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Original Research Article

Acute and sub-acute toxicity studies on an antidiabetic polyherbal formulation in mice

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Abstract

Purpose: *To investigate the acute and sub-acute toxicity of an Indonesian polyherbal formulation in experimental mice.*
Methods: *Young and healthy Swiss albino mice of both sexes (5 – 6 weeks old), weighing 24 – 25 g were used in this study. Acute toxicity test was conducted in the mice using OECD 425 method and AOT425StatPgm software to determine the polyherbal formulation's lethal dose (LD_{50}) value, while the sub-acute toxicity test was performed for 28 days using OECD 407 method, by measuring hematological and clinical biochemistry parameters, as well as examining the histology of the liver and kidneys. The acute toxicity test consisted of the following dose groups: 175, 550, 1750 and 5000 mg/kg, while sub-acute toxicity test dose groups were 200, 400 and 800 mg/kg. Each group consisted of 5 male and 5 female mice.*
Results: *Based on the results obtained, LD_{50} value of the polyherbal formula was >5000 mg/kg. Repeated doses for 28 days showed significant differences ($p < 0.05$) for white blood cells (WBC) and platelet parameters in male mice, and for mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and platelet parameters in female mice. However, the values were within normal limits. With regard to clinical biochemical parameters - aspartate aminotransferase (AST), Alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, and albumin - there were no significant differences ($p > 0.05$) between the test groups. Furthermore, no changes were observed in their histological features.*
Conclusion: *The evaluated polyherbal formulation can be considered safe for use in mice. However, clinical trials in diabetic patients at the proposed doses are required to ascertain the formulation's safety profile.*

Keywords: *Polyherbal, Antidiabetic, Acute and subacute toxicity, Safety*

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INTRODUCTION

The test for toxicity of traditional medicines in the preparation process is important in proving their safety for usage by humans. The presence of hazardous substances could affect organ function in humans or animals in a certain condition, amount, dose, and form. They also have the ability to cause unwanted effects either temporarily or permanently, even resulting to

death [1]. The World Health Organization (WHO) stipulated that the quality standard of traditional medicine has to meet several requirements including quality, safety, and efficacy [2].

Most Indonesians utilize conventional wisdom in the formulation of traditional medicines sourced from plants, based on the experience and skills that have been passed down from preceding generations [3]. The plants used as alternative medicine in curing diseases by Indonesian residents are the leaves of *yakon* (*Smallanthus sonchifolius*), salam (*Syzygium polyanthum*), *stevia* (*Stevia rebaudiana*), and tea (*Camellia sinensis*). Previous research reported that the polyherbal formula consisting of the leaves mentioned above, has the potential to function as an antidiabetic both *in vitro* and *in vivo* in a diabetic mouse model [4].

Generally, the safety of the individual herbs are well known. However, their combined effects are unclear. Therefore, it is essential to evaluate the safety and toxicity of herbs' combination, before usage. The preclinical toxicity studies are necessary for determining the safe dose for humans. This study did an evaluation of the acute (single dose for 14 days) and sub-acute (repeated doses for 28 days) toxicity for polyherbal formula in mice, and this was conducted according to the Organization for Economic Cooperation and Development (OECD) guidelines 423 and 407 respectively [5,6].

EXPERIMENTAL

Preparation of extract

The leaves for the polyherbal formulation were purchased from Balai Penelitian Tanaman Rempah dan Obat (Research Institute for Spice and Medicinal Plants - Balitro (Bogor, West Java, Indonesia). All the four plant materials were botanically authenticated and validated by Dr Atik Retnowati, a taxonomist at the Research Center for Biology, Indonesian Institute of Sciences, and voucher specimens were deposited at the herbarium of Laboratory of Pharmacognosy, Faculty of Pharmacy, Universitas Pancasila. The dried plant materials were separately ground to powder form. The leaves of yakon (*Smallanthus sonchifolius*), salam (*Syzygium polyanthum*), stevia (*Stevia rebaudiana*), and tea (*Camellia sinensis*) were washed thoroughly and dried under a shade.

The dried herbs were properly blended and formulated into powder form. The formulated powder was stored in an air-tight container and subjected to extraction in ethanol using the Soxhlet apparatus as described in the following. A 40 g sample was wrapped in a whatmann paper, and then inserted into the Soxhlet apparatus. After that, 400 mL of ethanol was added to the apparatus and then ran in the machine at a temperature of 70^{o}C, until the dripping (wet extract) was colorless, or up to 5 hours. The wet extract was thickened using a rotary evaporator at a temperature of 50 ^{o}C.

