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Acute/subchronic oral toxicity of *Brucea javanica* seeds with hypoglycemic activity

Ajmi Noor Shahida [a,c], Chee Yan Choo [a], Tin Wui Wong [b,c] *

a. MedChem Herbal Research Group, Faculty of Pharmacy

b. Particle Design Research Group, Faculty of Pharmacy

c. Non-Destructive Biomedical and Pharmaceutical Research Centre Universiti Teknologi MARA, 42300, Puncak Alam, Selangor, Malaysia.

Abstract

Brucea javanica (L.) Merr (Simaroubaceae) seeds are used as antidiabetic folk medicine. This study examined acute and sub-chronic toxicity of seed extracts with hypoglycemic activity. Dried powdered seeds were extracted using different polarity solvents: methanolic solution, *n*-hexane, chloroform and *n*-butanol. These extracts were orally administered to mice with blood glucose levels determined. The median lethal doses (LD_{50}), biochemical and histological profiles of liver and kidney of mice were assessed. Methanolic and butanolic extracts reduced blood glucose levels of mice at 32 mg/kg with no mortality, unlike inactive hydrophobic extract. The LD_{50} values of methanolic and butanolic extracts were 281.71 and 438.43 mg/kg respectively. The butanolic extract exhibited a similar activity as methanolic sample but had a lower level of acute toxicity. The inactive hydrophilic and toxic hydrophobic constituents of butanolic extract were removed via aqueous residual and hydrophobic solvent partitioning during extraction. Histology examination and blood tests of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, urea, billirubin and creatinine indicated that the butanolic extract did not induce liver and kidney toxicity upon 9-week consumption. Butanolic extract contained blood glucose lowering quassinoids bruceine D (10.3 %) and E (0.4 %). It is safe for treatment of diabetes mellitus in extract form.

Keywords: Acute toxicity, *Brucea javanica*, diabetes mellitus, Simaroubaceae, sub-chronic toxicity.

1. Introduction

Brucea javanica (L.) Merr is commonly known as Java brucea, "Ya-Dan-Zi" or Macassar kernel tree classified under the family of Simaroubaceae. It has been shown to exhibit anti-malarial,[1] amoebicidal,[2] cytotoxic and anti-leukemic,[3-11] anti-protozoan,[12]anti-HIV,[13] anti-inflammatory[14] and anti-babesial[15] effects.

In Malaysia, the seeds of *B. javanica* are used by the traditional practitioners for the treatment of diabetes.[16] An oral dose equivalent to 5 to 10 seeds a day is recommended for diabetic patients. *B. javanica* seeds can be consumed in

* Corresponding author
Email: wongtinwui@salam.uitm.edu.my

varying forms of liquid extract during diabetes treatment. Nonetheless, the scope of toxicity study hitherto is limited to acute toxicity tests of aqueous extract whereby its intra-venous administration to mice induces oligouria, hematuria and central depression (only for bruceantin), and oral administration of aqueous seed decoction to chicken is reported to have a LD_{50} value of 0.4 g/kg.[17] The safety aspect of seeds brings much concern as most of the chemical constituents isolated from this plant exhibit cytotoxic effect against various cancer cell lines.[3,10-11,14,18] Given that seeds are consumed orally for folk treatment of diabetes, the present study aims to evaluate the *in vivo* oral toxicity of various seed extracts at its effective hypoglycemic doses.

2. Experimentals

2.1. Materials

The dried seeds of *B. javanica* were purchased from the traditional medicine market in Malacca, Malaysia in 2007. The identity of *B. javanica* was authenticated by Emeritus Professor Dato' Dr. Abdul Latiff Mohamed from Universiti Kebangsaan Malaysia, Bangi, Malaysia. The voucher specimen (FP/UiTM/BJ/01/05) was deposited at the Faculty of Pharmacy, Universiti Teknologi MARA, Malaysia. Methanol, *n*-hexane, chloroform and *n*-butanol (Merck, Germany) were organic solvents employed in the processes of extraction. Polyethylene glycol 3000 (PEG 3000, Merck, Germany) and dimethyl sulphoxide (DMSO, Merck, Germany) were used as solubilizers for the preparation of extracts.

