Acute and Genetic Toxicity of Essential Oil Extracted from 

Litsea cubeba (Lour.) Pers.

MAN LUO,1,2 LI-KE JIANG,2 AND GUO-LIN ZOU1*

1College of Life Sciences, Wuhan University, Wuhan 430072, People’s Republic of China; and 2College of Life Sciences, Anhui Agricultural University, Hefei 230036, People’s Republic of China

ABSTRACT

Litsea cubeba oil is an aromatic essential oil extracted from the fresh fruits of Litsea cubeba (Lour.) Pers. It is used as a flavor enhancer in foods, cosmetics, and cigarettes; as a raw material in the manufacture of citral, vitamins A, E, and K, ionone, methyl ionone, and perfumes; and as an antimicrobial and insecticide. Based on the widespread use of L. cubeba oil, its insolubility in water, resulting in its partition in soil sediment, and its volatility when exposed to the atmosphere, risk of injury due to consumption and occupational exposure may be significant. In the present study, we studied the toxicity of L. cubeba oil with a battery of acute and genetic toxicity tests in Institute of Cancer Research mice and Sprague-Dawley rats. The oral, dermal, and inhalation 50% lethal dose and concentration (LD50 and LC50) of L. cubeba oil were determined. Results indicated that the oral LD50, the dermal LD50, and the inhalation LC50 are approximately 4,000 mg/kg of body weight, in excess of 5,000 mg/kg, and approximately 12,500 ppm, respectively. We therefore conclude that L. cubeba oil is slightly toxic. In addition, the genetic toxicity of L. cubeba oil was assessed with Salmonella Typhimurium, by determination of the induction of micronuclei in bone marrow cells, and by testing for chromosome aberration in spermatocyte cells of Institute of Cancer Research mice. The results of genetic toxicity testing of L. cubeba oil in vitro and in vivo were negative.

Litsea cubeba (Lour.) Pers. is indigenous to Eastern Asia, and the essential oil extracted from its fresh fruits is 70% citral (4). L. cubeba oil is a pale yellow, volatile essential oil with an intense lemon-like, fresh, sweet odor that is insoluble in water. China is the largest producer and exporter of L. cubeba oil in the world. In recent years, more than 4.4 million lb of L. cubeba oil has been produced per year, and three quarters of that production is exported to England, the United States, France, Germany, Holland, and other countries (2). L. cubeba oil is used as a fragrance enhancer in foods, cosmetics, and cigarettes and also as a raw material in the manufacture of citral, vitamins A, E, and K, ionone, methyl ionone (1), perfumes, and other essential oil mixtures. In China, the State Food and Drug Administration approves L. cubeba oil for use in foods as a flavoring substance and adjuvant (GB 2760-86). It is also used as a Chinese medicine for treating tracheitis and coronary heart disease. Additionally, L. cubeba oil is used as an antifungal agent and insecticide in the storage of grains, foods, archival documents, and/or clothing (13). The widespread use of L. cubeba oil consequently leads to exposure of manufacturing workers, consumers, or patients. There are many reports about the antimicrobial properties and mechanisms of action of L. cubeba oil and its main component, citral (6, 8–10, 12, 17–19, 21–24); however, there is little information about the safety of L. cubeba oil (14). To establish the safety data of L. cubeba oil, its toxicity characteristics were assessed in a battery of acute and genetic toxicity tests using Institute of Cancer Research (ICR) mice and Sprague-Dawley (SD) rats. We determined the oral, dermal, and inhalation 50% lethal dose and concentration (LD50 and LC50) of L. cubeba oil using the method reported by Holzhütter et al. (3) and the Globally Harmonized Classification System (GHS) (15) to biometrically evaluate its toxic class. In addition, the genetic toxicity of L. cubeba oil was assessed with Salmonella Typhimurium, by determination of the induction of micronuclei in bone marrow cells, and by testing for chromosome aberration in spermatocyte cells of ICR mice receiving L. cubeba oil by oral gavage.

MATERIALS AND METHODS

Test compounds. L. cubeba oil extracted from its fresh fruits was obtained in Guangxi, China, by distillation. The China standard GB/T 14156-93 (NO13) summarizes physical and chemical attributes of L. cubeba oil. In the present study, L. cubeba oil was dissolved in corn oil before use.

