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Toxic effects of *Litsea elliptica* Blume essential oil on red blood cells of Sprague-Dawley rats*

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Abstract: *Litsea elliptica* Blume leaves have been traditionally used as medicinal herbs because of its antimutagenicity, chemopreventative and insecticidal properties. In this study, the toxic effects of *L. elliptica* essential oil against Sprague-Dawley rat's red blood cells (RBCs) were evaluated. *L. elliptica* essential oil was given by oral gavage 5 times per week for 3 treated groups in the doses of 125, 250, and 500 mg/(kg body weight), respectively, and the control group received distilled water. Full blood count, RBC osmotic fragility, RBC morphological changes, and RBC membrane lipid were analyzed 28 d after the treatment. Although *L. elliptica* essential oil administration had significantly different effects on hemoglobin (Hb), mean cell hemoglobin concentration (MCHC), mean cell volume (MCV), and mean cell hemoglobin (MCH) in the experimental groups as compared to the control group (P<0.05), the values were still within the normal range. *L. elliptica* induced morphological changes of RBC into the form of echinocyte. The percentage of echinocyte increased significantly among the treated groups in a dose-response manner (P<0.001). The concentrations of RBC membrane phospholipids and cholesterol of all treated groups were significantly lower than those of control group (P<0.001). However, the RBC membrane osmotic fragility and total proteins of RBC membrane findings did not differ significantly between control and treated groups (P>0.05). It is concluded that structural changes in the RBC membrane due to *L. elliptica* essential oil administration did not cause severe membrane damage.

Key words: *Litsea elliptica*, Toxicity, Red blood cells, Morphology, Cell membrane, Echinocyte
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INTRODUCTION

Litsea elliptica Blume of the Lauraceae family is widely found in the region of South East Asia (Jiwajinda *et al*., 2002). Extracts of its leaves are used in the traditional native herbal medicine to treat conditions such as stomach ulcers, fever and headache (Grosvenor *et al*., 1995; Jiwajinda *et al*., 2002). A significant antimutagenic activity has also been reported (Nakahara *et al*., 2002). In Thailand, *L. elliptica* has been proven to reduce the incidence of gastric cancer (Bhamarapravati *et al*., 2003).

The search for new insecticides from natural products based on their highly biodegradable, environmentally friendly minimal use properties (Mittal and Subbarao, 2003) is needed. The essential oil of *L. elliptica* leaves displayed potential to be used as insecticide (Rohani *et al*., 1997). Furthermore, repellency properties against *Aedes aegypti* bites have also been reported (Jantan and Zaki, 1998).

The use of insecticides involves a major health concern due to their extensive applications in

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household products and public health programs. The toxic effects of insecticides on biological systems gained much attention from previous researches (Celik and Suzek, 2008; Nehez *et al*., 1994). Insecticides considered as toxicants can even cause damage to the biological systems of the non-target organisms (Celik and Suzek, 2008).

Hematological constituents provide information for evaluating the biotoxicity of certain compounds (Celik and Suzek, 2008). Suwalsky *et al*.(2006) studied the effects of *Ugni molinae* extract on the red blood cells (RBCs) and found that this plant extract affected the morphology and lipid membrane composition of the RBC. The interaction between the plant extract and the membrane of RBC could possibly alter its morphology.

The main aim of the present study was to examine the toxic effect of *L. elliptica* essential oil treatment on RBC changes. Hematological parameters, morphological changes, membrane lipid and protein compositions, and membrane stability of RBCs induced by different concentrations of *L. elliptica* essential oil were examined.

MATERIALS AND METHODS

Plants materials and extraction

The leaves of *L. elliptica* were collected from Bangi Forest Reserve, Selangor, Malaysia (longitude 3°0′0″ N and latitude 102°19′60″ E), with a voucher specimen numbered as FRI41999 deposited at the Herbarium of the Forest Research Institute Malaysia, Kepong. The leaves were dried at room temperature (25~28 °C) and were then grinded to smaller particles. Clevenger apparatus was used to extract the essential oil by steam distillation for at least 8 h.

Animals

Forty female Sprague-Dawley rats provided by Laboratory Animal Resources Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia, were used. The animals were 3 months old and weighed (200±20) g. Two rats were housed per cage and were fed standard diet (mouse pellet 702 P, Gold Coin Sdn. Bhd.) and tap water ad libitum. After acclimatization period, the rats were divided into four groups with 10 rats each. *L. elliptica* essential oil in the doses of 125, 250, and 500 mg/(kg body weight (BW)) was administered to the 3 treated groups, respectively, by oral gavage route 5 times per week for 28 d. The control group received distilled water.

