

LD₅₀ Estimations for Diabecine™ Polyherbal Extracts Based on In Vitro Diabetic Models of 3T3-L1, WRL-68 and 1.1B4 Cell Lines

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Safety data of newly developed active pharmaceutical ingredient is of regulatory requirement. The LD₅₀ value is important to estimate the suitable dosage of extract for acute toxicity study and determine its safe dosage. LD₅₀ estimations for aqueous and ethanol extracts of polyherbal Diabecine™ were determined due to its potential to be used as active ingredient in herbal supplement for diabetic patients. Diabecine™ is a proprietary herbal blend of *Curcuma xanthorrhiza*, *Cinnamomum zeylanicum*, *Andrographis paniculata*, *Orthosiphon stamineus* and *Eugenia polyantha* Wight traditionally used as herbal medicine for diabetes. The cytotoxicity towards three cell lines commonly used for in vitro diabetic study namely, 3T3-L1, WRL-68 and 1.1B4, was determined in this study using MTT assay method. The IC₅₀ values were determined and used to estimate LD₅₀ values using ICCVAM's regression formula: $\log LD_{50} \text{ (mg/kg)} = 0.372 \log IC_{50} \text{ (}\mu\text{g/mL)} + 2.024$. All the extracts showed non-toxic effects against 3T3-L1, WRL-68 and 1.1B4 cell lines at concentration of 0.5 to 100 $\mu\text{g/mL}$, but significantly cytotoxic to all the three cell lines at concentration of 1,000 $\mu\text{g/mL}$ and above. The IC₅₀ values for 3T3-L1, 1.1B4 and WRL-68 are in the range of 493.8 to 779.6 $\mu\text{g/mL}$, 258.4 to 386.7 $\mu\text{g/mL}$ and 630.9 to 2,785 $\mu\text{g/mL}$. The 1.1B4 cell line was shown to have the highest cytotoxic sensitivity to the extracts. The IC₅₀ values for 1.1B4 were used to estimate the LD₅₀ values for ethanol freeze-dried, ethanol spray-dried, aqueous freeze-dried and aqueous spray-dried extracts, which are 956.4 mg/kg, 969.4 mg/kg, 834.4 mg/kg and 880.4 mg/kg. The starting dose of each extracts for acute toxicity study could be 3.2 factors lower than the estimated LD₅₀ values, which was suggested to be 298.9 mg/kg, 302.9 mg/kg, 260.8 mg/kg and 275.1 mg/kg for ethanol freeze-dried, ethanol spray-dried, aqueous freeze-dried and aqueous spray-dried extracts.

1. Introduction

Natural extracts from the plant is the oldest form of pharmaceutical treatment and are in growing demand worldwide (Fernández-Ponce et al., 2013). They are widely relied by populations due to their effectiveness, negligible side effects, wide range of action and relatively low cost (Chawla et al., 2013). Some of these phytochemicals which are abundant in fruits, vegetables, spices and teas have been consumed for centuries to treat hyperglycemic condition. Due to the fact that 'natural' doesn't always mean safer and better compared to synthetically made medicine, there is a need to scientifically establish the efficacy and toxicity of these phytochemicals.

Diabecine™ is a herbal supplement for diabetes, with proprietary herbal blend of Javanese turmeric (*Curcuma xanthorrhiza*), cinnamon (*Cinnamomum zeylanicum*), the king of bitters (*Andrographis paniculata*), Java tea (*Orthosiphon stamineus*) and Indonesian bay leaves (*Eugenia polyantha* Wight). There is no documented

scientific publication currently available to validate the effectiveness and safeness of Diabecine™ except from consumers' testimonials. However, previous studies have shown the anti-diabetic activity of the crude extracts of *C. xanthorrhiza* (Adnyana et al., 2013), *C. zeylanicum* (Ranasinghe et al., 2012), *A. paniculata* (Zhang and Tan, 2000), *O. stamineus* (Sriplang et al., 2007), and *E. polyantha* (Studiawan and Santosa, 2005) on mice or rats.

Although Diabecine™ is being used traditionally for more than 50 y to treat conditions related to diabetes especially to lower blood glucose, there is no safety data available for Diabecine™.