Animals. Six-week-old male and female ICR mice that weighed 18 to 22 g were obtained from the Animal Experiment Center of Southeast University (Jiangsu, China) (National Certificate [NC] no. SCXK [Su] 2002-0014). Male and female SD rats that weighed 180 to 210 g were between 7 or 8 weeks old at the beginning of the study and were provided by the Animal Experiment Center of Anhui University of Medical Sciences (NC no. SCXK [Wan] 2002-0016). All animals were allowed ad libitum access to standard rodent chow diet (NC no. SYXK [Su] 2002-0049) and water and were group housed in acrylic cages at a temperature of 24°C, a relative humidity of 40 to 70%, and a 12-h light-dark cycle (8 a.m. to 8 p.m.). All procedures that used animals were reviewed and approved by the Jiangsu and Anhui

* Corresponding author. Tel: 86-27-87645674; Fax: 86-27-87669560; E-mail: xionglu@ustc.edu.cn.
Institutional Animal Care and Use Committee. (The animal program is fully accredited by the Jiangsu and Anhui Association for Assessment and Accreditation of Laboratory Animal Care.)

Acute toxicity analysis of *L. cubeba* oil: acute oral toxicity test. Acute oral toxicity testing began with exposure of three female ICR mice to *L. cubeba* oil at a starting dose of 2,000 mg/kg of body weight. Three female ICR mice were randomly selected for the acute oral toxicity test. After overnight fasting, the mice were given one dose of *L. cubeba* oil dissolved in corn oil via oral gavage at a dose of 2,000 mg/kg once (the drug volume equaled 10 ml/kg). The animals’ activity, behavior, and general health status were observed twice daily for a period of 14 consecutive days. Activity was monitored once a day with a three-point scale: 1, lazy, moving slowly; 2, intermediate; and 3, active moving or searching. Body weight and food consumption were recorded daily, and the specific growth rate (SGR) was expressed as the daily weight gain: 

\[
SGR = (weight_x - weight_y)/(time_x - time_y)
\]

The mice that died during the observation and the sacrificed 14-day survivors (euthanized by CO\textsubscript{2} asphyxiation) were examined postmortem. Based on the number of moribund and/or dead female animals in the postexposure group, the test was continued at a dose of 2,000 mg/kg with three male ICR mice when zero to one female animal was moribund and/or dead at 2,000 mg/kg; the testing also continued at a dose of 1,000 mg/kg with another three female ICR mice in which the number of moribund and/or dead female animals was two to three. The parameters for choosing the dose of oral exposure and the amount and sex of the mice were as detailed by Holzhütter et al. (3).

Acute dermal toxicity test. Three male SD rats were randomly selected to be exposed to a starting dose of 2,000 mg/kg of *L. cubeba* oil. An area of approximately 5 cm\textsuperscript{2} of back hair of each rat was shaved bare unilaterally. Twenty-four hours after shaving, a drop of *L. cubeba* oil was applied at a dose of 2,000 mg/kg to the shaved area for 4 h, using occlusive aluminum foil over plastic film as a dressing. The area was then cleaned with warm sterile water. The same observation period as in the acute oral toxicity test followed the *L. cubeba* oil application. Testing continued according to the outcome of the test at 2,000 mg/kg. One possibility for testing is to continue the experiment at a dose of 1,000 mg/kg on another three male mice, since two to three animals were moribund and/or dead at 2,000 mg/kg; the other possibility is to test at a dose of 2,000 mg/kg on three female mice with the same parameters as those used in the method of Holzhütter et al. (3).

