28-Day repeated dose oral toxicity study of *Litsea cubeba* essential oil in Sprague-Dawley rats

Siraprapa Tubtim¹* and Ake Wasiksiri²

¹Faculty of Pharmacy, Huachiew Chalermprakiet University, 18/18 Bangna-Trad Road, K.m 18, Bangplee District, Samutprakarn, 10540, Thailand.

²School of Pharmacy, Rangsit University, Muang Ake, Phathum Thani 12000, Thailand

*Corresponding author: Tel: +66 (0) 2312 6300 ext. 1622; E-mail address: Siraprapa.t@hcu.ac.th

Abstract:

*Litsea cubeba* essential oil is a raw material source for the isolation of citral that can be converted to a number of important derivatives. This citral-rich essential oil is also increasingly used for flavour and fragrance applications in many household products. As there have been little safety information about short term use of *Litsea cubeba* oil, this repeated dose oral toxicity study was carried out. Four groups of 6 male and 6 female Sprague-Dawley rats respectively received a daily dose of 0 (control), 1, 10 and 100 mg/kg/day of *Litsea cubeba* oil by gavage for 28 consecutive days. Daily cage-side observation on clinical signs of toxicity and weekly measurement of body weight were done. Clinical biochemistry and hematological examinations of blood sample were performed at the end of the treatment. Measurement of organ weight relative to body weight, gross necropsy and histological examination were done at the terminal sacrifice. All the rats survived to the end of the study period. No concrete evidences of toxicities attributable to treatment with *Litsea cubeba* oil were observed on clinical signs, serum biochemistry examination of liver and renal function, hematological examination and also histological evaluation of liver, kidneys, heart and spleen. Changes in relative liver and kidney weight, however, were detected in certain treated groups but considered not to be of toxicological significance. Male rats receiving *Litsea cubeba* oil exhibited significant reduction in body weight gain at weekly measurement (p < 0.05). Therefore, repeated administration of *Litsea cubeba* oil at the given dose to Sprague-Dawley rats under this study condition produced no toxicologically significant effects. However, some considerable effects as observed in this study should not be overlooked.

Keywords: Essential oil; *Litsea cubeba*; Toxicity; 28-day repeated dose
Introduction

*Litsea cubeba* (Lour.) Pers. (Family Lauraceae) is a native of Southeast Asia, now cultivated in Taiwan, Indonesia and the Republic of China. It is a deciduous small tree with slender branchlets, bearing bunches of 3-5 aromatic small fruits. It is one of 21 *Litsea* species indigenous to Thailand. It is usually found in the natural habitat at the altitude over 1200 metres among other plants in the evergreen forests. It is a well-known tree among the tribespeople in the northern hills of Thailand (Chiangmai, Mae Hong Sorn provinces) since they have used the fruits as a flavor in their soups.

The fruits, leaves and the bark of the root and branches have ethnomedical applications [1-5]. In the Republic of China, the berries are categorized as stomachic, carminative, and expectorant. They are used in a treatment of bronchitis and dyspepsia. The root bark and leaves are employed against athlete’s foot and other skin diseases. In Indonesia, the fruits are used to treat dysentery and other bowel troubles, and also to treat venereal diseases. In Malaysia, the fruits are used in herbal mixture to drink after confinement as a diuretic, and also genito-urinary antiseptic.

The pharmacological studies showed that the essential oil from *Litsea cubeba* Pers. has antifungal, antiyeast and antibacterial activities [6-7]. It also has antispasmodic and bronchodilator activity [5]. The fruits are also emmenagogue.

The skin of the fruits is thin, smooth and somewhat translucent with oil cells. The essential oil can be extracted by distillation of the fresh fruits at the ripening stage with water as soon as possible at low temperature without crushing. The oil is then separated from the distillate and dried with anhydrous sodium sulfate. The ripening fruits yield 2-9% of essential oil. The fruit essential oils from various geological locations are different in the composition and also the ratio of the components. The fruit essential oil from Thailand contains citral as the main constituent. Other monoterpenes compounds found in this oil are camphene, citronellal, citronellol, p-cymene, geranial, geraniol, limonene, linalool, neral, alpha-pinene and alpha-isopulegole. *Litsea cubeba* oil also consists of sesquiterpenes (such as beta-caryophyllene); phenylpropanoids (safrole and eugenol); ketones (methyl heptene ketone, beta; methyl heptyl ketone) and alkenes (hept-5-en-1-ol, 2, 6-dimethyl; hept-5-en-2-ol, 6-methyl).

