Evaluation of acute oral toxicity of lemon grass oil and citral in albino rats

Adheena Xavier, S Suja Rani*, R Shankar, AR Nisha, S Sujith, R Uma

ABSTRACT

Essential oils, which are the plant derived secondary metabolites have been reported for various traditional medicinal applications. Amongst them, lemongrass oil (LGO) derived from Cymbopogon spp. as well as its major constituent citral possess a myriad of therapeutic potentials. The present study has been undertaken to study the adverse effects of LGO and citral on acute oral exposure to Sprague Dawley rats to establish the preliminary safety of these compounds prior to their efficacy evaluation against fatty liver disease. The toxicity study was conducted as per OECD guidelines No. 420. The LGO and citral were solubilized in 1% tween 80 and administered orally in a sequential manner in one animal at 2000 mg/kg (sighting study) followed by four animals (main study). The animals were then monitored for any clinical abnormalities or mortality and body weight gain during the observational period of 14 days, after which the animals were sacrificed and examined for abnormal lesions. LGO was further subjected to gas chromatography-mass spectrometry (GC-MS) analysis to characterize its chemical constituents, which revealed alpha and beta citral as the two major constituents. The rats treated with LGO and citral survived throughout the study period and didn’t exhibit any clinical abnormalities. Moreover, body weight gain was comparable to the vehicle treated rats and necropsy revealed no pathological alterations. Thus, the present study indicated LGO and citral as safe compounds with an LD50 greater than 2000 mg/kg and could be labelled as category 5/unclassified in hazard category of Globally harmonized system for classification of chemicals.

Keywords: Lemongrass Oil, Citral, Sprague Dawley Rats, Tween 80, Acute Oral Toxicity, OECD 420, GC-MS Analysis.

INTRODUCTION

Medicinal plants have long been recognised as a source of therapeutic compounds and continue to be a valuable resource for finding new drug leads nowadays [1]. Diverse classes of plant derived secondary metabolites, possess many biological actions in terms of health control as well as treatment of different diseases [2]. Essential oils (EO), which are one of the plant secondary metabolites, have been reported for various applications especially in the fields of pharmaceuticals, food, perfume and cosmetic industries. They are validated to have antifungal, antibacterial, anti-inflammatory, antioxidant and other properties. Moreover, the therapeutic potential of many essential oils is nowadays has been extensively explored for the treatment and management of various disorders [4]. Amongst the large array of essential oils, lemon grass oil (LGO) and its major component citral have already been verified for its antioxidant and hypolipidemic effects [4-6].

_Cymbopogon citrates_ commonly known as ‘West Indian lemon grass’ or ‘lemon grass’ belonging to the family of _Poaceae_, is a tropical herb having an arsenal of therapeutic effects including antifungal, antibacterial, analgesic, anti-inflammatory and antioxidant activities apart from being used as a flavouring agent, perfume and as a preservative. It contains alcohols, ketones, aldehydes, terpenes and esters. The EO derived from lemon grass was also reported to have a plethora of pharmacological activities. Moreover, LGO possess different constituents, amongst which citral (3,7- dimethyl -2,6-octadienal) is the major component of LGO. Citral is a mixture of α and β citral and this mixture determines the quality of LGO and imparts the specific lemon like smell to the oil. Apart from citral, other components seen in LGO are geranial, neural, limonene, β-myrcene, geranyl acetate, linalool and many others [7-9]. Though, LGO has been characterised and used for its diverse activities, their effect on amelioration of fatty liver disease has not been explored and safety evaluation, being the primary essential step prior to efficacy assessment, acute oral toxicity evaluation of these compounds was undertaken in rats. Therefore, the present study aims at assessing the preliminary safety of LGO and citral in female Sprague Dawley (SD) rats as per Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TG) No. 420 (Acute oral toxicity – Fixed dose procedure). The chemical composition of LGO was also assessed by gas chromatography-mass spectrometry (GC-MS) analysis.
MATERIALS AND METHODS

Test substance collection

Lemongrass essential oil (95010000064) was procured from Synthase Industries Pvt. Ltd., Kerala and citral (95%; C83007) was purchased from M/s Sigma Aldrich, India. They were stored in a dry ventilated area, protected from light.

Experimental animals

Fifteen female albino SD rats, weighing 140-200 g were procured from Small Animal Breeding Station, Mannuthy. The animals were maintained in well ventilated polypropylene cages, under standard laboratory conditions of 12 h/12 h light/dark cycle at 25±2°C with 30-70% relative humidity and were acclimatized for a week prior to experimentation. They were provided ad libitum with drinking water and standard rat chow. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of College of Veterinary and Animal Sciences, Mannuthy (No. IAEC/CVASMTY 20/2020 dated 31.12.2020) and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

Experimental design

Acute oral toxicity evaluation of LGO and Citral

Acute oral toxicity test of LGO and citral was performed as per the OECD TG No. 420, Acute Oral Toxicity-Fixed Dose Procedure [10]. The LGO and citral was solubilized in 1% tween 80 [11,12], and administered orally in a sequential manner as one animal at 2000 mg/kg dose level (limit test of sighting study) followed by four animals at 2000 mg/kg in the main study [10].