Acute inhalation toxicity test. Exposure experiments were conducted at a starting concentration of 2,500 ppm of *L. cubeba* oil, with three male and three female rats, in a cabinet with six exposure chambers. The chambers were constructed of stainless steel and glass with a nominal internal volume of approximately 150 liters. On top of the cabinet, a small electric fan was positioned to promote *L. cubeba* oil volatilization and homogenization within the chamber. A glass beaker with the specified load of *L. cubeba* oil was placed on top of an electric heater that was installed at the bottom of the cabinet. During exposure, rats were individually restrained in perforated stainless steel and plastic cylinders with conical nose pieces. The restrainers were inserted into the faceplate of the exposure chamber so that only the nose of each rat extended into the chamber. The temperature in the cabinet was kept at 28 to 29°C, and 375 ml of *L. cubeba* oil was added to the beaker. After the heater operated for 10 min, three male and female SD rats were inserted into the cabinet and were exposed nose-only for 4 h. These rats were then observed for 2 weeks per the previous description. Another exposure test was performed at 500 ppm for 4 h with a group of three male and three female SD rats, since the number of moribund and/or dead animals in the 2,500 ppm for 4 h group was three to six rats. We also tested exposure at 5,000 ppm for 4 h, since the number of moribund and/or dead animals in the 2,500 ppm group was zero to two rats (as described by Holzhütter et al. (2)).

Genetic toxicity analysis of *L. cubeba* oil: *Salmonella* Typhimurium mutagenicity test. Testing was performed as described by Zeiger et al. (20). *L. cubeba* oil was incubated with *Salmonella* Typhimurium tester strains TA98, TA100, TA1535, and TA1537 either in buffer or S9 mix (metabolic activation enzymes and such cofactors as G-6-P and NADP\textsuperscript{+} from Aroclor 1254–induced rat liver) for 20 min at 37°C. Top agar supplemented with t-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37°C. With the solvent of dimethyl sulfoxide (DMSO), 50 mg, the essential oil was added into a 10-ml volumetric flask to prepare the 5 mg/ml of oil, and then the solution was fivefold diluted with DMSO in succession of four times. The five dosage groups of the oil were 0.008, 0.04, 0.2, 1, and 5 mg/ml, and the volume used in the test was 0.1 ml per plate. Dexon and 2-amino-phenazine (2-AF) were dissolved in water, and 1,8-chrysazin was solved with DMSO, with concentrations of 0.5, 0.1, and 0.5 mg/ml, respectively, and 0.1 ml of drug was added into a plate when used. Each trial consisted of triplicates of concurrent positive (Dexon, 2-AF, 1,8-chrysazin) and negative controls (water, DMSO) and of five doses of *L. cubeba* oil (0.8, 4, 20, 100, and 500 μg per plate). The high dose was limited by toxicity. All trials were repeated once.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain-activation combination. An equivocal response is defined as an increase in revertants that is not dose related, reproducible, or of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

Chromosomal aberration test of ICR mice spermatocyte. Each test consisted of a concurrent solvent control (corn oil, 10 mg/kg), a positive control (mitomycin, 2.5 mg/kg), and three doses of *L. cubaba* oil at 460 mg/kg (½ LD\textsubscript{50}), 925 mg/kg (¼ LD\textsubscript{50}), and 1,850 mg/kg (½ LD\textsubscript{50}), respectively. The five groups of male ICR mice of a concurrent solvent control, a positive control, and three doses of *L. cubaba* oil were fed by oral gavage once a day for 5 consecutive days. Five female ICR mice (positive control) were injected intraperitoneally at a dose of 4 mg/kg one time. On day 12 of the study, all animals were injected intraperitoneally with a dose of 4 mg/kg of Colcemid; incubation then continued for 6 h. After the mice were euthanized with CO\textsubscript{2}, the testicles were observed microscopically. Sections were then dyed, and more than 50 spermatogenous cells in each testicle were observed microscopically. Data were then recorded regarding metaphase chromosomal aberration in structure, polyploid, type, and number, and data analysis was undertaken with binomial distribution.

