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In-vivo toxicity studies of a mixture of *Hibiscus sabdariffa* L., *Clinacanthus nutans* L. and Stevia leaves in *Sprague Dawley* rats

(Kajian ketoksikan *in-vivo* campuran daun *Hibiscus sabdariffa* L., *Clinacanthus nutans* L. dan Stevia terhadap tikus *Sprague Dawley*)

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Abstract

Natural products from medicinal plants are widely used worldwide as they are claimed to generate new alternative medicines. Following its proven medicinal properties, a mixture of *Hibiscus sabdariffa* L., *Clinacanthus nutans* L.*, Stevia rebaudiana* in the ratio of 1:1:0.5 (HS:CN:Stevia; w/w/w) was shown to be effective therapy as diuretic and in chemoprevention. However, no study has been conducted to assess the toxic effect or the LD_{50} of this mixture. Therefore, a standard *in-vivo* toxicological assessment was carried out in a 28-day oral administration study. The doses tested were 1,000 mg/kg, 2,000 mg/kg and 5,000 mg/kg of body weight in *Sprague Dawley* rats. No mortality, adverse clinical signs and abnormal changes in body weight. Toxicologically significant changes were only seen in relative liver weight, red blood cell count, haematocrit, haemoglobin and total protein. However, the difference was toxicologically insignificant since the values were within normal physiological ranges. These observations suggest that the mixture is practically non-toxic in *Sprague Dawley* rats and the no-observed-adverse-effect level (NOAEL) is greater than 5,000 mg/kg.

Keywords: tocixity, *Hibiscus sabdariffa* L., *Clinacanthus nutans* L., *Stevia rebaudiana*, rats

Introduction

Natural products from medicinal plants are widely used worldwide as they are believed to be new alternative medicines for conventional therapy. Malaysia has numerous plants with high medicinal values such as *Hibiscus sabdariffa* L. (HS), *Clinacanthus nutans* L. (CN), *Stevia rebaudiana* and others. HS is also known as roselle, sorrel, mesta and karkade is an annual herbaceous shrub of the *Malvaceae* family (Lin et al. 2007). Traditionally, the roselle leaves (green reddish color with 10 – 15 cm length and have a sour taste) are used for medicinal purposes in China and India in controlling high blood pressure, reducing menstrual pain, pyrexia and liver damage (Lin et al. 2007). Previous studies have demonstrated that roselle leaves extract is non-toxic and posses hypoglycaemic, hypolipidaemic, antioxidant, oestrogenic effects and anticancer effects (Lin et al. 2012). Prasongwatana et al. (2008) reported that infusions of the leaves and calyx extracts could alleviate fever, act as cholerectic, diuretic and uricosuric substance. The infusions could also decrease the blood viscosity which revealed the effect

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of anti-hypertension, anti-obesity, anti-diabetic and anti-cholesterol.

CN or commonly known as Sabah snake grass or 'Belalai Gajah' in Malaysian language is a perennial herb in *Acanthaceae* family. It is widely known as a traditional medicinal plant in Southeast Asia such as Malaysia, Indonesia, Thailand and China. In Malaysia, the fresh leaves are usually boiled with water and consumed as herbal tea for treating cancer and diabetes (Muhammad Shahzad et al. 2015). In Indonesia and Thailand, CN is used to treat inflammation and viral infection (Yong et al. 2013), skin rashes, insects and snake bites, lesions caused by simplex herpes virus, diabetes mellitus, fever and diuretics (Lau et al. 2014). Several scientific reports proved that CN is non-toxic (Chavalittumrong et al. 1995), has anti-inflammatory, anti-hepatitis and anti-herpes activities (Yoosook et al. 1999, Wanikiat et al. 2008, Sittiso et al. 2010) and anti-cancer activity (Yong et al. 2013).

Stevia rebaudiana (Stevia) is a small perennial and woody shrub in the *Asteraceae* family and has been used as a bio-sweetener and sugar substitute (Gupta et al. 2013) to lower blood sugar level (Goyal et al. 2010). It is also known as sweet herb, sweet leaf, honey leaf, candy leaf or honey yerba (Carakostas et al. 2008). This magical natural herb is non-toxic which its secondary metabolites did not produce teratogenic, mutagenic or carcinogenic effects and no allergic reactions (Pol et al. 2007). Stevia has many pharmacological and therapeutic applications which could act as an efficient medication for curing chronic and non-chronic diseases like diabetes, cardiovascular disease, cancer, renal disease, obesity, inflammatory bowel disease and dental caries (Gupta et al. 2013).