The study was performed according to the Organization for Economic Co-operation and Development (OECD) test guideline 407 (OECD, 2000). The dose of 500 mg/(kg BW) was based on our previous preliminary study (Siti Nor Ain, 2008) in which we had determined the No-Observe-Adverse-Effect-Level (NOAEL). The study was duly approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). On Day 28, all animals were anesthetized with diethyl ether, and blood specimens were obtained by sinus orbital, transferred into heparinized tubes, and placed on ice for 2~4 h.

Hematological study

Freshly collected blood samples were analyzed using an automatic hematology analyzer: ABC vet. Isolab Sdn. Bhd. (France). Total RBCs, total white blood cells (WBCs), hemoglobin (Hb) concentration, haematocrit (Hct), mean cell hemoglobin (MCH), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), and platelet (Plt) were analyzed.

For osmotic fragility determination, the procedure by Darcie and Lewis (1994**)** was employed. Five triplicate sets of plain tubes containing 5 ml of sodium chloride solution (0~0.85% (w/v)) were prepared for determination of the osmotic fragility. A 50-μl fresh blood was added to the tubes, homogenized, and incubated for 30 min at room temperature, followed by a centrifugation at 2000 r/min for 5 min. The absorbance of the supernatant was measured using spectrometry at 540 nm. The haemolysis of RBCs in each tube was expressed as a percentage against the maximum value of absorbance of distilled water.

For morphological examination by a scanning electron microscope (SEM), the procedure by Przybylska *et al*.(1998) was followed with slight modification. RBCs were washed three times by adding 9 portions of 0.1 mol/L sodium-potassium phosphate buffer (pH 7.4) to 1 portion of whole blood, and centrifuged at 3500 r/min for 20 min. The washed RBCs were fixed with 2.5% (w/v) glutaraldehyde in the same buffer to achieve about 50% of final haematocrit for 1 h, and then were allowed to settle on standard microscopic cover glasses for 1 h. The cover glasses

were washed twice with the same buffer. Samples were postfixed with 1% (w/v) osmium tetraoxide for 1 h and dehydrated with ascending ethanol series (30%, 50%, 70%, 85%, and 95%; v/v) in 100% acetone. Subsequently, all samples were dried with CO_2, coated with gold-palladium, and examined by an SEM (Tecnai G2, FEI, USA). In each slide, 50 RBCs were classified and mean morphological index (MI) was calculated.

Extraction of lipids was performed according to a previous protocol (Folch *et al*., 1957). The total cholesterol of RBC membrane was estimated by ferric chloride, which was measured colorimetrically at 560 nm and expressed in mg/ml. In another sample of dried extract, the phospholipids were determined using ammonium ferrothiocyanate measured at 485 nm (Cynamon *et al*., 1984).

Statistical analysis

All values are expressed as mean±standard deviation (SD). Analysis of variance (ANOVA) was employed to analyze the data. Comparisons were carried out using Scheffe pos-hoc test. Differences were considered statistically significant when $P<0.05$.

RESULTS

There were no differences in RBCs, WBCs, Hct, or Plt values between all the treated and control groups. All treated groups showed a significant increase in Hb concentration compared to the control group ($P<0.05$). Groups treated with 125 and 500 mg/(kg BW) of *L. elliptica* essential oil had significantly lower levels of MCH and MCHC compared to the control group ($P<0.05$). The group treated with 250 mg/(kg BW) showed significantly lower level of MCV compared to the control group ($P<0.05$) (Table 1).

Fig.1 shows four curves of osmotic fragility in all groups. *L. elliptica* essential oil did not cause significant change in hemolysis compared with the control group (Fig.1).