Mouse bone marrow micronucleus test of ICR male mice. Preliminary range studies were performed. Factors that affect dose selection included chemical solubility and toxicity; the limiting
factor was toxicity. Based on the just determined oral LD$_{50}$ value of *L. cubeba* oil on ICR strain mice, the dose range was defined to include 185 mg/kg (2 $LD_{50}$), 740 mg/kg (4 $LD_{50}$), and 1,850 mg/kg (5 $LD_{50}$). Test procedures were similar to the details described by Shelby et al. (16). Five male ICR mice of each dose group were fed by oral gavage twice at 30-h intervals with *L. cubeba* oil dissolved in corn oil; the dosing volume was 10 ml/kg. In the solvent control group, only corn oil was administered to five male ICR mice by oral gavage, and five male ICR mice (positive control mice) received oral gavage of 40 mg/kg of cyclophosphamide dissolved in water (2 mg/ml). The mice were euthanized with CO$_2$ 24 h after the second oral administration, and blood smears were prepared from bone marrow cells obtained from their femurs. Air-dried smears were fixed and stained; 2,000 polychromatophilic erythrocytes were scored for the frequency of micronucleated cells in each of five animals per dose group, and the ratios of polychromatophilic erythrocytes to normal chromatophils were calculated. The results were tabulated as the mean $\pm$ standard error of the mean (SEM) of the pooled results from all animals within a treatment group. The frequency of micronucleated cells among polychromatophilic erythrocytes was analyzed by a statistical software package that tested for increasing trend over the dose group using a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group (5).

**Statistical analysis.** Data were analyzed statistically by the Student’s $t$ test or by Welch normal approximation compared with the $t$ test for different significance between control and experiment data. The data were expressed as mean $\pm$ SEM.

**RESULTS**

Gas chromatography–mass spectrometry analysis of test compounds. *L. cubeba* oil obtained in Guangxi, China, was analyzed by gas chromatography–mass spectrometry (25). Gas chromatography separated 37 peaks, and mass spectrometry identified 31 of them with National Institute of Standards and Technology mass spectral data. The identified constituents covered 94.15% of the peak area of the *L. cubeba* oil. Its major chemical ingredients are citral (66.80%), consisting predominantly of geranial (35.22%) and neral (31.58%), limonene (11.04%), 6-methyl-5-hepten-2-one (4.31%), β-linalool (2.90%), myrcene (1.66%), α-pinene (1.01%), β-pinene (0.95%), α-terpineol (0.81%), sabine (0.49%), and β-caryophyllene.

**Oral acute toxicity of *L. cubeba* oil.** Since no moribund or dead female mice were observed after exposure to a starting dose of 2,000 mg/kg, the oral exposure test was conducted at a dose of 2,000 mg/kg with three male mice. On day 6 of the study, one male mouse was euthanized after it was observed to be in a moribund state. According to biometric evaluation of the oral acute toxicity class (ATC) method for the starting dose or concentration of the GHS, the oral LD$_{50}$ values of *L. cubeba* oil were found to be approximately 4,000 mg/kg, placing it in hazard class 5, which is the lowest toxic class in the GHS (Table 1).

After oral exposure via gavage, all mice appeared dysphoric and overactive (activity scale 3). Thirty minutes later, the mice gradually became motionless (activity scale 1); 24 h later, most returned to normal levels of activity. Only one male exhibited clinical signs during the experimental period, including totter, lethargy, and decreased food consumption. This mouse was removed from the study on day 6 due to a significantly lower mean body weight gain for 5 days compared with those of the group and also due to its moribund signs. During the 2-week observation period, feed consumption of exposed ICR mice was less than that of the controls through day 5. However, food consumption returned to control values by day 10 and increased a little compared with the control during days 11 to 15. Necropsy was performed on the moribund mouse after euthanasia; the results revealed inflammation and hemorrhage of the stomach, hyperplasia of the squamous mucosa, and slight hyperplasia and squamous metaplasia of the respiratory epithelium. At day 15, macroscopic examination did not reveal any abnormal signs in the size and appearance of visceral organs in other mice exposed to a dose of 2,000 mg/kg of *L. cubeba* oil.

**Dermal acute toxicity of *L. cubeba* oil.** Since none of the male rats exposed to a starting dose of 2,000 mg/kg were dead or moribund, the dermal exposure test was again performed at a dose of 2,000 mg/kg with three female rats. Note that none of the rats were moribund or dead in either the exposure period or the 2-week observation. According to the GHS, for the dermal ATC method with starting doses, the dermal LD$_{50}$ of this material exceeds 5,000 mg/kg, and the toxic class of *L. cubeba* oil is above category class 5 (Table 1).