Since these medicinal plants are claimed to be non-toxic, cheap and readily available, these herbs were combined as a herbal mixture in the ratio of 1:1:0.5 (HS leaf: CN: Stevia; w/w/w) in order to maximise the effectiveness of their therapeutic effects as diuretic and in chemoprevention. As several studies have proven the tremendous medicinal values of these plants, it is necessary to take into account the safety of the herbal mixture. Therefore, there is a pressing need to clarify the toxicological profile of this natural product. In this study, a comprehensive safety evaluation of herbal mixture was carried out in 28-day repeated oral administration study in *Sprague Dawley* rats.

Materials and methods

Sample preparation

The HS, CN and Stevia leaves were dried using commercial oven dryer at 40 °C until the moisture content reached below 10 %. Dried samples were then ground using Waring blender (Waring, Connecticut) for 2 – 3 s and sieved (0.5 mm). The herbal mixture was prepared by mixing the leaves at ratio of 1:1:0.5 (HS:CN:Stevia; w/w/w) in sachets. The extract of the herbal mixture was prepared by infusing the mixed dried leaves with boiling water for 10 min. The extract were prepared at three different concentrations (1,000, 2,000 and 5,000 mg/kg) for the oral toxicity study.

Experimental animals

A total of twenty female *Sprague Dawley* rats weighing approximately 200 g each and 5 – 6 weeks old were randomly assigned into four groups; a control and three treatment groups (n = 5). Individual body weights were recorded and detailed physical examinations on the fur, skin and health condition were performed twice during the 7 days period of acclimatisation to ensure the status of healthy animals. The animals were housed in a system controlled environment in the light-dark cycle (12 – 12 h, lights on 7:00 –19:00), temperature (24 ± 2 °C) and a relative humidity (30 – 70 %) during the study.

The animals were provided *ad libitum* with a standard pellet (Specialty Feeds, Australia) and distilled water.

Sub acute oral toxicity study

A sub-acute or 28-days repeated dose oral toxicity study was performed according to Ryu et al. (2004). During the oral administration period, *Sprague Dawley* rats were respectively dosed with distilled water (control group, C) or herbal mixture at doses of 1,000 mg/kg of body weight (low dose, LD), 2,000 mg/kg of body weight (medium dose, MD) and 5,000 mg/kg of body weight (high dose, HD) via drinking bottles as they can access *ad libitum*. On average, each rat usually consumed about 100 ml of sample per day. Any remaining sample left will be measured. General appearance or behaviour of each rat was observed daily during the 28-days study and the body weight was recorded weekly.

Haematology and serum biochemistry analysis

The animals were fasted for approximately 12 h and blood samples were withdrawn from the *vena cava* under light ether anesthesia. Samples of blood for haematology and biochemistry analyses were withdrawn. An aliquot of blood per animal (approximately 20 µl) was treated in a 3 ml ethylen-diamino-tetracetic-acid (K_3-EDTA) tube (Bacton Dickinson, BD Vaccutainer) to analyse haematological indexes. The blood sample was analysed for complete blood profile: red blood cell (RBC), white blood cell (WBC), platelet (PLT), haematocrit (HCT), and haemoglobin (Hb) level. The measurements were performed in a haematology analyser (Medonic CA530, Italy).

For serum biochemical blood analysis, one aliquot of blood per animal was placed in a 5 ml Z-serum tube (Bacton Dickinson, BD Vaccutainer) and centrifuged at 3,000 rpm for 20 min. The serum was analysed for aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, bilirubin, total protein (TP), albumin (Alb), globulin, glucose (Glu), urea, creatinine (Cr), total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) by using blood clinical analyser (Vitalab Selectra E, Italy). All reagents for the tests were obtained from Randox (Randox Laboratories Ltd, Antrim, United Kingdom).

Statistical analysis

All data for obtained were expressed as mean ± standard deviation (SD) and subjected to one-way analysis of variance (ANOVA) using SAS System, ver. 9.0 statistical software. When statistically significant differences were indicated ($p < 0.05$), the Duncan New Multiple Range Test (DMRT) was employed for comparisons between control and treated groups.