Animals

Young and healthy Swiss albino mice of both sexes (5 – 6 weeks old), weighing about 24 – 25 g, were used in this study. The animals were purchased from Bogor Agricultural University, West Java, Indonesia, and maintained under standard environmental conditions (23 – 25 ^{0}C) in Java, Indonesia, and fed on a pelletized diet, water, *ad libitum*. The animals were acclimatized to the laboratory environment for a week prior to the commencement of the study. The protocol used was approved by the Animal Ethical Committee, Faculty of Medicine, UPN Jakarta, Indonesia (approval no. 19-05-0496). All procedures performed in studies involving animal subjects were in accordance with the ethical standards of the institutional and/or national research committee as well as the 1964 Declaration of Helsinki and its later amendments [7].

Acute toxicity study

The mice were made to fast for 24 h prior to the commencement of the study. Ten mice (five males and five females) were used and each were given a single dose of 175, 550, 1750, and 5000 mg/kg of Polyherbal product (P.O). They were observed strictly and individually for the first 0.5, 4, and 24 h, then daily for 30 days. After this, they were observed for altered autonomic effects (lacrimation, salivation piloerection) central nervous system effects (tremors, convulsion, drowsiness), skin (fur), body weight, feed and water consumption, and mortality [5]. The determination of the LD_{50} value was carried out using AOT425StatPgm software.

Sub-acute toxicity study

Forty Wistar mice were divided into 4 groups of 10 each (5 males and 5 females). Three of the groups consisted of experimental doses of 200, 400, and 800 mg/kg/day respectively, while the fourth was the control group which was fed with normal feed and water, and weighed weekly for behavioral changes, feed and water consumption, and general morphological

difference. On the 28th day of treatment, the animals were anesthetized by i.p. administration containing 5 ml/kg of solution of 1 % chloralose in 25 % urethane (w/v). Their blood samples were collected using cardiac puncture and put into EDTA sample tubes for hematological analysis, and into heparinized tubes to obtain serum for biochemical analysis. The serum was acquired after centrifugation and allowing the previously extracted blood from all mice model to congeal for 30 min. After sacrificing the experimental animals, their vital organs (kidneys and Liver) were carefully excised and examined [6-12].

Histopathology Analysis

The histopathology method was inspired by Maynard and El-Nageh [2]. The retrieved kidneys and liver were submerged inside a 10% neutraled buffered formaldehyde (BNF) for 48 hours for fixation. After that, the fixated tissue was cut in a 0.5-1 cm width and placed inside an embedding cassette and then processed inside a tissue processor with time setting. The embedding liquid was a combination of 90% absolute ethanol, 5% methanol, and 5% isopropanol for around 2 hours.

After embedding, the tissue was placed into a mold and submerged in paraffin. After the paraffin had solidified, it was removed from the mold, cut with a microtome with the thickness of 4-5 ck, and then hematoxylin and eosin (H-E) was used for staining. Kidney and liver histopathology analysis was done by comparing the histology tissue from the treatment and control. The observed features are vacuolization, fattening degeneration of the organ, as well as necrotic and hydrophic degeneration. The microscopic observation was expressed in scoring, and descriptively analyzed [13].

Biochemical analysis

Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were measured using Bayererase (AST) and Alan (Bayer/Siemens, Germany). Kreatinin and Ureum was measured using the procedure for DiaSys Creatinin FS dan DiaSys Urea FS (GmbH, Germany).

Statistical analysis

The results of the study are expressed as the mean ± standard error of the mean (S.E.M). Statistical analysis of the data was carried out with one-way analysis of variance (ANOVA), followed by Tukey post hoc multiple comparison test. Significant differences were set at $p < 0.05$.

RESULTS

Effect of extract on mouse body weight

The body weight of the treated mice remained significantly unchanged (Table 1). During the sub-acute toxicity experimental period, all groups showed gradual and normal increase in their weight difference ($p > 0.05$) between the treated and control groups.

The initial mean body weight of the control group was 27.00 ± 3.08 g for males and 21.80 ± 1.79 g for females, while the final mean body weight was 27.80 ± 3.03 g for males and 21.80 ± 1.09 g for females.