Determination of the inhibitory concentration that kills 50 % of the cells (IC_{50}) using in vitro cytotoxicity study is one of the ways to estimate the starting dose for in vivo acute toxicity study. The IC_{50} values can be converted to estimate the lethal dose that kills 50 % of the animals (LD_{50}) following the regression formula given by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): $\log LD_{50} \text{ (mg/kg)} = 0.372 \log IC_{50} \text{ (}\mu\text{g/mL)} + 2.024$ (ICCVAM, 2006). According to the Organization for Economic Co-operation and Development (OECD) Guideline 425 (OECD, 2008), the starting dose is recommended to be 3.2 factors lower than the calculated LD_{50} value of each compound under investigation. The guideline also suggested using two specific cell lines, which are NHK and 3T3 for general cytotoxicity evaluations of chemicals. However, in this report, three cell lines commonly used for in vitro diabetes investigation were used. The cells are mouse pre-adipocytes (3T3-L1), human hepatocytes (WRL-68) and human pancreatic beta cells (1.1B4). 3T3-L1 is a clonal subline isolated from 3T3 mouse embryonic fibroblasts and can be differentiated to adipocytes as they progress from a rapidly dividing to a confluent and contact inhibited state (Green and Meuth, 1974). WRL-68 cell line was derived from an unexpected spontaneous transformation when human embryo liver tissue was trypsinised and kept in Eagle's Minimum Essential Medium mixed with 10 % bovine serum at 37 °C for a few month (Apostolov, 1976). 1.1B4 cell line was established by electrofusion of freshly isolated human pancreatic beta cells with the immortal human PANC-1 epithelial cell line (McCluskey et al., 2011).

This study aims to estimate the suitable in vivo starting dose for acute toxicity study of four types of Diabecine's polyherbal extracts, namely, i) ethanol polyherbal extract-freeze dried (EPH-FD), ii) ethanol polyherbal extract-spray dried (EPH-SD), iii) water polyherbal extract-freeze dried (WPH-FD) and iv) water polyherbal extract-spray dried (WPH-SD) using 3T3-L1, WRL-68 and 1.1B4 cell lines. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is used to measure the in vitro cytotoxic effects of these extracts on the cells. The IC_{50} values were converted to LD_{50} values based on the regression formula and the starting dose was calculated using the lowering factor of 3.2 from the LD_{50} estimation.

2. Materials and Methods

2.1 Cell lines and materials

3T3-L1 mouse pre-adipocytes and WRL-68 human hepatocytes were purchased from American Type Culture Collection (ATCC, Manassas, USA) and 1.1B4 human pancreatic beta cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin (PS), fetal calf serum (FCS) and fetal bovine serum (FBS) were purchased from Gibco, Invitrogen. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (USA). Polyherbal Diabecine, in its raw coarsely ground mixture, was supplied by Naturemedic Supply, Malaysia.

2.2 Plant material extraction and drying

2.2.1 Water extraction method

Approximately 1.5 kg of coarse herbs was soaked in 20 L of filtered water in jacketed vessel for 3 h and regularly stirred. The extract was collected by filtration process. After extraction process, the extract was ready to be dried.

2.2.2 Ethanol extraction method

Approximately 1.5 kg of coarse herbs was soaked in 20 L of 70 % food grade ethanol in jacketed vessel for 3 h and regularly stirred. The extract was collected by filtration process. The weight of the wet herbs and volume of extraction was measured. The ethanol was eliminated by using rotary evaporator at 60 °C. After extraction process, the extract was ready to be dried.

2.2.3 Spray drying method

The condition of the inlet and outlet temperature was 185 °C and 108.5 °C. The extract was fed into the dryer by a peristaltic pump at flow rate of 15.0 g/min. The duration of drying was 9 h and 45 min. The dried powder

extracts were produced and stored in containers with tight caps at around 4 °C in the cold room until further analysis.

2.2.4 Freeze drying method

This method was used to remove the moisture from a frozen extract using vacuum. The extract was put in a freezer at -20 °C to produce a frozen extract. Then, the frozen extract was put into the drying chamber of the freeze dryer (Labconco). The equipment was set to -50 °C and vacuum pressure was applied to facilitate the removal of moisture content on the frozen extract. After the dried powder extract was produced, the powders were stored in containers with tight caps at around 4 °C in the cold room until further analysis.