The essential oil from *Litsea cubeba* Pers. is a pale yellow, mobile oil of intensely citrus type fragrance resembling partly lemongrass oil and partly lemon peel oil. Thus, it leads to the idea of exploitation as a flavour in various products. Because of its more widespread use and little safety information, this toxicity testing was performed in animal model to provide information on the effect of repeated administration of *Litsea cubeba* oil. Based on the Organization for Economic Cooperation and Development (OECD) Guideline [8], We conducted a 28-day repeated dose oral toxicity study of *Litsea cubeba* oil in Sprague-Dawley male and female rats.

Materials and Methods

**Test materials**

*Litsea cubeba* Pers. was collected from the road-side forests at Doi Suthep of Chiangmai province. Experiments showed that the right stage of the fruits to be harvested was the ripening stage when the fruits turned from yellowish to reddish. The fruit essential oil was extracted by water distillation. *Litsea cubeba* oil is a clear faint yellowish oil of citrus type fragrance resembling partly lemongrass oil and partly lemon peel oil. The specific gravity is 0.7995, the refractive index is 1.480 at 30.4°C and the optical rotation is -29.8 at 30°C. It was stored in stopped container, well-filled, and protected from light and heat in a cool and dry place. The *Litsea cubeba* oil was also stored away from incompatible materials such as oxidizing agents, strong acids and strong bases. The *Litsea cubeba* essential oil was diluted with soybean oil (Thai Vegetable Oil Public Company Limited) before use.
Animals

Healthy Sprague-Dawley male and female rats were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhonpathom province. Female rats were nulliparous and nonpregnant. Initial body weights were 272-315 grams for males and 211-267 grams for females.

Methods

The study was carried out based on the 1993 OECD guidelines. All the rats were acclimatized to the laboratory conditions for 2 weeks prior to the study at room temperature of 23 ± 1 °C. Before the test, the rats were randomly assigned to the treatment and control groups, 12 rats per group (6 males and 6 females). The rats were kept in suspended stainless steel cages, each consisting of 2-3 rats. Commercial pelleted food from Charoen Pokphand Animal Food Co.Ltd was given with an unlimited supply of drinking water. Three dose levels (1, 10, 100 mg/kg body weight) of *Litsea cubeba* essential oil diluted with soybean oil were orally administered to the rats by gavage using stomach tubes, one dose per group. Dosing was performed everyday for 28 days. Control rats were dosed with soybean oil at equivolume as the treatment groups and handled in an identical manner.

Clinical observations

General clinical observations for morbidity and mortality were made everyday. Daily cage-side observations did not demonstrate any changes in the skin, fur, eyes, respiratory system and behavioral patterns. The animals were weighed prior to the study and at weekly intervals.

Clinical pathology determinations

At the end of the study period, blood samples were collected from orbital sinus of the rats under ether anesthesia for hematological and clinical biochemistry examinations. Hematological parameters including hematocrit value, hemoglobin concentration, number of red blood cells, white blood cells and platelets were investigated using automatic cell counter, Coulter MAX.M. Clinical biochemistry determinations of liver and renal function were carried out using automated analyzer, DADE Dimension AR. Parameters for liver function test were aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities. Parameters for renal function test were urea nitrogen and serum creatinine concentrations.

Gross necropsy and histological examinations

After blood sample collection at the end of the study period, all the rats were sacrificed. Major organs and tissues were examined for grossly visible lesions. The liver, kidneys, heart and spleen were weighed wet as soon as possible after dissection and preserved in 10% buffer formalin for further histopathological examination. The microscopic evaluations were completed by an independent pathology laboratory.

Statistics

Mean values and standard deviation (SD) of all quantitative data from each treatment group were calculated and compared with that of the respective control groups. Student’s t-test was used to calculate statistical significance of the differences. The p < 0.05 level was taken as significant.

Result

Clinical signs of toxicity

It was observed that the animals given *Litsea cubeba* oil were generally healthy. Daily cage-side observations did not demonstrate any changes in the skin, fur, eyes, respiratory system and behavioral patterns. All the rats survived to the end of the study.