2.2. Experimental animal

Male *Mus musculus* mice (Kebayan Enterprise, Malaysia), weighed between 25 and 30 g, were used in examination of toxicity and blood glucose lowering profiles of *B. javanica* extracts in accordance to that of reported by Zia *et al.* (2001) and Lamela *et al.* (1985).[19-20] The mice were housed in a standard environment at an ambient temperature of 25 ± 1°C and a relative humidity of 65 ± 5 % on a 12 hour light/dark cycle. They were given free access to standard pelletized food (GoldCoin Enterprise, Malaysia) and water *ad libitium*. The mice were acclimatized for at least one week and subjected to 12 hours of fasting prior to experiment unless otherwise stated. All experiments were conducted following national ethics approval in accordance to the international guidelines (OECD Environment, Health and Safety).

2.3. Preparation of extracts

The seeds of *B. javanica* were oven dried at 40 ± 1°C (Memmert, Germany) and ground into powder using the cutter mill. The powdered seeds were subjected to an extraction process using 80% v/v aqueous methanolic solution for a period of 48 hours at 24 ± 1°C. The formed extract was filtered and the filtrate was evaporated to dryness under a reduced pressure at 40 ± 1ºC using the rotary evaporator (Buchi Rotavapor, Switzerland). When necessary, the methanolic extract was further processed by means of sequential partition with *n*-hexane, chloroform and *n*-butanol using the deionized water as co-extractant. Practically, the dried yield of methanolic extract was 12.1% w/w of the powdered seeds. The dried yields of hexane, chloroform, butanolic and aqueous residual extracts were 2.9, 5.2, 18 and 67% w/w of the methanolic extract respectively. All batches of each extract were pooled and stored at 4°C till further testing.

2.4. Evaluation of hypoglycemic activity

The mice were divided into groups of 10 each. The extracts, dissolved using 0.2% w/v of PEG 3000 solution or 2% w/v DMSO solution, were administered orally to mice with pure PEG 3000 or DMSO solution serving as control sample.

DMSO solution was used as solubilizer for hexane and chloroform extracts as PEG 3000 was not able to solubilize hydrophobic constituents of these extracts. The blood was withdrawn from tail vein immediately prior to the extract administration (0 hour) and every 2 hours after extract administration for 8 hours. Blood glucose levels of mice at interval longer than 8 hours were not examined owing to severe hypoglycemia following prolonged fasting. The blood glucose concentration of mice was examined using the glucometer (Ascensia Elite, Bayer Corporation, USA). Positive control was developed with mice receiving glibenclamide at 3 mg/kg body weight of mouse. The reduction extent of blood glucose concentration was defined as quotient of the difference between the blood glucose concentration at 0 hour and at a specified time to that at 0 hour, expressed in the unit of percentage.

2.5. Evaluation of toxicity

2.5.1. Acute toxicity

The blood glucose lowering extracts were subjected to acute toxicity tests using mice. Groups of 10 mice were administered with extracts at doses of 128, 192, 256, 320 and 512 mg extract/kg body weight of mouse with standard pelletized food and water *ad libitum* following the oral administration of extracts. The mice were kept under observation for 24 hours after receiving the extracts for any overt signs of acute toxicity such as diarrhea, tremor, changes in color of eyes or fur, eating behavior and level of activity, as well as, death. The acute toxicity of extracts was also expressed by LD_{50} value of which denoted the dose of extract/kg body weight of mouse which resulted in 50 % mortality of mice in 24 hours. The LD50 values of extracts were calculated from the log dose-probit relationship described by Finney 1971.[21] The percentage of mortality was derived from the quotient of number of mice died at 24 hours to that of number of mice at 0 hour. In the groups of mice which showed 0 and 100 % mortality, the percentages of mortality were defined as:

$$\text{Percentage of mortality} = 100\ (0.25/n) \quad (1)$$
$$\text{Percentage of mortality} = 100\ [(n - 0.25)/n] \quad (2)$$

respectively where n was the number of mice involved in the study. The percentages of mortality from various groups of mice were then transformed into probits and were plotted against log doses. The dose which corresponded to a probit value of 5 was taken as the LD_{50} value of extract.