The morphological changes of rat RBCs were observed under SEM (Fig.2). All the treated groups

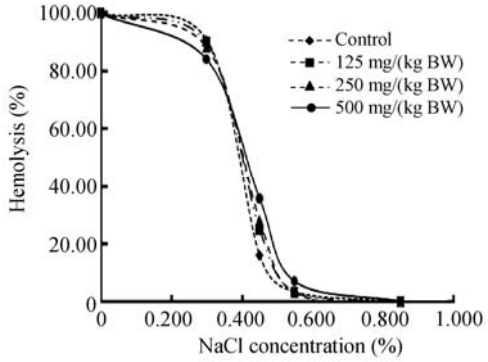

Fig.1 Percentages of osmotic fragility of RBC membrane against sodium chloride at different concentrations show no significant difference between all groups

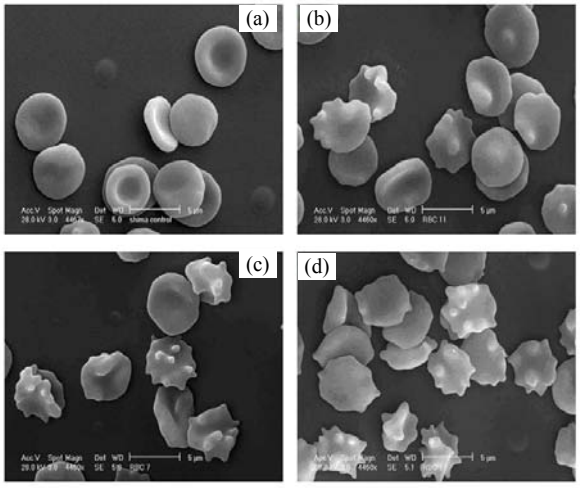

Fig.2 Effects of *L. elliptica* essential oil on the morphology of rat RBCs. SEM images of (a) untreated RBCs and RBCs treated with (b) 125 mg/(kg BW), (c) 250 mg/(kg BW), and (d) 500 mg/(kg BW). The echinocytes can be observed in (b), (c) and (d)

Table 1 Hematological parameters measured after 28 d of treatment in the groups treated with various dose of *L. elliptica* essential oil and the control group

Group	RBC ($\times10^{12}$ L^{-1})	WBC ($\times10^{9}$ L^{-1})	Hb (g/dl)	Hct (L/L)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Plt ($\times10^{9}$ L^{-1}l)
Control	7.82±0.15	10.23±0.74	13.45±0.32	0.40±0.01	51.60±0.65	17.18±0.18	33.26±0.41	655.90±63.72
L. elliptica essential oil (mg/(kg BW))								
125	7.92±0.11	12.09±1.20	14.62±0.22*	0.39±0.01	49.80±0.39	18.48±0.13*	37.14±0.15*	432.50±141.44
250	8.27±0.18	11.53±0.72	14.71±0.21*	0.40±0.01	48.80±0.63*	17.80±0.25	36.46±0.13*	491.90±119.00
500	8.16±0.16	12.64±1.39	14.82±0.27*	0.41±0.01	50.30±0.47	18.19±0.16*	36.14±0.27*	424.40±70.82

* Significantly different as compared to the control group ($P<0.05$)

showed morphological changes in the form of echinocytes. The percentage of echinocytes significantly increased among the treated groups in a dose-response manner (P<0.001). The percentages of echinocyte formation in 250 and 500 mg/(kg BW) groups were significantly higher when compared to 125 mg/(kg BW) group (P<0.01). The percentage of echinocyte formation with 500 mg/(kg BW) dose had significantly higher values compared to the dose of 250 mg/(kg BW) (P<0.001) (Fig.3).

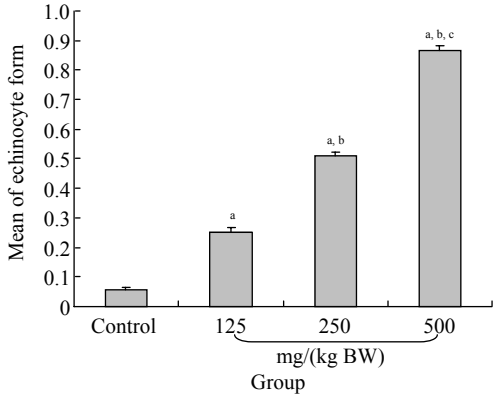

Fig.3 Means of echinocyte form for the control and treated groups

[a] Significant difference compared to the control group (P< 0.001); [b] Significant difference compared to 125 mg/(kg BW) group (P<0.001); [c] Significant difference compared to 250 mg/(kg BW) group (P<0.001)

The mean concentration of RBC membrane phospholipids was observed (Fig.4). The treated groups with doses of 125, 250, and 500 mg/(kg BW) had significantly lower level of membrane phospholipid contents compared to the control group (P<0.001). The mean concentrations of RBC membrane phospholipids of 250 and 500 mg/(kg BW) groups were significantly lower (P<0.001) than that of 125 mg/(kg BW) group. The mean concentration of RBC membrane phospholipids in the group of 500 mg/(kg BW) was significantly lower (P<0.001) than that in 250 mg/(kg BW) group. Similarly, the cholesterol content of RBCs in all the treated groups also showed patterns similar to the phospholipids (Fig.5).