Clinical pathology determinations

Clinical biochemistry determinations

As detailed in Table 1, after 28 days of *Litsea cubeba* oil administration to male rats, there were no statistically significant differences between the control group and each treatment group in urea nitrogen, serum creatinine concentration and also activities of
AST, ALT and ALP. In female rats, the only statistically significant differences ($p < 0.05$) between the treatment groups and the control group were found in the case of urea nitrogen concentration and ALP activity. Serum urea nitrogen in females of the 1 mg/kg/day dose was statistically significantly lower compared with the control group ($p < 0.05$). The other dose levels did not produce similar effects. There were also significant increases ($p < 0.05$) in ALP activities of female rats at two highest doses.

**Hematological determinations**

As detailed in Table 2, no significant differences in any of the hematological parameters were found in male rats at any of the doses. Female rats given 10 mg/kg/day of *Litsea cubeba* oil, however, demonstrated statistically significant decrease in hematocrit value, hemoglobin level and red blood cell numbers in comparison with the control group. The number of white blood cells was also higher in female rats at 10 mg/kg/day dose. These significant changes in hematological parameters did not occur in females of 1 and 100 mg/kg/day doses. The number of platelets in treated male and female rats at any dose level was similar to that of the respective control groups.

**Body weight gain**

Weekly measurement of body weights of all the rats was performed in this study. The control group receiving only soybean oil and also all treated male and female rats gained weight during the study period. As depicted in Figures 1 and 2, male rats given 10 and 100 mg/kg/day doses exhibited lower body weight gain ($p < 0.05$) when compared with the control group at the first and second week of the treatment. This reduction in body weight gain was more pronounced at the third and fourth weeks. At the end of the test period, all treated male groups had lower body weight gain than the control group ($p < 0.05$). At the second week of treatment, female rats receiving the two highest doses demonstrated significant ($p < 0.05$) but transient decreases in body weight gain in comparison with the control group. After three and four weeks of treatment, body weight gain of female rats at any dose level was not statistically significantly different from the control group.

**Postmortem observations**

**Gross necropy examinations**

Gross necropsy observations immediately after organ dissection did not reveal any pathological abnormalities. As depicted in Figures 3 and 4, there are significant decreases ($p < 0.05$) in liver weight (relative to body weight) of male rats at the 1 mg/kg/day dose and 100 mg/kg/day dose. On the contrary, significant increase ($p < 0.05$) in relative liver weight was observed in female rats at the 100 mg/kg/day dose. Significant increases in relative kidney weight were also evident in male rats at the 100 mg/kg/day dose and in female rats at the 10 and 100 mg/kg/day dose. Measurement of relative spleen weight did not exhibit differences between treated male or female rats and the respective control groups. *Litsea cubeba* oil also did not produce change in relative heart weight of male rats but only at the intermediate dose level in female rats was a higher heart weight apparent.

**Histological examinations**

The histopathological results of the four major organs of all the rats studied were summarized as the followings:

**Liver** : Both of the control and *Litsea cubeba* oil-treated groups showed similar results. Cell structure and formation appeared normal except for the occurrence of small vacuoles in the cytoplasm of some cells. A small number of fat droplets were also observed.

**Kidneys** : In control group, there were no considerable changes at the glomerulus and epithelial cell of the tubules except for the occurrence of hyaline casts within the lumen of certain tubules, in the cortex and medulla. In some rats, cystic formation was observed in some area of the cortex together with the infiltration of adipose tissue in the cortex and medulla. The similar changes were found in most of the *Litsea cubeba* oil-treated groups. However, in 10 and 100 mg/kg group, a little more hyaline casts were detected. Vacuolar degeneration was also observed in some epithelial cells of certain tubules.
Table 1  Clinical biochemistry parameters after 28-day repeated doses of *Litsea cubeba* oil in male and female rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0 mg/kg of <em>Litsea cubeba</em> oil (Control)</th>
<th>1 mg/kg of <em>Litsea cubeba</em> oil</th>
<th>10 mg/kg of <em>Litsea cubeba</em> oil</th>
<th>100 mg/kg of <em>Litsea cubeba</em> oil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>28.70 ± 1.74</td>
<td>33.53 ± 6.19</td>
<td>31.74 ± 3.09</td>
<td>28.36 ± 2.54</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.57 ± 0.05</td>
<td>0.55 ± 0.04</td>
<td>0.58 ± 0.05</td>
<td>0.53 ± 0.06</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>189.20 ± 32.58</td>
<td>228 ± 55.32</td>
<td>183.67 ± 40.96</td>
<td>178.00 ± 55.12</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>56.60 ± 9.04</td>
<td>56.17 ± 3.66</td>
<td>53.5 ± 2.35</td>
<td>47.33 ± 7.94</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>459.40 ± 100.51</td>
<td>385.17 ± 45.16</td>
<td>456 ± 107.41</td>
<td>432.60 ± 88.22</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>31.58 ± 2.87</td>
<td>27.07 ± 2.78</td>
<td>36.35 ± 9.82</td>
<td>28.37 ± 3.17</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.67 ± 0.05</td>
<td>0.63 ± 0.05</td>
<td>0.69 ± 0.18</td>
<td>0.66 ± 0.06</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>207.67 ± 24.91</td>
<td>218.83 ± 39.26</td>
<td>228.33 ± 75.90</td>
<td>222.50 ± 60.81</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>57.83 ± 4.67</td>
<td>61.00 ± 11.59</td>
<td>73.20 ± 15.80</td>
<td>80.00 ± 35.55</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>296.33 ± 18.59</td>
<td>367.50 ± 132.27</td>
<td>407.00 ± 73.24</td>
<td>441.83 ± 76.90</td>
</tr>
</tbody>
</table>