Throughout the experimental period, no noticeable behavior or activity changes were observed in any rats, and no treatment-related illness or death occurred. There was no observable difference in the animals’ hair luster between the exposed and control groups. No alteration in food consumption was observed in any rat, and no body weight loss was observed in the rats exposed to *L. cubeba* oil; in fact, the SGR values increased throughout the 14-day studies (Table 2). Slightly higher, but not statistically significant, body weights were noted in all rats compared with those of controls at day 15.

The oil-induced skin irritation by *L. cubeba* oil changed from mild erythema and moderate edema to marron, roughness, and scab within 1 week of exposure. Histological examination revealed significant hyperplasia of the squamous epithelium, spinous cells from derma and hypoderm, and additionally hyperplasia of the horny layer. Basal cells were active, and displayed slight hyperplasia of collagenous fibers, capillary blood vessels, and fibroblasts.

**Table 1.** LD$_{50}$ and LC$_{50}$ values and toxicity classification of *Litsea cubeba* oil.

<table>
<thead>
<tr>
<th>Species</th>
<th>Route of exposure</th>
<th>LD$<em>{50}$/LC$</em>{50}$</th>
<th>Toxic class in GHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR mouse</td>
<td>Oral</td>
<td>$\sim$4,000 mg/kg</td>
<td>5$^a$</td>
</tr>
<tr>
<td>SD rat</td>
<td>Inhalation</td>
<td>$\sim$12,500 ppm</td>
<td>5</td>
</tr>
<tr>
<td>SD rat</td>
<td>Dermal</td>
<td>$\geq$5,000 mg/kg</td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$ Class 5 is the lowest toxic class in GHS.
TABLE 2. Specific growth rate (SGR) and survival of animals exposed to Litsea cubeba oil

<table>
<thead>
<tr>
<th>Route of exposure</th>
<th>No./sex of animals</th>
<th>Days 0–5 (mean ± SEM)</th>
<th>Days 6–10 (mean ± SEM)</th>
<th>Days 11–15 (mean ± SEM)</th>
<th>Body wt change at 15 days compared with control (mean ± SEM)</th>
<th>Survival ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral (ICR mice)</td>
<td>3/M</td>
<td>-0.4 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>-2.4 ± 0.3</td>
<td>83.3%</td>
</tr>
<tr>
<td>Inhalation (SD rats)</td>
<td>3/M</td>
<td>-2.6 ± 0.1</td>
<td>4.2 ± 0.2</td>
<td>4.2 ± 0.8</td>
<td>-9.2 ± 0.8</td>
<td>100</td>
</tr>
<tr>
<td>Dermal (SD rats)</td>
<td>3/F</td>
<td>1.4 ± 0.3</td>
<td>2.0 ± 0.5</td>
<td>5.2 ± 0.3</td>
<td>4.8 ± 0.1</td>
<td>100</td>
</tr>
</tbody>
</table>

a Death of animals only happened during the 2-week observation period after exposure; the survival ratio = number of surviving animals/total of oral exposure experiment = 5/6 = 83.3%.

b Data are from animals in group exposed to 5,000 ppm of L. cubeba oil; data of the 2,500 ppm group are not shown.

c Death of animals only happened during the L. cubeba oil exposure period; the survival ratio = number of surviving animals/total of inhalation exposure experiment = 11/12 = 91.6%.

In histological findings, because slight hyperplasia of derma and hypoderm was observed. No ulceration and necrosis, no adverse effects on skin accessory, and no other abnormal physiological responses were exhibited.

Inhalation acute toxicity of L. cubeba oil. None of the rats exposed to a starting concentration of 2,500 ppm died. Therefore, the inhalation exposure test was continued at a concentration of 5,000 ppm with another six rats, three male and three female SD rats. The only difference for this group was that 750 ml of L. cubeba oil was added into the beaker; other test parameters remained as in the previous group. One female rat died during the exposure period. According to the GHS, of the inhalation ATC methods for starting a concentration, the inhalation LC50 value was approximately 12,500 ppm; thus, the hazard of L. cubeba is class 5 (Table 1).