2.5.2. Sub-chronic toxicity

The mice were divided into two groups of 20 each. Group 1 received 0.2% w/v pure PEG 3000 solution whereas group 2 were administered with extract which contained 32 mg butanolic extract/kg body weight of mouse once a day for 9 weeks via the oral route without prior fasting unless otherwise stated. These mice were subjected to observation daily for behavioral changes or symptoms of toxicity as described under the section of acute toxicity. In addition, the food consumption pattern and body weight of mice were monitored weekly. At the end of every 3 weeks, the mice were fasted for 12 hours and their biochemical parameters of blood such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, billirubin and creatinine were determined using the diagnostic analyser (Reflotron Plus, Roche, USA) to reflect the state of health of both liver and kidney which represented the major organs of metabolism. At the end of 9 weeks, these mice were sacrificed with both kidney and liver removed for histology examination. The kidney and liver tissues were subjected to cryosectioning by means of cryostat (CM1850 UV, Leica, Germany) and the

microtomed samples were stained using hematoxylin (BDH, UK) and eosin (Sigma Aldrich, Germany) reagents. The stained samples were examined under a compound microscope at 10´ magnification (DM 2000, Leica, Germany).

2.6. Statistical analysis

Two-way ANOVA or Student's t-test was used to evaluate all data expressed in mean and standard deviation by SPSS version 14. The level of significance for all statistical evaluation was set at $p < 0.05$.

3. Results and Discussion

The extent of blood glucose concentration of mice receiving 32 mg crude methanolic extract/ kg of their body weight was significantly reduced by 41.14 ± 14.57 % against control at 8 hours (Table 1a; ANOVA: $p < 0.05$). This was comparable to the extent of blood glucose reduction in the positive control, glibenclamide. The mice which received 10, 50, 70 and 90 mg methanolic extract/kg body weight exhibited a less marked extent of blood glucose concentration reduction throughout 8 hours against control. Using a higher dose at 128 mg methanolic extract/kg body weight of mouse, the mice became inactive within the first 2 hours, and some were found dead after 24 hours of administration.

Feeding 32 mg/kg body weight of mouse with hexane, chloroform and aqueous soluble fractions exhibited no significant blood glucose lowering activity of these extracts in mice against control (Table 1b; ANOVA: $p > 0.05$). Nonetheless, the butanol soluble fraction exhibited significant blood glucose lowering activity. A total of 80 % of mice fed with chloroform extract were dead within 24 hours of administration. In comparison to mice which received chloroform extract, butanolic extract gave no cases of mortality to mice. It was presumed that the biologically toxic constituents were contained in the hydrophobic extract. The hydrophilic constituents, namely those soluble in the butanolic extract, were safe for consumption.

Further evaluation of butanolic extract using doses of 32, 64 and 128 mg extract/kg body weight of mouse exhibited a significant reduction in their blood glucose concentration when compared to control at 8 hours (Table 1c; ANOVA: $p < 0.05$), but with no sign of mortality in mice after 24 hours of consumption. In the absence of constituents present in hydrophobic extract, the toxicity of butanolic extract was greatly reduced. On the other hand, the blood glucose lowering activity was retained by the containment of hydrophilic constituents in the butanolic extract. Other hydrophilic constituents, which were insoluble in butanolic extract and inactive with respect to blood glucose lowering effect in mice, were removed as aqueous residual extract.

From the log dose-probit relationship evaluation, the blood glucose lowering methanolic and butanolic extracts of *B. javanica* were found to have LD_{50} values of 281.71 and 438.43 mg extract/kg body weight of mouse respectively. Similar to observation noted during the blood glucose lowering response test, the level of acute toxicity of methanolic extract was higher than butanolic extract. A lower dose of methanolic extract was required to cause 50 % mortality in mice than butanolic extract. At high doses of 256, 320 and 512 mg methanolic extract/kg body weight, the mice developed changes of behavior at a faster rate than those of receiving butanolic extract. These mice were physically less active, and exhibited a reduced tendency for eating, a higher intensity of tremor as well as a higher level of mortality. Practically, the LD_{50} values of both methanolic and butanolic extracts were 9 and 14 folds higher than

Table 1(a). Extent of blood glucose concentration reduction in mice receiving (a) various doses of methanolic extract (n = 10), (b) 32 mg/kg body weight of methanolic, butanolic, hexane, chloroform and aqueous residual extracts (n = 10), and (c) various doses of butanolic extract of *B. javanica* (n = 10).