2.3 Cells culture maintenance

3T3-L1 mouse pre-adipocytes, WRL-68 hepatocytes and 1.1B4 pancreatic beta cells were cultured and maintained based on protocol provided by depositor. 3T3-L1 and WRL-68 were grown in DMEM supplemented with 10 % fetal calf serum (FCS) and 10 % fetal bovine serum (FBS) with additional 1 % of antibiotic solution (100 units/mL penicillin and 0.1 g/L streptomycin) (PS) at 37 °C in a humidified atmosphere of 5 % CO₂. 1.1B4 were cultured in RPMI-1640 medium supplemented with 10 % FBS and 1 % PS at 37 °C in a humidified atmosphere of 5 % CO₂. Cells at exponential growth phase were used for experimental work.

2.4 MTT assay

The viability of 3T3-L1, WRL-68 and 1.1B4 cells in the presence of EPH-FD, EPH-SD, WPH-FD, and WPH-SD extracts was measured using MTT assay according to the method of Mosmann (1983) and Twentyman and Luscombe (1987). At 80 - 90 % confluency, 3T3-L1, WRL-68 and 1.1B4 cells were suspended using routine sub-culture methods. 10,000 cells/well were seeded in 96-well plates and incubated at 37 °C under a humidified atmosphere of 5 % CO₂ for 24 h. Next, cells were further incubated for another 24 h at 37 °C in the absence or presence of EPH-FD, EPH-SD, WPH-FD and WPH-SD extracts at various concentrations (0.5, 1, 5 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, 500 µg/mL, 1,000 µg/mL, 5,000 µg/mL and 10,000 µg/mL). 20 µL of MTT solution (5 mg in 1 mL PBS, sterile filtered) was added to each well. Plates were wrapped in aluminium foil and incubated for 4 h at 37 °C. Subsequently, the medium was gently aspirated from the wells and 200 µL of DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. The absorbance was immediately measured at 570 nm using the ELx800 Absorbance Microplate Reader (BioTek Instruments, USA). Samples were run in 6 replicates per plate. Wells without treatments were used as a control.

2.5 IC₅₀ calculation

Median inhibitory concentrations (IC₅₀) were statistically analysed by GraphPad Prism Version 6 software (GraphPad Software, 2016). Non-linear regressions of log (inhibitor) vs response with four parameters were selected for IC₅₀ estimation. The bottom and top parameters were constrained to 0 % and 100 %.

2.6 Estimation of LD₅₀ based on in vitro IC₅₀ value

The in vivo LD₅₀ of acute oral toxicity was estimated from in vitro IC₅₀ by using the following formula: $\log LD_{50} = 0.372 \times \log IC_{50} (\mu\text{g/mL}) + 2.024$ (ICCVAM, 2006).

2.7 Statistical analysis

Results were expressed as mean \pm standard error of the mean (SEM) of at least three independent experiments. Differences between treated samples and control were analysed using unpaired two-tailed Student's t-test with correction for multiple comparisons using the Holm-Sidak method. Nonlinear regression analyses were used to calculate IC₅₀ values and their 95 % confidence intervals. The IC₅₀ values were compared with one another using the extra sum-of-squares F test. For all analyses, P-values < 0.05 were considered statistically significant and indicated with an asterisk (*) in the figures.

3. Results and Discussions

3.1 Diabecine polyherbal extracts effect on cells viability

In the present study, cytotoxicity of Diabecine polyherbal extracts on 3T3-L1, 1.1B4 and WRL-68 cell lines was determined with the MTT assay (Figure 1). There were no significant differences ($P > 0.05$) in the viability of 3T3-L1, 1.1B4 and WRL-68 cells after 24 h exposure of Diabecine polyherbal extracts from 1 - 100 µg/mL. However, the cells viability of the three cells types was significantly decreased ($P < 0.05$) when treated with concentration of 1,000 µg/mL of the extracts. WPH-FD extract was shown to significantly enhance proliferation of WRL-68 cells at 5 to 50 µg/mL via mechanisms yet unknown.