Effect of extract on biochemical parameters

The oral administration of polyherbal for 28 days at a dosage of 200 and 400 mg/kg did not cause significant changes in hematological parameters when compared to the control group mice. However, at 800 mg/kg dose, the platelet count decreased in both male and female mice.

This might be due to certain phytochemicals present in the individual drugs of the polyherbal formulation that dissolved in the solvent based on its polarity [14]. The phytochemical in plants could have toxic effect on the body, and one of the prerequisites for herbal medicine is preclinical tests that include acute, subacute, and chronic toxicity [15].

There were no significant differences in the liver and kidney function parameters, which included the AST, ALT, and albumin tests between the normal and the dosage group ($p > 0.05$). Furthermore, there were no significant differences in the renal function parameters, which included the measurement results of BUN and creatinine, between the normal and the dosage group ($p > 0.05$).

This showed that the polyherbal formula administration did not affect the liver and kidneys function both in male and female mice.

Effect on tissue histology

In the sub-acute toxicity study, there were no detectable abnormalities in the tissue histology examination of the kidneys and liver sections of mice.

Table 1: The ethanolic effect comparison of the Formulation Polyherbal on Body Weight of treated and control mice during sub-acute toxicity study

Parameter	Dose	Mean ± SEM	
		Male	Female
Body weight initial week 1	Control	27.00±3.08	21.80±1.79
	200mg/kg	26.60±3.21	20.80±1.30
	400mg/kg	26.00±2.55	22.80±3.11
	800mg/kg	28.00±2.24	28.20±3.11
Body weight initial week 2	Control	28.00±3.32	21.20±1.64
	200mg/kg	27.40±2.41	22.00±1.58
	400mg/kg	26.80±1.64	23.60±3.05
	800mg/kg	28.40±2.70	26.60±3.36
Body weight initial week 3	Control	27.80±3.03	21.80±1.09
	200mg/kg	27.20±1.92	22.80±1.92
	400mg/kg	26.60±0.55	24.20±3.70
	800mg/kg	28.20±2.49	26.60±2.70

Values are expressed as mean ± SEM (N = 5)

Table 2: Effect of Polyherbal on hematological parameters of male and female mice in sub-acute toxicity

Parameter	Sex	Group			
		control	200mg/kg	400mg/kg	800mg/kg
WBC (10^3µL)	Female	6.18±2.47	5.54±2.26	7.12±2.34	6.06±0.84
	Male	11.36±4.55	9.86±1.74	7.74±1.46	7.64±2.28
RBC (10^6µL)	Female	7.67±1.62	8.14±1.62	8.02±0.94	7.08±0.96
	Male	7.74±1.28	8.08±0.86	8.22±0.65	9.38±1.46
Hemoglobin (g/dL)	Female	12.00±2.72	13.08±2.29	12.64±1.67	11.88±1.63
	Male	12.92±1.39	13.08±1.61	12.82±0.62	12.92±1.72
Hematocrit (%)	Female	36.40±7.62	39.94±6.84	39.34±5.42	36.86±4.66
	Male	39.58±4.66	40.54±4.20	40.10±2.25	41.92±6.05
Platelet count (10^3µL)	Female	652.4±181.8	692.2±191.1	727.6±122.6	642.6±142.3
	Male	856.4±218.6	822.2±186.6	919.2±82.58	807.3±97.5
MCV (fL)	Female	47.50±1.51	49.32±2.65	48.96±1.40	49.30±2.76
	Male	52.02±4.80	50.24±3.23	48.84±1.38	48.02±2.01
MCH (Pg)	Female	15.50±0.65	16.14±1.14	15.74±0.3	15.36±1.24
	Male	16.90±1.56	16.02±1.39	15.62±0.79	15.98±0.98
MCHC (g/dL)	Female	32.86±1.39	32.74±0.65	32.16±0.31	31.16±0.89
	Male	32.48±0.44	32.24±1.06	32.00±0.89	33.22±0.98

Values are expressed as mean ± SEM, N=5/group

Table 3: Effect of Polyherbal on biochemical parameters of male and female mice in Sub-acute toxicity