	Extent of blood glucose concentration reduction (%)			
	Time (hour)			
Dose (mg/kg BW[a])	2	4	6	8
Control (PEG 3000)	1.60 ± 9.60	14.58 ± 18.48	22.75 ± 13.89	18.33 ± 6.88
10	-2.64 ± 22.41	13.64 ± 21.74	21.20 ± 17.34	23.86 ± 16.06
32	7.12 ± 16.71	21.85 ± 19.06	30.07 ± 19.51	41.14 ± 14.57*
50	-14.44 ± 15.03	-0.61 ± 18.15	22.26 ± 18.87	25.07 ± 18.86
70	-1.39 ± 12.47	17.17 ± 17.11	37.76 ± 18.81	36.19 ± 18.50
90	4.03 ±15.93	14.64 ± 18.43	14.32 ± 16.15	25.76 ± 20.11
Glibenclamide	28.16 ± 6.78*	41.28 ± 7.99*	48.12 ± 14.50*	47.22 ± 6.43*

* $p < 0.05$, in relation to control. a BW: body weight of mouse. The values represent mean ± standard deviation.

Table 1(b).

	Extent of blood glucose concentration reduction (%)			
	Time (hour)			
Sample	2	4	6	8
Control (PEG 3000)	1.60 ± 9.60	14.58 ± 18.48	22.75 ± 13.89	18.33 ± 6.88
Methanol	7.12 ± 16.71	21.85 ± 19.06	30.07 ± 19.51	41.14 ± 14.57*
Butanol	-7.56 ± 17.50	14.80 ± 12.48	20.59 ± 14.35	39.14 ± 13.50*
Aqueous residue	-25.94 ± 12.50	-21.62 ± 14.23	-6.11 ± 16.89	4.20 ± 12.19
Control (DMSO)	4.72 ± 12.55	16.61 ± 16.09	27.46 ± 14.40	25.26 ± 11.12
Hexane	-3.41 ± 13.39	10.12 ± 9.39	18.79 ± 20.70	15.91±19.80
Chloroform	-2.68 ± 31.08	6.27 ± 36.71	24.46 ± 28.08	23.02 ± 18.74

Table 1(c).

	Extent of blood glucose concentration reduction (%)			
	Time (hour)			
Dose (mg/kg BW)	2	4	6	8
Control (PEG 3000)	1.60 ± 9.60	14.58 ± 18.48	22.75 ± 13.89	18.33 ± 6.88
10	-6.71 ± 19.22	-11.08 ± 12.85	-5.52 ± 15.07	3.24 ± 15.38
32	-7.56 ± 17.50	14.80 ± 12.48	20.59 ± 14.35	39.14 ± 13.50*
64	-1.71 ± 26.45	11.72 ± 25.91	20.79 ± 13.17	37.02 ± 14.97*
128	-9.49 ± 16.55	18.60 ± 12.95	23.75 ± 11.30	32.31 ± 16.31*

their effective blood glucose lowering dose at 32 mg extract/kg body weight of mouse respectively. On the note that butanolic extract demonstrated potential blood glucose lowering property with a greater level of safety than other extracts, sub-chronic toxicity test was conducted over a period of 9 weeks using butanolic extract at the lowest effective dose of 32 mg extract/kg body weight of mouse with control receiving pure PEG 3000 solution.

Generally, the body weight of mice increased with time (Table 2). There was no substantial difference in eating habit between the control and extract-fed mice. In addition, there were no overt signs of toxicity such as diarrhea, tremor, changes in color of eyes or fur and level of activity in all batches of mice. Similar to control, all extract-fed mice survived at the end of 9 weeks. The blood levels of ALT, AST and ALP of extract-fed mice increased with time (Table 2). Nonetheless, such observation was not an attribute of liver and/or kidney damage following the administration of extract. In mice receiving pure PEG 3000 solution, a similar rise in the blood levels of ALT, AST and ALP was noted. There was no significant difference in the degree of changes in blood levels of ALT, AST and ALP over 3 to 9 weeks between the control and extract-fed mice (Student's t-test: $p > 0.05$). In the case of creatinine, urea and bilirubin, it was noted that the blood levels of these biochemical indicators were consistently low in both control and extract-fed mice over a period of 9 weeks,below the detectable limit of Reflotron device (Table 2). In conjunction with the findings of ALT, AST and ALP, as well as, overt signs of toxicity experienced by mice, it was envisaged that the administration of mice with butanolic extract at the dose of 32 mg/kg body weight of mouse had no substantial negative impact on the physiological state of both liver and kidney. This was further supported by histological examination of liver and kidney tissues of mice. There was no substantial difference in the lobular structures of liver and kidney between mice receiving pure PEG 3000 solution and butanolic extract dissolved in PEG 3000 solution at the dose of 32 mg/kg body weight of mouse (Fig. 1a and b). Apparently, the histological variation only elicited in tissues of liver and kidney when the butanolic extract was administered to mice at a high dose of 128 mg/kg body weight of mouse (Fig. 1c).