Results and Discussion

Sub-acute oral toxicity study

Clinical observations and body weight assessment

No abnormalities in terms of clinical signs including death were seen in rats from any groups. *Figure 1* shows a non-significant increment in body weight between the control and treated groups. The results showed that even the treated rats have gained positive body weight signifying the absence of toxic effects of the mixture at all doses. Body weight changes have been used as an indicator of adverse effects of drugs and chemicals (Teo et al. 2002; Hilaly et al. 2004).

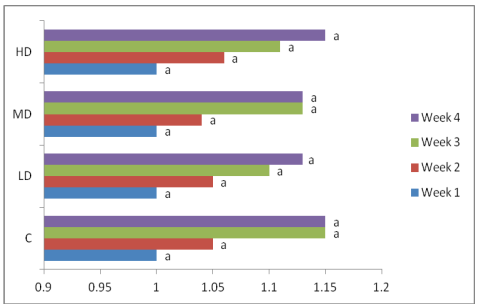

Figure 1. Normalised mean body weight of rats at the end of the study period

Relative organ weights

Organ weight is the most sensitive indicator of drug toxicity whereby significant differences in organ weights may occur in the absence of any gross morphological changes (Piao et al. 2013). Organ weights are usually presented as relative values and expressed as a percentage of body weight (Wolfsegger et al. 2009). In this study, the actual and relative organ weights at necropsy following 28 days of toxicity study. The results for relative organ weights (liver, heart, lung, kidney and spleen) were shown in *Table 1*. Thus, this result suggested a within normal function of the organs. The relative liver weight of the low dose treated group (6.42%) decreased significantly (p <0.05) as compared to control group (7.96%) while the medium and high dose treated group showed no significant difference.

Mumoli et al. (2006) and Giannini et al. (2005) suggested that, hepatotoxicity is associated with increased of liver weight, aminotransferases and alkaline phosphatase enzyme level in plasma. While, reduction of body weight gain and internal organ weight are generally considered as a sensitive indicator of toxic effect (Thanabhorn et al. 2006). Nevertheless, in this study, the reduction of liver weight is not conclusively attributed to toxicity since the blood enzymatic profiles (*Table 2*) were within normal reference range (Petterino and Argentino-Storino 2006). Meanwhile, the gross examination at necropsy did not reveal any experiment-related changes. The gross interpretation showed no lesion, inflammation, bleeding, abnormal enlargement or spots of internal organs been observed in all treated rats as compared to control.

Haematology

Table 3 shows no significant differences in PLT and WBC (p >0.05) between groups and such values were in the normal reference range (Petterino and Argentino-Storino 2006). While, RBC in the MD and HD group significantly (p <0.05) increased as compared to the control. The HD group had a significant (p <0.05) increase in HCT and Hb concentration compared to control group. However, the result did not show any toxicity-related changes since the RBC and HCT values were within the normal reference range (Han et al. 2010). This again, suggested no adverse effect of herbal mixture on the haematology values of the treated rats.

Serum biochemistry

Table 2 shows concentration of ALT, AST, bilirubin and protein in *Sprague Dawley* rats during the study period. Blood liver enzymes often measured and used as an indicator of liver damage and overall liver health (Giannini et al. 2005). The ALT and AST are liver marker enzymes involved in the catabolism of amino acids. The measurement of serum levels of these two enzymes is a standard feature of clinical chemistry investigations in regulatory toxicity studies (Ennulat et al. 2010). The ALT is considered as liver specific enzyme present in greatest concentration in the hepatocytes of most species (Hall 2001). Previous study also showed that an increased level of ALT activity in the serum is accepted as an indication of hepatic toxicity in rats whenever the damage is more than 70%

(Ennulat et al. 2010; Witthawaskul et al. 2003). It is possible that in this study, there are damages within the liver in the treated group, but it did not exceed 70% for a significant rise in ALT to be seen. Future work on the histology of the liver will give a better assessment on the actual toxic nature of this mixture.

There was no significant increased in the concentration of bilirubin in all treated groups compared to control group (*Table 2*).