Ten minutes after inhalation exposure to 2,500 ppm of oil, animals were actively moving. After 30 min, animals were moving slowly. After 4-h exposure, three female and three male SD rats returned to normal activity levels when fresh air was blown into the chamber, and no abnormal signs were monitored during the 2-week observation period. In an inhalation exposure experiment of 5,000 ppm, after approximately 10 min some animals became restless and jumpy and exhibited coiled tails and 30 min later all animals were lethargic. After 4 h of exposure, five animals (two female and three male SD rats) revived and gradually recovered activity by 24 h, but at 48 h one female rat died of coma.

In the group of three male and three female rats exposed to 2,500 ppm, the animals’ feed intake and body weight gain showed no significant difference compared with those of the controls. However, in the 5,000 ppm group, in which five mice survived (three male and two female SD rats), feed consumption was less than that of the control for the first 5 days and was more than that of the control for days 6 through 14. The mean body weight gain of these five animals exposed to 5,000 ppm was less than those of the control for days 1 to 5 and increased from day 6 onward; however, the mean body weight of the five survivors was less than those of the controls at the end of the 2-week observation period (Table 2). Necropsy of the dead female rat revealed clinical findings, including pulmonary inflammation and pulmonary hemorrhage as evidenced by blood clot and cytoplasmic vacuolization of hepatocytes, hyperplasia of the squamous mucosa, and squamous metaplasia of the respiratory epithelium. For all surviving rats, histological examinations did not uncover any significant differences in the size and appearance of visceral organs compared with those from the control group.

Genetic toxicity of the L. cubeba oil. The results indicate that whether in the S9 mix or in the buffer trial, the number of revertant colonies was similar to those in the concurrent solvent control, and there was no dose-related response (Table 3). The number of revertant colonies in positive controls was above 2.7 times that of the concurrent solvent control. Based on these data, L. cubeba oil had no mutagenic effects on the four tester strains.

The incidences of micronucleated cells in the three dose groups were 1.8, 1.6, and 1.8%, respectively, which was not significantly different from those of the concurrent solvent (1.8%; P > 0.05) but had remarkable differences when compared with positive controls (49.6%; P < 0.01) (Table 4). A small decrease in the frequency of micronucleated polychromatolytic erythrocytes was noted in the 740 mg/kg group, but this was not statistically significant. Thus, L. cubeba oil does not appear to result in bone marrow micronuclei.

The incidence of chromosomal aberration in the L. cubeba oil dose group (0.2, 0.6, and 0.4%) was not statistically significant from the concurrent solvent group (0.4%), nor was there a dose-dependent relationship among the groups (P > 0.05) (Table 5). However, the rate value of the positive control group (4.6%) was much higher than that of the concurrent solvent control (P < 0.01). The type of fragment dominant in chromosomal aberrations and ring-like quadrivalent also appeared in positive controls. Therefore, L. cubeba oil did not lead to chromosomal aberration.

DISCUSSION

We performed acute and genetic toxicity studies of L. cubeba oil because there is widespread human exposure.
TABLE 3. Mutagenicity of Litsea cubeba oil in Salmonella Typhimurium