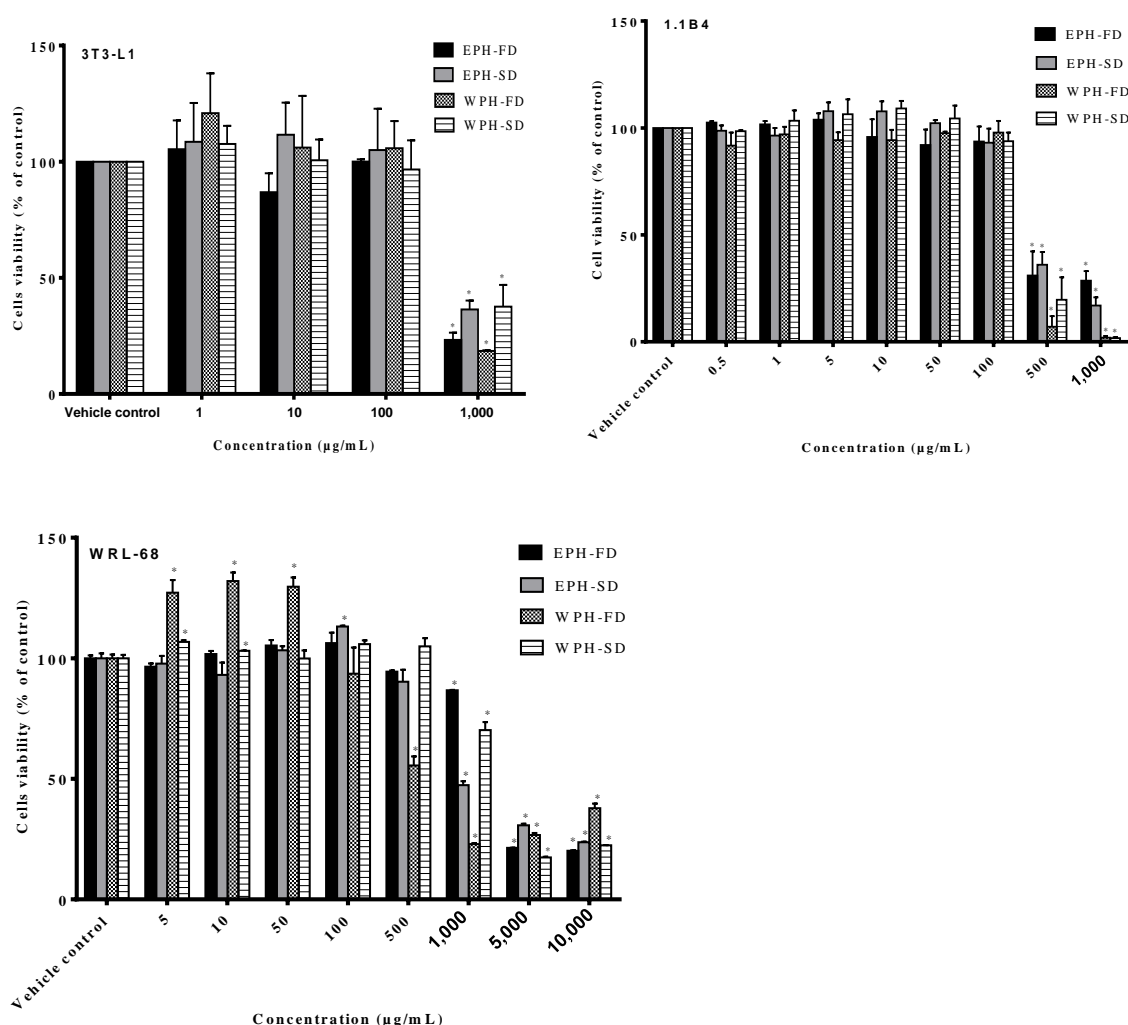


Figure 1: Viability of 3T3-L1, 1.1B4 and WRL-68 cells relative to vehicle control after treatment with Diabecine extracts at various concentrations for 24 h as determined by MTT assay

The IC_{50} of EPH-FD, EPH-SD, WPH-FD and WPH-SD extracts on 3T3-L1, 1.1B4 and WRL-68 cells are listed in Table 1. As the MTT assay shows highest cytotoxic sensitivity in 1.1B4 cells (lowest IC_{50} values) compared to WRL-68 and 3T3-L1 cells after 24 h exposure to the Diabecine polyherbal extracts, the IC_{50} values for 1.1B4 cells were chosen to estimate the LD_{50} values.