Table 1. The percentage of relative organ weight of rats at the end of the study period (Mean ± SD)

Analysis	*n*	Control	LD	MD	HD
Kidney (%)	5	1.4 ± 0.12^{a}	1.4 ± 0.23^{a}	1.3 ± 0.14^{a}	1.5 ± 0.20^{a}
Heart (%)	5	0.9 ± 0.12^{a}	0.8 ± 0.11^{a}	0.7 ± 0.08^{a}	0.9 ± 0.15^{a}
Lung (%)	5	1.4 ± 0.32^{a}	1.2 ± 0.36^{a}	1.3 ± 0.12^{a}	1.4 ± 0.14^{a}
Spleen (%)	5	0.5 ± 0.11^{a}	0.4 ± 0.12^{a}	0.4 ± 0.06^{a}	0.4 ± 0.05^{a}
Liver (%)	5	7.9 ± 0.77^{a}	6.4 ± 0.67^{b}	7.1 ± 0.36^{ab}	7.6 ± 0.99^{a}

Values bearing similar superscript/s do not differ at $p > 0.05$

Table 2. Selected serum biochemistry of rats at the end of the study period (Mean ± SD)

Analysis	Control	LD	MD	HD
ALT (U/l)	52.3 ± 6.12a	58.8 ± 15.92a	51.3 ± 8.76a	53.2 ± 8.79a
AST (U/l)	110.0 ± 12.54a	116.0 ± 20.86a	116.8 ± 17.96a	114.7 ± 17.13a
Bilirubin (µmol/l)	3.1 ± 0.79a	3.5 ± 0.79a	3.9 ± 0.56a	3.6 ± 1.11a
Globulin (g/l)	30.7 ± 3.67a	35.3 ± 5.01a	35.7 ± 3.44a	34.0 ± 3.90a
Albumin (g/l)	48.5 ± 2.60a	49.0 ± 0.72a	50.6 ± 1.13a	50.5 ± 1.83a
Total Protein (g/l)	79.9 ± 4.73a	84.4 ± 5.62ab	86.0 ± 2.64b	84.5 ± 2.84ab

Values bearing similar superscript/s do not differ at $p > 0.05$

Table 3. Haemogram of rats at the end of the study period (Mean ± SD)

Analysis	Control	LD	MD	HD
RBC (10^{12}/l)	8.3 ± 0.78^{a}	8.9 ± 0.36^{ab}	9.3 ± 0.44^{b}	9.3 ± 0.82^{b}
HCT (%)	43.6 ± 3.62^{a}	46.6 ± 2.59^{ab}	47.8 ± 2.57^{ab}	48.8 ± 4.10^{b}
PLT (10^{9}/l)	1474.4 ± 229.05^{a}	1576.0 ± 132.23^{a}	1395.7 ± 246.91^{a}	1464.4 ± 209.84^{a}
WBC (10^{9}/l)	5.6 ± 2.93^{ab}	5.1 ± 0.25^{b}	4.3 ± 0.26^{b}	8.5 ± 1.60^{a}
Hb (g/l)	163.2 ± 14.75^{a}	177.3 ± 7.37^{ab}	175.0 ± 9.84^{ab}	182.2 ± 13.15^{b}

Values bearing similar superscript/s do not differ at $p > 0.05$

A small elevation in plasma bilirubin concentration is an important indicator of liver damage in laboratory animals (Rasekh et al. 2008). These results indicated that based on gross and selected enzymes (Petterino and Argentino-Storino 2006) and bilirubin (Han et al. 2010) parameters, there were no adverse effect of herbal mixture on the liver of *Sprague Dawley* rats.

The serum protein profile showed no significant changes (*Table 2*) except for slight significant ($p < 0.05$) increase on total protein level in MD group (86.02 g/l) compared to the control (79.90 g/l). A decreased concentration of total protein, albumin and globulin are associated with cirrhosis or liver malfunction (Patel et al. 2008). A high protein level showed the possibility of the liver and kidney malfunction where protein were not completely digested or absorbed. A lower protein level usually related to dehydration or myeloma multiple (Petterino and Argentino-Storino 2006). A cirrhotic liver is unlikely to happen in this study as the duration was quite short.

Table 4 shows the creatinine and urea concentration as a measure of the kidney status. A high level of urea and creatinine in blood indicates the occurence of severe kidney damage since the kidney has failed to filter the urea nitrogen out of circulation into the urine (Hassan et al. 2007; Rhionani et al. 2008). No evidence of kidney failure or malfunction were registered since the concentration of urea and creatinine are within normal limits and comparable in all (Petterino and Argentino-Storino 2006).

Table 5 shows the serum lipid profile TC, HDL, LDL and TG indicated no significant changes in all treated rats.