Parameter	Sex	Group			
		Control	200mg/kg	400mg/kg	800mg/kg
AST (µ/L)	Female	59.16±4.26	60.96±2.15	61.90±2.44	62.34±4.40
	Male	60.46±4.10	59.82±3.91	60.38±2.96	61.34±4.50
ALT (µ/L)	Female	27.40±2.04	28.34±1.70	29.42±2.52	30.72±2.41
	Male	28.80±1.09	28.62±0.78	30.54±1.31	30.70±1.39
BUN (mg/dL)	Female	15.56±0.94	14.44±1.27	14.60±1.20	16.42±1.54
	Male	14.74±1.54	15.16±1.75	15.98±1.47	16.22±0.68
Albumin (g/dL)	Female	3.85±0.34	4.09±0.39	4.20±0.37	4.25±0.26
	Male	3.89±0.30	3.89±0.48	4.01±0.35	4.06±0.44
Creatinine (mg/dL)	Female	1.43±0.36	1.44±0.40	1.73±0.26	1.82±0.28
	Male	1.44±0.34	1.45±0.44	1.66±0.59	1.88±0.26

Values are expressed as mean ± SEM, N=5/group

DISCUSSION

Polyherbal formula consists of *yacon* (*Smallanthus sonchifolius)*, stevia *(Stevia rebaudiana*), bay (*Syzygium polyanthum*), and tea (*Camelia sinensis*) leaves. Preliminary research showed that the combination of the leaves mentioned above had antidiabetic effects *in vitro* and *in vivo* [4].

Preclinical test results provide information about the pharmacological, pharmacokinetic, pharmacodynamic, and the toxicity effect of the medicines on humans [12]. In this study, the acute and sub-acute toxicity test of polyherbal

formula as an antidiabetic was carried out in the preclinical test, according to the Organization for Economic Cooperation and Development (OECD) guidelines 423 and 407 respectively [5,6].

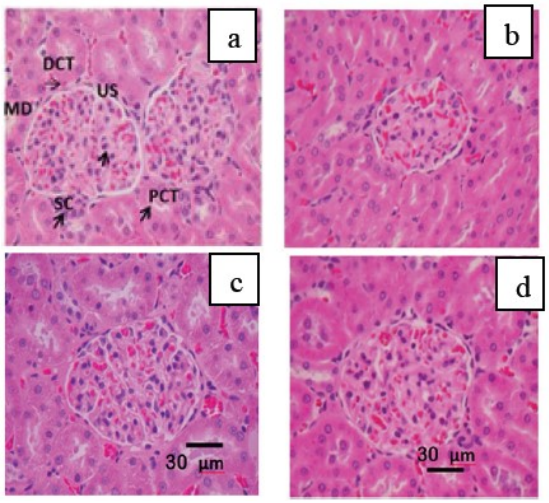

Figure 1: Histology of kidney sections of control male mice: (a) control group; (b) 200 mg/kg; (c) 400 mg/kg and (d) 800 g/kg of Polyherbal in 1 28-days subacute toxicity, PCT = proximal convoluted tubule, DCT = distal convoluted tubule, MD = acula densa, G = glomerulus, US = urinary space, SC = squamous cell, and P = podocyte. No abnormality was found in all

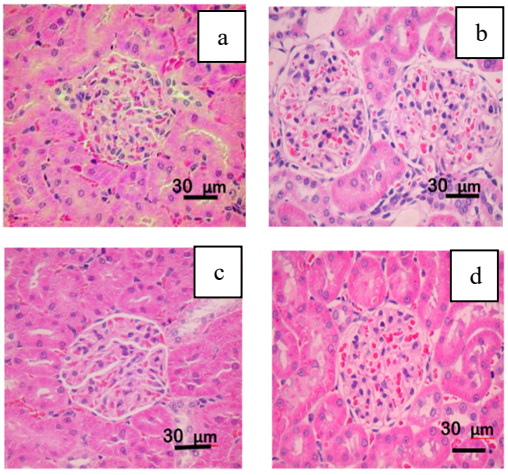

Figure 2: Histology of kidney sections of control female mice: (a) control group; (b) 200 mg/kg; (c) 400 mg/kg and (d) 800 g/kg of Polyherbal in 1 28-days subacute toxicity, PCT = proximal convoluted tubule, DCT = distal convoluted tubule, MD = macula densa, G = glomerulus, US = urinary space, SC = squamous cell, and P = podocyte, no abnormality was found in all