Table 2. Sub-chronic toxicity profiles of mice receiving 32 mg/kg body weight of butanolic extract of *B. javanica* (n = 20).

Sample	Time (week)	Body weight (g)	Creatinine (mmol/L)	Bilirubin (μmol/L)	Urea (mmol/L)	ALT (U/L)	AST (U/L)	ALP (U/L)
Control (PEG 3000 solution)	3	31.32 ± 2.23	< 44.20	< 8.55 – 68.70	< 3.33 – 7.06	39.43 ± 26.20	85.25 ± 38.70	111.08 ± 46.42
	6	31.93 ± 4.14	< 44.20	< 8.55 – 99.50	3.33 – 8.44	76.99 ± 34.53	210.41 ± 67.01	129.82 ± 58.07
	9	33.65 ± 4.35	< 44.20	< 8.55 – 153.00	3.61 – 8.59	63.87 ± 19.28	215.75 ± 42.01	113.64 ± 39.30
Butanolic extract	3	32.99 ± 2.86	< 44.20	< 8.55 – 56.10	< 3.33 – 6.25	28.51 ± 11.28	76.28 ± 10.68	76.98 ± 34.29
	6	33.82 ± 3.93	< 44.20	< 8.55 – 85.30	< 3.33 – 7.77	47.31 ± 15.04	168.56 ± 48.12	86.04 ± 18.57
	9	34.28 ± 4.64	< 44.20	10.00 – 108.00	< 3.33 – 5.85	59.21 ± 37.73	157.93 ± 43.30	55.88 ± 17.72

The values represent mean ± standard deviation.