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dosage/plate</th>
<th>TA98 -S9</th>
<th>+ 10% S9</th>
<th>TA100 -S9</th>
<th>+ 10% S9</th>
<th>TA1535 -S9</th>
<th>+ 10% S9</th>
<th>TA1537 -S9</th>
<th>+ 10% S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.1 ml</td>
<td>34 ± 2</td>
<td>33 ± 2</td>
<td>160 ± 12</td>
<td>168 ± 10</td>
<td>160 ± 21</td>
<td>173 ± 21</td>
<td>269 ± 21</td>
<td>280 ± 28</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.1 ml</td>
<td>31 ± 2</td>
<td>34 ± 1</td>
<td>154 ± 10</td>
<td>160 ± 13</td>
<td>165 ± 24</td>
<td>189 ± 23</td>
<td>259 ± 26</td>
<td>273 ± 28</td>
</tr>
<tr>
<td>Sample</td>
<td>0.8 µg</td>
<td>32 ± 3</td>
<td>35 ± 2</td>
<td>151 ± 13</td>
<td>159 ± 16</td>
<td>167 ± 24</td>
<td>180 ± 20</td>
<td>268 ± 28</td>
<td>275 ± 25</td>
</tr>
<tr>
<td>4 µg</td>
<td>31 ± 2</td>
<td>33 ± 3</td>
<td>156 ± 12</td>
<td>165 ± 15</td>
<td>157 ± 20</td>
<td>171 ± 22</td>
<td>273 ± 24</td>
<td>287 ± 23</td>
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<tr>
<td>20 µg</td>
<td>30 ± 1</td>
<td>32 ± 2</td>
<td>160 ± 19</td>
<td>177 ± 91</td>
<td>169 ± 22</td>
<td>184 ± 23</td>
<td>265 ± 22</td>
<td>274 ± 27</td>
<td></td>
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<tr>
<td>100 µg</td>
<td>33 ± 3</td>
<td>34 ± 3</td>
<td>165 ± 12</td>
<td>172 ± 18</td>
<td>163 ± 22</td>
<td>178 ± 23</td>
<td>272 ± 21</td>
<td>284 ± 25</td>
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</tr>
<tr>
<td>500 µg</td>
<td>20 ± 4</td>
<td>24 ± 2</td>
<td>153 ± 93</td>
<td>160 ± 11</td>
<td>167 ± 19</td>
<td>181 ± 23</td>
<td>263 ± 22</td>
<td>278 ± 23</td>
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<tr>
<td>DMSO</td>
<td>0.1 ml</td>
<td>31 ± 1</td>
<td>32 ± 2</td>
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<tr>
<td>4 µg</td>
<td>30 ± 2</td>
<td>35 ± 3</td>
<td>150 ± 17</td>
<td>162 ± 16</td>
<td>175 ± 19</td>
<td>178 ± 22</td>
<td>274 ± 24</td>
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<td>32 ± 2</td>
<td>34 ± 3</td>
<td>148 ± 14</td>
<td>153 ± 19</td>
<td>158 ± 20</td>
<td>179 ± 23</td>
<td>263 ± 26</td>
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<td>31 ± 3</td>
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<td>180 ± 27</td>
<td>261 ± 23</td>
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<tr>
<td>Dexon</td>
<td>50 µg</td>
<td>2,052 ± 232</td>
<td>1,387 ± 208</td>
<td>2,126 ± 108</td>
<td>1,555 ± 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-AF</td>
<td>10 µg</td>
<td>3,118 ± 238</td>
<td>2,326 ± 278</td>
<td>2,374 ± 303</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,8-Chrysazin</td>
<td>50 µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>763 ± 96</td>
<td></td>
</tr>
</tbody>
</table>

a n = 3.
b When the concentration was higher than 500 µg, the bacterium growth was restrained.
from its use as a food and fragrance additive, as a raw material in the manufacture of citral, and as an ingredient in antimicrobials and insecticide. Adopting the oral, dermal, and inhalation ATC method for the starting doses and concentrations of the GHS, the LD50 and LC50 values of L. cubeba oil were determined, and the toxicity classes of L. cubeba oil were defined. The results indicated that L. cubeba oil has a slight toxicity. L. cubeba oil was not mutagenic in Salmonella Typhimurium strains TA98, TA100, TA1535, or TA1537, with or without metabolic S9 activation. In cytogenetic tests using ICR mouse spermatoocyte cells, chromosomal aberrations were not significantly increased after ICR mice were exposed to L. cubeba oil by oral gavage at doses of 460, 925, or 1,850 mg/kg. Negative results were obtained in an in vivo bone marrow micronucleus test in male ICR mice treated by gavage with 185, 740, or 1,850 mg/kg. In conclusion, L. cubeba oil administered in high concentrations via the oral or inhalation exposure route had some adverse effects on animal growth. No adverse body weight effects of L. cubeba oil by dermal exposure on animal body weight were found.

L. cubeba oil was found to be irritating to SD rats treated dermally with 2,000 mg/kg of the oil for 4 h, because slight skin irritation was observed. After 1 week, derma and hypoderm were still slightly hyperplasmic, which indicated that the organism did not catabolize L. cubeba oil.