* Values of clinical biochemistry parameters were presented as mean ± standard deviation (SD) (n=6)

* = Statistically significant difference from controls (p < 0.05)

Table 2  Hematological parameters after 28-day repeated doses of *Litsea cubeba* oil in male and female rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0 mg/kg of <em>Litsea cubeba</em> oil (Control)</th>
<th>1 mg/kg of <em>Litsea cubeba</em> oil</th>
<th>10 mg/kg of <em>Litsea cubeba</em> oil</th>
<th>100 mg/kg of <em>Litsea cubeba</em> oil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of red blood cells (X10^{12}/L)</td>
<td>7.93 ± 0.83</td>
<td>7.72 ± 1.48</td>
<td>7.20 ± 1.55</td>
<td>7.96 ± 1.12</td>
</tr>
<tr>
<td>Hemoglobin concentration (g/dl)</td>
<td>15.98 ± 1.46</td>
<td>15.42 ± 2.92</td>
<td>14.17 ± 2.94</td>
<td>15.42 ± 2.14</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>44.26 ± 3.97</td>
<td>42.07 ± 8.60</td>
<td>38.77 ± 8.54</td>
<td>43.70 ± 6.27</td>
</tr>
<tr>
<td>No. of white blood cells (X10^{9}/L)</td>
<td>10.30 ± 1.69</td>
<td>8.68 ± 0.74</td>
<td>11.22 ± 2.52</td>
<td>9.42 ± 2.46</td>
</tr>
<tr>
<td>No. of platelets (X10^{9}/L)</td>
<td>721.25 ± 151.54</td>
<td>568.67 ± 170.08</td>
<td>662.80 ± 106.90</td>
<td>616.50 ± 222.21</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of red blood cells (X10^{12}/L)</td>
<td>7.57 ± 0.31</td>
<td>7.00 ± 1.46</td>
<td>6.06 ± 1.04*</td>
<td>6.86 ± 1.35</td>
</tr>
<tr>
<td>Hemoglobin concentration (g/dl)</td>
<td>15.50 ± 0.45</td>
<td>13.95 ± 2.90</td>
<td>12.10 ± 1.91*</td>
<td>13.93 ± 2.35</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42.30 ± 1.67</td>
<td>38.12 ± 8.24</td>
<td>32.12 ± 5.41*</td>
<td>37.80 ± 7.35</td>
</tr>
<tr>
<td>No. of white blood cells (X10^{9}/L)</td>
<td>5.38 ± 1.28</td>
<td>5.73 ± 1.92</td>
<td>7.44 ± 0.69*</td>
<td>5.78 ± 1.45</td>
</tr>
<tr>
<td>No. of platelets (X10^{9}/L)</td>
<td>617.75 ± 118.34</td>
<td>544.00 ± 12.73</td>
<td>747.67 ± 179.96</td>
<td>692.00 ± 188.09</td>
</tr>
</tbody>
</table>

* Values of hematological parameters were presented as mean ± standard deviation (SD) (n=6)

* = Statistically significant difference from controls (p < 0.05)
Heart: No cell death, necrosis were observed in the myocardial cells of the control group. No inflammation or fibrosis occurred. In 10 and 100 mg/kg groups, a small number of adipose cells and fibrous tissue infiltrated some area of the myocardial cells.