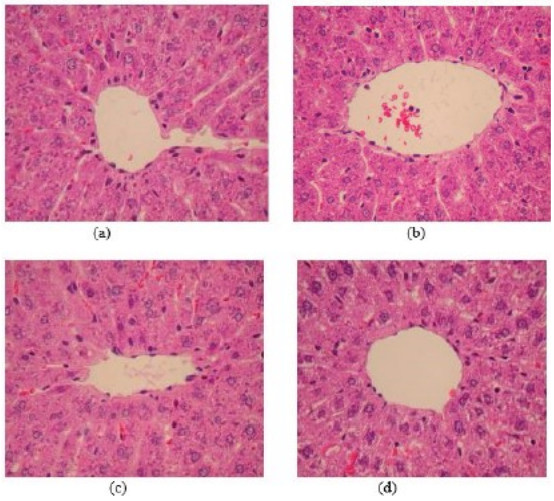

Figure 3: Histology of liver sections of control male mice: (a) control group; (b) 200 mg/kg; (c) 400 mg/kg and (d) 800 mg/kg of Polyherbal in 1 28-days subacute toxicity, no abnormality was found in all histological slides. CV=central vein, EC=endothelial cells, H= hepatocytes, S=hepatic sinusoidal, KC=kupffer cells

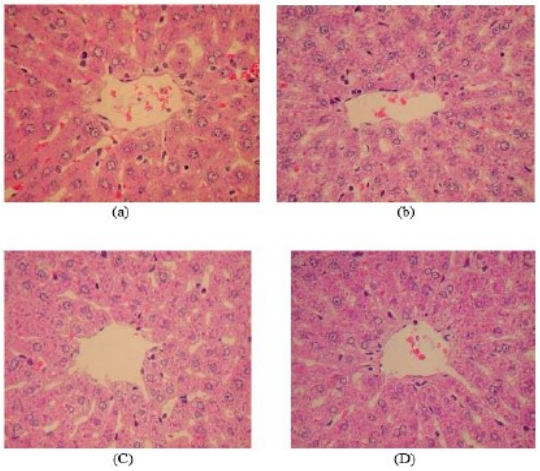

Figure 4: Histology of liver sections of control female mice: (a) control group; (b) 200 mg/kg; (c) 400 mg/kg and (d) 800 mg/kg of Polyherbal in 1 28-days subacute toxicity. No abnormality was found in all histological slides. CV=central vein, EC=endothelial cells, H= hepatocytes, S=hepatic sinusoidal, KC=kupffer cells

The observation results of the animals' behaviour at each dose level showed no symptoms of tremor, salivation, convulsions, diarrhea, or coma. Furthermore, no allergic reactions in the animals given doses of 175 and 550 mg/kg. The motor activity of the test animals was normal until the 14th day after the treatment, while the eyes,

skin, mucous membranes, and right reflex were still normal. Based on the observations, LD_{50} value of the polyherbal formula extract was >5000 mg/kg. According to the Organization for Economic Cooperation and Development (OECD) guidelines for acute oral toxicity, an LD_{50} dose of 5000 mg/kg and above was categorized as unclassified, hence the drug was found to be safe [5]. In the present study, the acute toxicity effect of antidiabetic polyherbal formulation had reported that no toxicity or mortality were observed up to a dose of 5000 mg/kg.

To complement the acute toxicity result, the oral sub-acute toxicity was conducted on the mice by administering antidiabetic polyherbal formulation once daily for 28 days. It was observed that the mean body weight of male and female mice from day 0 to 28 fluctuated. However, there was no significant change in the body weight for each group. This indicated that there were no toxicity symptoms or conditions that cause appetite loss, since significant change in body weight is an early indicator of a toxic effect from the test preparation given. Changes in body weight could be associated with the fat accumulation and physiology adaptive response to the herbal medicine, rather than the toxicity when the changes are fluctuating [16].