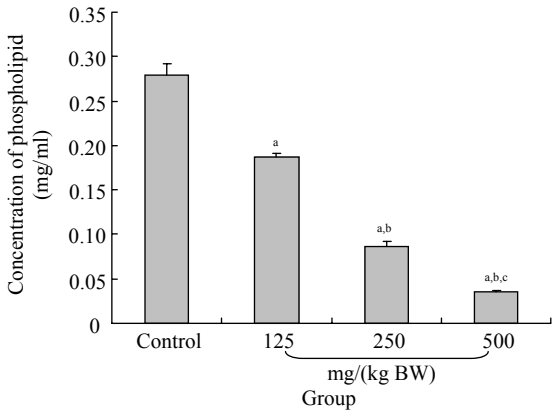

Fig.4 Concentrations of erythrocyte membrane phospholipids for the control and treated groups

[a] Significant difference compared to the control group (P< 0.001); [b] Significant difference compared to 125 mg/(kg BW) group (P<0.001); [c] Significant difference compared to 250 mg/(kg BW) group (P<0.001)

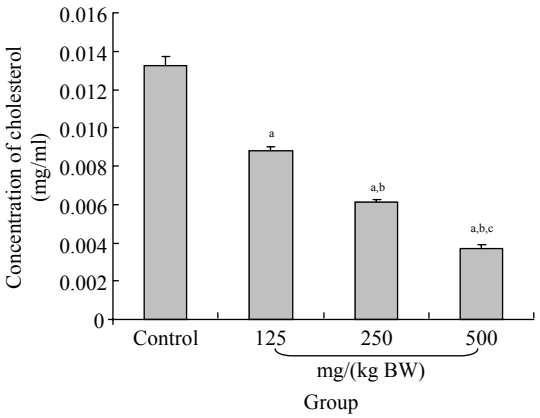

Fig.5 Concentrations of erythrocyte membrane cholesterol for the control and treated groups

[a] Significant difference compared to the control group (P< 0.001); [b] Significant difference compared to 125 mg/(kg BW) group (P<0.001); [c] Significant difference compared to 250 mg/(kg BW) group (P<0.001)

DISCUSSION

Phytotherapeutic products contain bioactive principles with potential to cause adverse effects (Bent and Ko, 2004). Even some of the roots of the plants may contain deposited toxic elements (Shi *et al*., 2008). Hence, it is important to determine the adverse effects of various plant extract. Due to poor pharmacovigilance services, it is often difficult to determine the frequency of such adverse effect caused by natural products (Eisenberg *et al*., 1998). Thus, all the natural products used in therapeutics must be submitted for efficacy and safety tests by the same methods employed for new synthetic drugs (Talalay and Talalay, 2001).

It is necessary to determine the dose of *L. elliptica* essential oil to be used in animal and human

studies (Da Silva *et al*., 2002; Janbaz *et al*., 2002; Álvarez *et al*., 2004; Hasumura *et al*., 2004). Based on the acute toxicity study of the *L. elliptica* essential oil (Siti Nor Ain, 2008), the doses to be evaluated in 28-d repeated dose studies were found to be 125 mg/(kg BW) (1/4 NOAEL), 250 mg/(kg BW) (1/2 NOAEL), and 500 mg/(kg BW) (1 NOAEL).

Regarding the hematological parameters, the data showed significant differences in Hb, MCV, MCH, and MCHC between the treated and control groups. However, the values of Hb, MCH and MCHC were still within the normal range, i.e., Hb, 13.1~16.7 g/dl; MCV, 51~63 fl; MCHC, 32.4~37.8 g/dl; and MCH, 17~22 pg (Sanderson and Philips, 1981). These results show that *L. elliptica* essential oil has no significant adverse effects on the RBC parameters. We observed no toxicity in the other systems of the body.

