the cytotoxicity by phenolic compounds in the formulation was also reported in the brown slime cap as well as mango fruit extracts on HepG2 and other cell lines (Navarro *et al*., 2019; Zhang *et al*., 2020). This result indicated that the phenolic compounds in the formulation were likely responsible for the less toxic effects on cell lines used in this study through anti-oxidative mechanisms.

It can also be suggested that the antidiabetic properties of the *Jamu pahitan* used by *Klinik Wisata Kesehatan Jamu* Kalibakung were mediated by direct improvement for glucose uptake in skeletal muscle and the indirect anti-oxidative action. The antioxidative mechanisms can play an important role, for instance, during diabetes, the ROS are excessively generated beyond the scavenging capacities of endogenous antioxidant defenses, therefore, leading to the progression and complications of the disease. The indirect actions of antioxidant properties of the formulation can ameliorate the pathophysiological changes in diabetes. The intake of exogenous antioxidants was proven to lower the blood glucose levels in diabetic patients and improve insulin sensitivity in rats, as demonstrated in zinc supplements (Bandeira *et al*., 2013; Gerber & Rutter, 2017).

Although, it exerted lower antioxidant and glucose uptake stimulatory activities, the water extract of the formulation showed a safer profile on the skeletal muscle and hepatic cells. Therefore, the consumption of formulation decoction, as suggested by the clinic, is safe to use. However, the ethanol extract is more suitable when it is further developed to obtain a preparation with better antidiabetic activities. The bioactivity-guided fractionation process can be beneficial to obtain active fractions of the formulation extract with better efficacy and less toxicity profile as reported in jackfruit extract (Daud *et al*., 2018).

CONCLUSION

This study concluded that the crude drugs of *Jamu pahitan* used by *Klinik Wisata Kesehatan Jamu* Kalibakung for treating diabetic patients had good quality. The water extract formulation indicated a considerable high *in-vitro* antioxidant activity, which stimulated muscle glucose uptake

and was safe for the skeletal muscle and hepatic cells. Furthermore, the study emphasized that consumption of the formulation in decoction form, as prescribed by the clinic, was safe and effective for alleviating diabetes. This result is in line with the traditional use of *Jamu pahitan*, which were likely through the mechanism of glucose uptake stimulation in skeletal muscle and the supportive anti-oxidative action.

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