Spleen: In the control group, no noticeable changes were observed in the white pulp and red pulp of the spleen. However, the infiltration of red blood cells at the marginal zone of the white pulps was detected. A small number of neutrophils were also found at certain large vessels. Similar results were found in the 1 and 10 mg/kg groups. However, a little more infiltration of neutrophils and dilatation of vessels were observed in high-dose male group. The increase in active atypical cells of lymphocyte was also detected in high-dose female group.

Discussion

*Litsea cubeba* oil, the citral-rich essential oil, is increasingly used for flavour and fragrance applications such as household products (spray, fresheners, etc). Its major use, both in the People's Republic of China and international markets, is as a raw material source for the isolation of citral and it may be converted by the chemical industry to a number of important derivatives such as isocitral, pseudoionone and pseudomethylionone [9]. Although small volume of this essential oil is normally used for flavour and fragrance applications, repeated exposure still carries the possibility of health hazard. Because of its more widespread use and little safety information, multidose toxicity testing in animal model was performed in this study to provide information of repeated exposure to *Litsea cubeba* oil.

Daily observation of skin, fur, respiratory system and behavioral patterns showed no signs of toxicity to rats. No mortality occurred during the study period. Since liver and kidneys play significant roles in various metabolic and excretory processes, emphasis was placed on the effects *Litsea cubeba* oil might have on the functions of these organs. Clinical biochemistry determination of liver function through the assessments of AST and ALT activities showed no significant differences between treatment and control groups in both male and female rats. Although ALP activity was significantly higher in the two highest dose groups, this increase occurred only in the females. Although significant decreases in relative liver weight were noted in some treated male groups, this effect was not dose-dependent. Moreover, the opposite effect was found in female rats. Since there were no significant microscopic correlates for these changes in relative liver weight in both male and female rats, changes in relative liver weight in certain treated groups were considered unrelated to treatment.

For renal toxicity assessment, there were statistically significant increases in relative kidney weight of male rats receiving 100 mg/kg/day of *Litsea cubeba* oil and female rats at 10 and 100 mg/kg/day doses. Clinical biochemistry determinations of renal function through assessment of urea nitrogen and serum creatinine, however, demonstrated no significant changes in almost all treated groups. Although significant decrease in urea nitrogen was observed in females at 1 mg/kg/day dose, it was considered to have no toxicological significance because it was an isolated finding and not dose-related. No correlated gross necropsy and histological findings were also observed in these organs. The changes in relative kidney weight, therefore, were again considered unrelated to treatment.

For toxicity assessment of other organs, the significant increase in relative heart weight was observed in only female rats at 10 mg/kg/day dose. This was the isolated finding and not dose-related. Although histological examination revealed some changes in cell structure of the hearts of certain groups, they were not significantly different from the controls. Thus, the effect on heart was considered not to be of clinical significance. No significant effects of *Litsea cubeba* oil on spleen were found. The relative spleen weight of treated groups was not significantly different from the control groups. Although some structural changes of spleen were observed in some groups, they were comparable to the control group.
Figure 1  Body weight gain of male rats given daily doses of *Litsea cubeba* oil for 28 days

Figure 2  Body weight gain of female rats given daily doses of *Litsea cubeba* oil for 28 days
**Figure 3** Relative organ weights after 28-day repeated doses of *Litsea cubeba* oil in male rats

* = Statistically significant difference from controls (p < 0.05)

**Figure 4** Relative organ weights after 28-day repeated doses of *Litsea cubeba* oil in female rats

* = Statistically significant difference from controls (p < 0.05)
No hematological changes after 28 days of treatments were detected in all treated groups except for the females at 10 mg/kg/day dose. However, the decreases in hematocrit, hemoglobin concentration and number of red blood cells and also the increase in number of white blood cells in this group did not show any dose-responsiveness and should not be regarded as treatment-related. 

The only significant effect of Litsea cubeba oil could be detected on body weight gain of male rats. Weekly measurement of body weight revealed significant reduction in body weight gain of male rats. No clear evidence of reduction in body weight gain was observed in female rats.

## Conclusion

Repeated administration of Litsea cubeba essential oil at dose levels of 1, 10, and 100 mg/kg/day for 28 consecutive days to male and female Sprague-Dawley rats under this study condition did not produce toxicologically significant effects. However, some considerable effects as observed in this study should not be overlooked.

## Acknowledgements

We wish to thank the Department of Medical Sciences, Ministry of Public Health for funding this research and express utmost gratitude for Professor Dr. Sasri Punyarajun who gave us the opportunity to do this research.

## Reference