According to World Health Organization (WHO, 1995), alpha cypermethrin, carbendazim, and chlorpyrifos used as insecticides are reported to cause anemia in rats at various doses. Based on the hematological parameter results obtained from this study, *L. elliptica* essential oil treatment did not cause anemia in all the treated rats. Since *L. elliptica* essential oil did not affect hematological parameter values, it has an advantage over the other synthetic insecticides mentioned above for future utilization.

The normal mammalian RBC is a flexible biconcave disk in shape. Studies had shown that RBCs respond to various treatments by various agents by altering their morphological features (Przybylska *et al*., 1998). A previous study reported that *Balbisia peduncularis* extract caused changes in the normal erythrocyte morphology observed under an SEM (Suwalsky *et al*., 2008). The same study also observed the formation of echinocytes, stomatocytes, and spherocytes (Suwalsky *et al*., 2008).

Interestingly, an earlier study reported that the plasma membrane changes following administration of guava extract (Abreu *et al*., 2006). The bilayer couple theory of membrane explains the interaction of amphiphatic drug membrane with human RBCs (Sheetz and Singer, 1974). An in vitro study performed by Suwalsky *et al*.(2008) showed that *Aristotelia chilensis* aqueous extracts were able to induce morphological changes of RBCs from normal discoid shape to echinocyte form. The changes explained the interaction of the extract with the membrane's outer phospholipids monolayer.

The present study shows that *L. elliptica* essential oil induced a morphological change from the normal discoid shape to an echinocytic form in rat RBCs. Morphological alterations induced by *L. elliptica* essential oil could be explained by the lipid bilayer structure. Shape alterations evolved from a differential expansion of amphiphatic molecules into two monolayers of cell membranes. *L. elliptica* essential oil is thought to be preferentially intercalated in the outer hemi-leaflet of the lipid bilayer and, at sublytic concentrations, could cause erythrocytes transformation from discoid to echinocytic forms.

The percentages of RBC echinocyte forms treated by *L. elliptica* essential oils were shown in a dose-response manner. As the dose increased, the morphological changes were getting more prominent in the treated groups due likely to the interaction of *L. elliptica* essential oil compounds with the RBC membrane. This evidence was supported by the reduction in the concentrations of cholesterol and phospholipids at the erythrocytes membrane treated by *L. elliptica* essential oil. The cholesterol and phospholipids concentrations had inverse correlation with the echinocytes formation. Therefore, the lower cholesterol and phospholipids concentrations possibly caused greater morphological changes in the RBCs.

L. elliptica essential oil has the potential as a botanical insecticide (Hidayatulfathi *et al*., 2003; 2004). The lipophilicity of most pesticides makes lipid-rich membranes the important targets of their interaction with living organism, such as RBC membrane (Suwalsky *et al*., 2006). It is likely that *L. elliptica* essential oil had acted in the same manner on the rat RBC membrane.

Osmotic fragility is a sensitive marker of changes in osmotic pressure characteristic of RBCs and it is related to many pathological conditions (Kolanjiappan *et al*., 2002). Osmotic fragility test is used to study structural disorders (Mahieu *et al*., 2000) and rigidity of the RBC membrane (Jokinen *et al*., 2004), and has been modified due to changes in the structure and greater rigidity, which lead to hemolysis. The interaction of certain chemicals with membrane component could stimulate changes in lipid distribution (Oteiza, 1994). This could be responsible for the

cationic flow disturbances and would result in the rigidity changes of the membrane. An earlier study by Shiva Shankar Reddy *et al*.(2007) showed that the interaction between aluminum on Na-K-ATP_{ase} and Ca-ATP_{ase} activities could lead to greater membrane rigidity and changes in osmotic fragility test.

In this study the alteration of membrane cholesterol and phospholipids compositions was detected and the morphological changes in the structure were observed. However, the osmotic fragility test showed no significant difference in all the treated groups. According to Udden (2005), the changes in osmotic fragility test are the late end-point of RBC membrane damage, which could perhaps explain that the structural changes that occurred in the RBC membrane due to *L. elliptica* treatment did not cause severe membrane damage.

CONCLUSION

In conclusion, the 28-d oral supplementation of *L. elliptica* essential oil altered the morphology and membrane lipid content of RBC, but did not cause severe structural damage. Future extensive studies are recommended to evaluate the safety assessment of *L. elliptica* essential oil before it could be developed into a household insecticide.

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