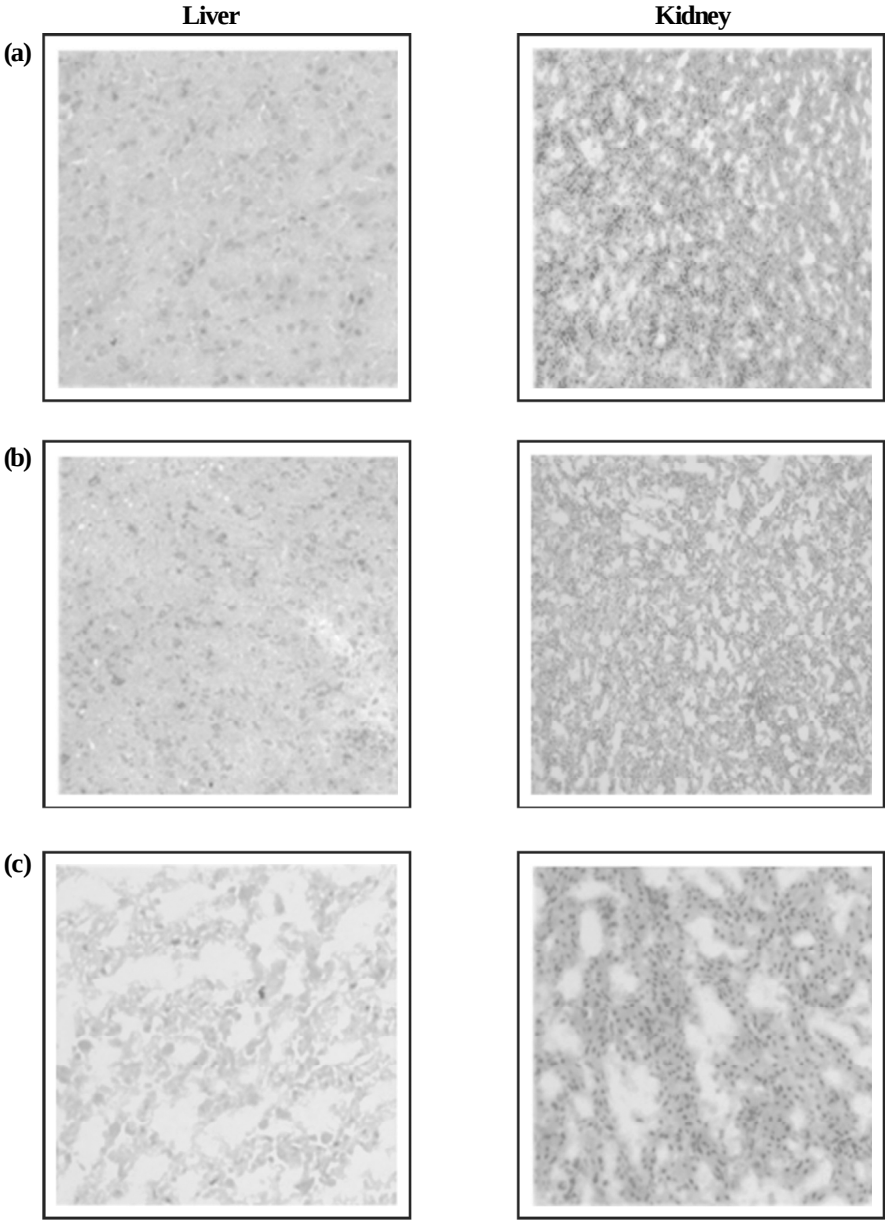

Fig.1: Longitudinal histology sections of liver and kidney of mice receiving a) PEG 3000 solution, b) 32 mg/kg and c) 128 mg/kg body weight of butanolic extract of *B. javanica* for 9 weeks. Magnification factor: 10´.

The butanolic extract of *B. javanica* seeds with blood glucose lowering property was shown to be not unsafe for use. Through bioactivity guided fractionation, The butanolic extract had been identified to contain both bruceines D and E, and both bruceines D and E quassinoids were found to be responsible for the hypoglycemic effect(Fig. 2).[22] Bruceines Dand E were unambiguously elucidated through 1D and 2D nuclear magnetic resonance, mass,

infrared and ultraviolet spectroscopy techniques. The methanolic extract contained bruceine D (0.2 %) and E (0.009 %).

The butanolic extract contained a higher amount of both bruceine D (10.3 %) and E (0.4 %). Both quassinoids were not detected in the hexane, chloroform and residual extracts. Bruceines D and E were relatively less toxic than bruceantin, a quassinoid isolated from the same plant and was withdrawn from human clinical trial due to lack of efficacy.[23]

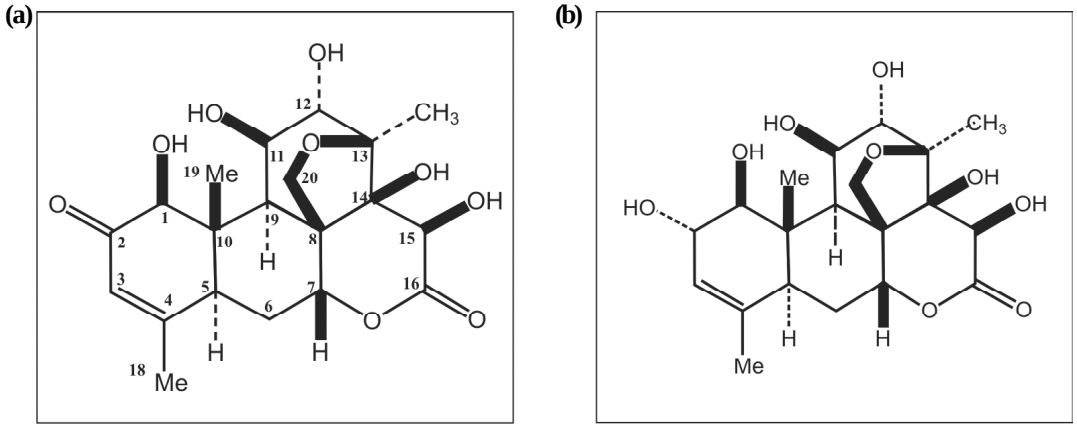

Fig. 2: Chemical structures of bruceines a) D and b) E.