The hematological examination which included the parameters of WBC (White Blood Cell), RBC (Red Blood Cell), HGB (Hemoglobin), HCT (Hematocrit), MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration), and PLT (Platelet or Trombocyte) levels were measured after the test animals were treated for 28 days. Overall, the results showed that there were no significant differences in male and female mice, with most of the parameters ($p > 0.05$) amongst the groups. Interestingly, significant differences ($p < 0.05$) were observed in the WBC and PLT variable for male and MCHC, PLT, and MCV for female mice. The significant difference was observed between 200 mg/kg with 400 mg/kg, and normal with 400 mg/kg in male mice. Meanwhile the platelet variable was significantly different in 800 mg/kg group compared to normal, 200 mg/kg, and 400 mg/kg. The significant difference of MCV was observed between 200 mg/kg with 400 mg/kg; MCHC was observed between 800 mg/kg with control and 200 mg/kg; platelet was observed between 800 mg/kg with control, 200 mg/kg, and 400 mg/kg in female mice. Regarding RBC parameter, all the treatments showed no significant differences with control after 28 days of polyherbal administration. Some plant isolates or extract could influence the bone marrow that is responsible for blood cell production [17], yet our polyherbal formula did not induce any anemic reaction to the mice model. The result suggests that the polyherbal formulation did not endanger the bone marrow in all of the administered doses (Table 2).

There were no significant differences between the normal and the dose group ($p> 0.05$) in the liver function parameters, namely, AST (Aspartate aminotransferase), ALT (Alanine aminotransferase), and albumin. Higher ASR and ALT level than control indicates interference of the hepatocyte function, so both AST and ALT will be released into the bloodstream. Lower albumin level is associated with interference of hepatocyte synthesis function, especially in chronic hepatocyte lesions. Moreover, there were no significant differences between the normal and the dose group ($p > 0.05$) in the measurement results of BUN (blood urea nitrogen) and creatinine. Normal kidney function will maintain constant kreatinine level in the bloodstream, and any increase in kreatinine indicates impaired kidney function. BUN assessment could be used to assess the kidney function, kidney disease progression, and hemodialysis process. Increased BUN in blood is associated with damage in glomelurus but also could be caused by lack of nutrition or presence of hepatotoxin. This showed that the polyherbal formula did not affect the function of the liver and kidneys both in male and female mice [16,17].

Histology examinations of organs from animals of all groups treated with polyherbal formula, and those of control animals showed normal architecture (Figures 1-4). This suggests no detrimental changes and no morphological disorder was induced by the oral daily administration of this extract for 28 days, since the oral dose of 800 mg/kg/day of Polyherbal administered for 28 consecutive days was the highest dose used in this study, and did not induce any hematological, anatomical and histopathological signs of toxicity.

A study by Barcellona et al. [18] found that the administration of 10% extract of *S. sonchifolius* with the dose of 14 g / kg and 0,32 g /kg did not cause mortality to mice. Long-term administration for 90 days everyday with the dose of .07, 0.14 and 0.28g/kg did not induce any significant observable changes in hematology, biochemical, and histopathology parameters. Another study by Hsu et al. [19] also observed the insignificant sub-acute toxicity and changes in body weight, hematology, serum, urine, and histopathology parameter after 28 days of *C. sinensis* extract treatment with 625, 1250 and 2500mg/kg doses

to mice. Similar result was observed at the toxicity study of S. rebaudiana leaf extract at doses of 500, 1000, and 2000mg/kg per day for 90 days [20]. *S. polyanthum* leaf extract administration at 100, 400 and 1000mg/kg per day for 90 days to rats produced similar result but with necrotic and liver fattening to female rats [21]. Based on those arguments, the effect of singular plants is not enough to reach the therapeutic effect for diabetic patients. The combination of those plants in a certain ratio in polyherbal formulation could give a better result with less toxicity.

CONCLUSION

The present findings suggest that polyherbal formulation from yakon (*S. sonchifolius*), tea (*C. sinensis*), salam (*S. polyanthum*) and stevia (*S. rebaudiana*) is non-toxic, since no marked changes in hematological, biochemical, and histopathological parameters were observed. Therefore, at normal therapeutic doses, Formula polyherbal is considered to be safe for long-term treatment of diabetic conditions. This study finally emphasized that the antidiabetic Polyherbal Formulation from yakon (*S. sonchifolius*), tea (*C. sinensis*), salam (*S. polyanthum*) and stevia (*S. rebaudiana*) was very safe up to 5000 mg/kg bw dose in animal model. However, this is the first study to investigate the toxicity of Polyherbal in mice, and a subchronic toxicity test should also be conducted to establish the adverse effects of a repeated administration of antidiabetic polyherbal formulation.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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