**TABLE 5. Induction of chromosomal aberration ICR mouse spermatoocytes with Litsea cubeba oil by oral gavage**

<table>
<thead>
<tr>
<th>Concentration (mg/kg)</th>
<th>No. of aberrations</th>
<th>Mean ± SEM/ cell (%)</th>
<th>No. of cells with aberrations</th>
<th>Mean ± SEM cells with aberrations (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>10</td>
<td>5.2 ± 2.4</td>
<td>2</td>
<td>0.4 ± 0.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Oil</td>
<td>460</td>
<td>5.6 ± 2.1</td>
<td>1</td>
<td>0.2 ± 0.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>925</td>
<td>5.4 ± 1.5</td>
<td>3</td>
<td>0.6 ± 0.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>1,850</td>
<td>4.8 ± 1.9</td>
<td>2</td>
<td>0.4 ± 0.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>2.5</td>
<td>13.4 ± 5.2</td>
<td>23</td>
<td>4.6 ± 1.1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

In each test group, the number of mice scored was 5 and the total cells scored were 500.
oil. During the second observation week, the irritated skin was mending, which suggested that the lesion caused by L. cubeba oil in high concentrations was a type of germ-free inflammation reaction (also evidenced by the fact that L. cubeba oil only affected the tissue that it penetrated). So, when L. cubeba oil damaged tissue cells, it possibly killed or restrained pathogenic microorganisms from growing at the same time.

The mechanism for the L. cubeba oil to damage cells of the stomach, lungs, and skin might resemble the mechanism by which citral damages Aspergillus flavus cells (11). It is possible that L. cubeba oil injured the cell membrane, resulting in a selective permeability change, so that after entering the cell, L. cubeba oil not only changed its subcellular state, but also changed the cell’s normal physiologic function and disturbed its metabolism, leading to cell death.

The objective of the acute toxicity study on L. cubeba oil was to evaluate the toxicity of the test compound and to provide information for further study. Although L. cubeba oil shows a slight acute toxicity, chronic toxicity studies of 14 weeks and 2 years duration are necessary to assess the safety of L. cubeba oil more comprehensively. Since the most significant human exposure to L. cubeba oil occurs through ingestion as a food additive, dosed feeding is the preferred route of exposure for rodent studies. Previous studies demonstrated that L. cubeba oil had significant antimicrobial activity against bacteria, yeast, and mold (19, 22, 24). These results indicated the potential consumer benefits of L. cubeba oil as a topical antimicrobial agent for treating skin infections or as an antifungal agent in the storage of grain, food, clothes, book, and archives. In view of this potential consumer use, there should be considerable interest in studying the dermal and inhalation route of exposure for studying rodent chronic toxicity in the future.

In the present study, the acute toxicity of L. cubeba oil was determined by methods first described by Holzhuêtter et al. (3), which use significantly fewer animals; also significantly fewer animals die when using this method compared with the conventional LD$_{50}$ and LC$_{50}$ tests. We determined the oral LD$_{50}$ value of L. cubeba oil by gavage on ICR mice with the improved Horn method (7) in a previous study (Table 6, unpublished data). In comparing Table 6 and Table 1, it was found that two oral LD$_{50}$ values from completely different methods were close to each other: 3,690 mg/kg and approximately 4,000 mg/kg, respectively. However, in the experiment performed using the improved Horn method (7), 40 ICR mice (20 male and 20 female) were used, and the animal mortality rate reached 45.0%, whereas in the present study only six ICR mice (three male and three female) were used, and the animal mortality rate was 16.7%. Therefore, the methods described by Holzhuêtter et al. (3) and adopted in our studies were more in agreement with the current international concern of reduction, refinement, and replacement in animal studies.

In conclusion, L. cubeba oil is a low-toxic essential oil. Although high concentrations of L. cubeba oil are obviously damaging to the cells of the stomach, lung, and skin, these lesions did not elicit a pathogenic pathological response and only appeared where L. cubeba oil contacted the tissue. Therefore, L. cubeba oil is likely to be safe for human consumption.

**ACKNOWLEDGMENTS**

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**REFERENCES**