4. Conclusions

Butanolic extract, which contained both bruceines D and E, was found to have a higher LD50 value at 438.43 mg/kg than methanolic extract (281.71 mg/kg). It did not induce liver and kidney toxicity upon 9 weeks of consumption. Its toxic constituents can be removed through hydrophobic partitions. The butanolic extract is potentially safe for use as an extract in the treatment of diabetes mellitus.

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References

1) O'Neill MJ, Bray DH, Boardman P, Chan KL, Phillipson JD, Warhurst DC, Peters W. (1987) *J. Nat. Pdt.* : 41–48.

2) Wright CW, O'Neill MJ, Phillipson JD, Warhurst DC. (1988) *Antimicrob. Agents Chemother.*32: 1725-1729.

3) Lee K-H, Imakura Y, Sumida Y, Wu R-Y, Hall IH. (1979) *J. Org. Chem.*44(13): 2180 – 2185.

4) Lee KH, Hayashi N, Okano M, Nozaki H, Juichi M. (1984) *J. Nat. Pdt.*47: 550-551.

5) Cassady JM, Suffness M. (1980) *Terpenoid*

antitumor agents, Academic Press: New York; 201-269.

6) Sakaki T, Yoshimura S, Tsuyuki T, Takahashi T, Honda T. (1986) *Chem. Pharm. Bull.* : 4447–4450.

7) Anderson MM, O'Neill MJ, Phillipson JD, Warhurst DC. (1991) *Planta Med.* 57(1): 62-64.

8) Fukamiya N, Okano M, Miyamoto M. (1992) *J. Nat. Pdt.* (4): 468 - 475.

9) Luyengi L, Suh N, Fong HHS, Pezzuto JM, Kinghorn AD. (1996) *Phytochem.*: 409-412.

10) Su B-N, Chang LC, Park EJ, Cuendet M, Santarsiero BD, Mesecar AD, Mehta RG, Fong HS, Pezzuto JM, Kinghorn AD. (2002) *Planta Med.* 68: 730-733.

11) Kim IH, Takashima S, Hitotsuyanagi Y, Hasuda T, Takeya K. (2004) *J. Nat. Pdt.*67: 863-868.

12) Sawangjaroen N, Sawangjaroen K. (2005) *J. Ethnopharmacol.*98: 67–72.

13) Okano M, Fukamiya N, Tagahara K, Cosentino M, Lee TTY, Morris-Natshke S, Lee KH. (1996) *Bioorg. Med. Chem. Lett.* 6: 701-706.

14) Hall IH, Liou YF, Lee KHS, Chaney G, Willingham Jr W. (1983) *J. Pharm. Sci.* 72(6): 626-630.

15) Subeki, Matsuura H, Takahashi K, Nabeta K, Yamasaki M, Maede Y, Katakura K. (2007) *J. Nat. Pdt.*70: 1654-1657.

16) Shamsul K, Tajuddin AM, Mazina MY. (2003) *Tumbuhan ubatan tradisional Malaysia,* Universiti Pertanian Malaysia Publisher: Malaysia; 17.

17) Li X, Wei W. (2002) *Combinations and Applications: Herbs for clearing heart,* Donica Publishing; 118-119.

18) Polonsky J, Varenne J, Prange T, Pascard C. (1980) *Tetrahedron Lett.* 21: 1853-1856.

19) Zia T, Nazrul Hasnain S, Hasan SK. (2001) *J. Ethnopharmacol.* 75: 191-195.

20) Lamela M, Cadavid I, Gato A, Calleja JM. (1985) *J. Ethnopharmacol.*14: 83-91.

21) Finney DJ. (1971) *Probit analysis: Estimation of the median effective dose (3rd edition),* Cambridge University Press: London;20 – 47.

22) NoorShahida A, Wong TW, Choo CY. (2009) *J. Ethnopharmacol.* 124(3): 586-591.

23) Cragg GM, Newman DJ. (2005) *J. Ethnopharmacol.* 100: 72-79.