The blood glucose concentration (*Figure 2*) also showed comparable levels between all groups. This likely suggested that the oral administration of the herbal mixture did not affect the general metabolism of glucose. All of the presented results showed the absence of toxic effects in all tissues as the measured profiles were within normal physiological ranges (Petterino and Argentino-Storino 2006; Han et al. 2010). Likewise, the comparable values between treated groups are evidence of an absent of a dose-response toxic effect.

Conclusion

In conclusion, this 28-days oral toxicity study reveals that daily doses of 1,000, 2,000 and 5,000 mg/kg of the mixture did not elicit toxicity in *Sprague Dawley* rats. The dose of 5,000 mg/kg/day was identified as the no-observed-adverse-effect level (NOAEL) in this study.

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Table 4. Kidney function assay of rats at the end of the study period (Mean ± SD)

Analysis	Control	LD	MD	HD
Urea (mmol/l)	7.6 ± 1.21[a]	8.2 ± 0.54[a]	8.0 ± 1.01[a]	7.8 ± 1.17[a]
Creatinine (µmol/l)	63.6 ± 4.33[a]	61.0 ± 3.39[a]	63.7 ± 4.46[a]	64.8 ± 3.03[a]

Table 5. Serum lipid profile of rats at the end of the study period (Mean ± SD)

Analysis	Control	LD	MD	HD
TC (mmol/l)	1.9 ± 0.22[a]	1.7 ± 0.57[a]	1.4 ± 0.19[a]	1.5 ± 0.34[a]
HDL (mmol/l)	0.7 ± 0.09[a]	0.8 ± 0.26[a]	0.7 ± 0.15[a]	0.7 ± 0.17[a]
LDL (mmol/l)	0.6 ± 0.15[a]	0.5 ± 0.31[a]	0.3 ± 0.11[a]	0.5 ± 0.16[a]
TG (mmol/l)	1.2 ± 1.00[a]	0.8 ± 0.27[a]	0.8 ± 0.23[a]	0.8 ± 0.23[a]

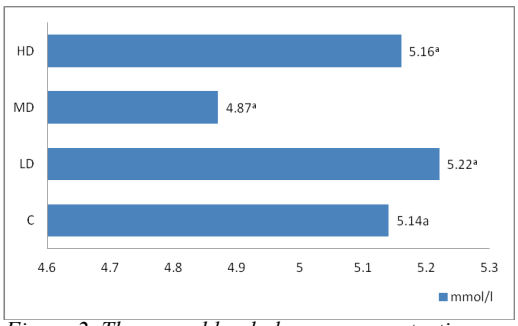

Figure 2. The mean blood glucose concentration of rats at the end of the study period

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Abstrak

Produk semula jadi daripada tumbuhan ubatan kini digunakan secara meluas di seluruh dunia sebagaimana ia dipercayai untuk menjana ubat-ubatan alternatif baru. Berdasarkan bukti nilai perubatannya, satu campuran *Hibiscus sabdariffa* L. (HS), *Clinacanthus nutans* L. (CN) dan *Stevia rebaudiana*, dalam nisbah 1:1:0.5 (HS:CN:Stevia; w/w/w) menunjukkan terapi yang berkesan sebagai diuretik dan kemopreventif. Walau bagaimanapun, tiada kajian telah dijalankan untuk menilai kesan toksik atau LD_{50} campuran ini. Oleh itu, satu penilaian toksikologi *in-vivo* piawai telah dijalankan dalam kajian pengambilan oral selama 28 hari. Dos yang diuji ialah 1,000 mg/kg, 2,000 mg/kg dan 5,000 mg/kg daripada berat badan tikus *Sprague Dawley*. Tiada sebarang kematian, tanda-tanda klinikal yang buruk dan perubahan luar biasa pada berat badan tikus. Perubahan toksikologi ketara hanya dilihat dalam berat relatif hati, sel darah merah, hematokrit, hemoglobin dan jumlah protein. Walau bagaimanapun, sebarang perubahan adalah tidak signifikan selagi nilainya berada dalam julat fisiologi yang normal. Pemerhatian ini menunjukkan bahawa campuran ini boleh dikatakan tidak toksik terhadap tikus *Sprague Dawley* dan aras selamat (tiada kesan buruk diperhatikan, NOAEL) adalah lebih besar daripada 5,000 mg/kg.