Table 1: IC_{50} values of Diabecine polyherbal extracts on 3T3-L1, 1.1B4 and WRL-68 cells

Extracts	3T3-L1		1.1B4		WRL-68	
	IC_{50} (µg/mL)	95 % CI (µg/mL)	IC_{50} (µg/mL)	95 % CI (µg/mL)	IC_{50} (µg/mL)	95 % CI (µg/mL)
EPH-FD	493.8	192.5 – 1,267	372.9	274.4 – 506.8	2,785	2,343 – 3,311
EPH-SD	779.6	225.1 – 2,699	386.7	312.9 – 477.8	1,948	1,324 – 2,866
WPH-FD	501.5	103.2 – 2,436	258.4	164.5 – 406.0	630.9	319.3 – 1,247
WPH-SD	716.3	326.8 – 1,570	298.5	211.1 – 422.2	2,059	1,746 – 2,960

3.2 LD_{50} estimation for in vivo study based on in vitro IC_{50} data

Using the regression formula: $\log LD_{50} = 0.372 \times \log IC_{50} (\mu\text{g/mL}) + 2.024$, the LD_{50} values of rat were estimated from the IC_{50} values obtained from the in vitro cytotoxic MTT assay on 1.1B4 cells (Table 2).

Table 2: Estimated LD₅₀ values based on *in vitro* IC₅₀ data on 1.1B4 cells

Extracts	IC ₅₀ (µg/mL)	LD ₅₀ (mg/kg)
EPH-FD	372.9	956.4
EPH-SD	386.7	969.4
WPH-FD	258.4	834.4
WPH-SD	298.5	880.4

Toxicology testing of herbal formulated supplement is essential to estimate the safe dosage required for efficacy and efficiency study, optimisation of product and also to evaluate potential adverse effect. Assessment of *in vitro* toxicity assay and genotoxicity is undoubtedly important to measure toxicology effect at molecular levels. The liver plays multiple important roles, including detoxification of toxic substances (Grant, 1991), production of biles for digestion of fats and in glucose homeostasis (Sherwin, 1980). The pancreatic beta cells play important role in the regulation of glucose homeostasis through synthesis and secretion of insulin, and its impairment leads to diabetes (Leibiger et al., 2008). Adipose tissue plays important role in lipid and glucose homeostasis, and its dysfunction leads to insulin resistance and type 2 diabetes (Guilherme et al., 2008). Taking into account of these characteristics of liver, pancreatic beta cells and adipose tissue, cytotoxicity of Diabecine™ polyherbal extracts were assessed *in vitro* via MTT cell viability assay on WRL-68 hepatocyte, 1.1B4 pancreatic beta cell and 3T3-L1 pre-adipocyte cell lines. As this assay suggested that exposure to concentrations of 100 µg/mL of the Diabecine™ polyherbal extracts are not cytotoxic towards WRL-68, 1.1B4 and 3T3-L1 cells, further *in vitro* studies on these cells with the extracts can be done at 100 µg/mL and below.

Traditionally, the safety of substances is determined through *in vivo* acute oral toxicity test by estimating the dose that produces lethality in 50 % of the animals tested (LD₅₀) (Botham, 2004). According to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2006), the LD₅₀ value of rat can be estimated from the IC₅₀ value obtained from *in vitro* cytotoxicity assay, by using the regression formula described previously. This approach enables the estimation of a starting dose closer to the actual LD₅₀ value for *in vivo* acute oral toxicity studies. Hence, this will reduce the numbers of animals needed for range finding, as well as reduce the numbers of animals that die or need to be humanely killed.

According to OECD Guideline 425 (OECD, 2008), the starting dose for acute oral toxicity study is recommended to be a step (factor 3.2 or one half log) below the estimated LD₅₀ value. Based on the estimated LD₅₀ values as shown in Table 2, the starting dose of each extracts for acute toxicity study was suggested to be 298.9 mg/kg, 302.9 mg/kg, 260.8 mg/kg and 275.1 mg/kg for EPH-FD, EPH-SD, WPH-FD and WPH-SD extracts.

The limitation of this method on predicting the LD₅₀ value for Diabecine polyherbal extracts is that the accuracy of correctly predicting the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) acute oral toxicity classification category for the extracts could be around 30 % as reported on other substances tested in the validation study (ICCVAM, 2006). The low level of accuracy could be due to the differences between cell cultures and whole animals in regards to the absorption, distribution, availability, metabolism and excretion of tested compounds.

4. Conclusion

This study revealed that 1.1B4 pancreatic beta cells has the highest cytotoxic sensitivity to the Diabecine polyherbal extracts compared to WRL-68 hepatocytes and 3T3-L1 pre-adipocytes. The LD₅₀ values for EPH-FD, EPH-SD, WPH-FD and WPH-SD extracts were estimated to be 956.4 mg/kg, 969.4 mg/kg, 834.4 mg/kg and 880.4 mg/kg. The starting dose of each extracts was calculated to be 298.9 mg/kg, 302.9 mg/kg, 260.8 mg/kg and 275.1 mg/kg for EPH-FD, EPH-SD, WPH-FD and WPH-SD extracts.

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Reference

- Adnyana I.K., Yulinah E., Yuliet, Kurniati N.F., 2013, Antidiabetic activity of aqueous leaf extracts of *Guazuma ulmifolia* Lamk., ethanolic extracts of *Curcuma xanthorrhiza* and their combinations in alloxan-induced diabetic mice, *Research Journal of Medicinal Plants* 7, 158-164.
- Apostolov K., 1976, Cell Lines, US Patent 3935066.
- Botham P.A., 2004, Acute systemic toxicity—prospects for tiered testing strategies, *Toxicology in Vitro* 18, 227-230.
- Chawla R., Thakur P., Chowdhry A., Jaiswal S., Sharma A., Goel R., Sharma J., Priyadarshi S., Kumar V., Sharma R., Arora R., 2013, Evidence based herbal drug standardization approach in coping with challenges of holistic management of diabetes: a dreadful lifestyle disorder of 21st century, *Journal of Diabetes & Metabolic Disorders* 12, 35.
- Fernández-Ponce M.T., Casas L., Mantell C., Martínez de la Ossa E.J., 2013, Potential use of mango leaves extracts obtained by high pressure technologies in cosmetic, pharmaceuticals and food industries, *Chemical Engineering Transactions* 32, 1147-1152.
- GraphPad Software Inc., 2016, GraphPad Prism, California, United States.
- Grant D.M., 1991, Detoxification pathways in the liver, *Journal of Inherited Metabolic Disease* 14, 421-430.
- Green H., Meuth M., 1974, An established pre-adipose cell line and its differentiation in culture, *Cell* 3, 127-133.
- Guilherme A., Virbasius J.V., Puri V., Czech M.P., 2008, Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes, *Nature Reviews Molecular Cell Biology* 9, 367-377.
- ICCVAM, 2006, ICCVAM Test Method Evaluation Report: In Vitro Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests, NIH Publication No. 07-4519, National Institute for Environmental Health Sciences, Research Triangle Park, North Carolina, United States.
- Leibiger I.B., Leibiger B., Berggren P.O., 2008, Insulin signaling in the pancreatic beta-cell, *Annual Review of Nutrition* 28, 233-251.
- McCluskey J.T., Hamid M., Guo-Parke H., McClenaghan N.H., Gomis R., Flatt P.R., 2011, Development and functional characterization of insulin-releasing human pancreatic beta cell lines produced by electrofusion, *The Journal of Biological Chemistry* 286, 21982-21992.
- Mosmann T., 1983, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *Journal of Immunological Methods* 65, 55-63.
- OECD, 2008, Guideline for Testing of Chemicals, 425, Acute Oral Toxicity – Up-and-Down Procedure, Organization for Economic Co-operation and Development, Paris, France.
- Ranasinghe P., Perera S., Gunatilake M., Abeywardene E., Gunapala N., Premakumara S., Perera K., Lokuhetty D., Katulanda P., 2012, Effects of *Cinnamomum zeylanicum* (Ceylon cinnamon) on blood glucose and lipids in a diabetic and healthy rat model, *Pharmacognosy Research* 4, 73-79.
- Sherwin R.S., 1980, Role of liver in glucose homeostasis, *Diabetes Care* 3, 261-265.
- Sriplang K., Adisakwattana S., Rungsipipat A., Yibchok-Anun S., 2007, Effects of *Orthosiphon stamineus* aqueous extract on plasma glucose concentration and lipid profile in normal and streptozotocin-induced diabetic rats, *Journal of Ethnopharmacology* 109, 510-514.
- Studiawan H., Santosa M.H., 2005, Uji aktivitas penurunan kadar glukosa darah ekstrak daun *Eugenia polyantha* pada mencit yang diinduksi aloksan, *Media Kedokteran Hewan* 21, 62-65.
- Twentyman P.R., Luscombe M., 1987, A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity, *British Journal of Cancer* 56, 279-285.
- Zhang X.F., Tan B.K., 2000, Anti-diabetic property of ethanolic extract of *Andrographis paniculata* in streptozotocin-diabetic rats, *Acta Pharmacologica Sinica* 21, 1157-1164.