All the animals were observed for mortality and clinical signs for the first 10 min, 30 min, 1 h, 2 h and 4 h after dosing and thereafter twice daily for mortality and once a day for clinical signs, for 14 days. The clinical signs observed during the first 4 h of the drug administration included gross behavioural changes like hyperactivity, grooming, convulsions, sedation and loss of righting reflex along with changes in respiration, salivation, urination and defecation, if any. Body weight of animals was recorded individually before dosing and at weekly intervals thereafter.

After 14 days, all surviving animals were euthanized and subjected to detailed gross pathology examination of the visceral organs. As liver was the major site for metabolism of the xenobiotic agents, representative samples of liver were collected in 10% neutral buffered formalin (NBF), processed and embedded in paraffin for histopathological evaluation. The paraffin embedded tissues were sectioned and stained with hematoxylin and eosin as per the technique followed by Bancroft and Cook [13], and were examined under light microscope.

The chemical composition of LGO was analyzed by GC-MS using Shimadzu Model Number: QP2010S (Software: GCMS Solutions) fitted with an ELITE-5MS Capillary column (30 m x 0.25 mm ID, 0.25 m thickness). The column temperature was kept at 80°C for 4 minutes before increasing to 280°C at a rate of 5°C/min for 6 minutes. The injector and interface temperatures were 200°C and 280°C, respectively. The temperature of the ion source was 200°C. Over a scan range of 50–500 m/z, an electron ionization device with an ionization energy of 70 eV was used for GC-MS detection. Carrier gas was Helium at flow rate of 1 mL/ min in split 1:50 with injection volume of 1 µL. NIST 11 and WILEY 8 were the libraries used.

RESULTS

At the limit dose of 2000 mg/kg, vehicle (tween 80), LGO and citral treated rats did not show either mortality or any adverse signs of intoxication immediately following dosing as well as during the 14 days of observation period. The general behavioral pattern of animals treated with vehicle (tween 80), LGO and citral appeared normal and they did not show any abnormalities in the feed and water intake during the entire experiment period. The overall weight gain of the LGO and citral treated rats was found to be comparable to that of vehicle (tween 80) treated rats. Necropsy was done in all the experimental animals at the end of study which revealed, no major gross pathological changes with LGO and citral administration at 2000 mg/kg dose levels similar to tween 80 treated group (tables 1 and 2). Moreover, all the animals showed normal structure and architecture of hepatocytes on histological examination of liver, suggesting the preliminary safety profile of LGO and citral on acute oral administration (Fig. 1). Thus, LGO and citral were found to be safe at the tested limit dose level of 2000 mg/kg, indicating an oral LD50 value of greater than 2000 mg/kg body weight for both LGO and citral. Hence it can be labelled as Category 5/unclassified in the hazard category according to globally harmonized system (GHS).

Chromatogram obtained on phytochemical analysis of LGO using GC-MS is presented in Fig. 2, while the phytoconstituents acquired on GC-MS analysis is enlisted in table 3. The GC-MS analysis revealed ten peaks, among which the two peaks yielded 84% of the total constituents, which were pertinent to the two major chemical constituents of LGO such as alpha citral (46.61 per cent) and beta citral (37.39 per cent).

Figure 1: Sections of liver from treated groups after acute oral toxicity test (H & E)

(A) 200X showing normal architecture of liver after tween 80 treatment (B) 200X showing normal architecture of liver after LGO treatment (C) 200X showing normal architecture of liver after citral treatment
Table 1: Clinical signs, mortality and gross pathology findings observed in female SD rats treated with LGO and citral

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Study</th>
<th>Dose (mg/kg)</th>
<th>Mortality (death/total)</th>
<th>Cage side observations</th>
<th>Gross pathology findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>Sighting</td>
<td>2000</td>
<td>0/1</td>
<td>Nil</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>Main</td>
<td>2000</td>
<td>0/4</td>
<td>Nil</td>
<td>NAD</td>
</tr>
<tr>
<td>LGO</td>
<td>Sighting</td>
<td>2000</td>
<td>0/1</td>
<td>Hypoactive</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>Main</td>
<td>2000</td>
<td>0/4</td>
<td>Hypoactive animal</td>
<td>NAD</td>
</tr>
<tr>
<td>Citral</td>
<td>Sighting</td>
<td>2000</td>
<td>0/1</td>
<td>Hypoactive</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>Main</td>
<td>2000</td>
<td>0/4</td>
<td>Hypoactive animals</td>
<td>NAD</td>
</tr>
</tbody>
</table>

n= Number of animals; NAD = No abnormality detected; LGO: Lemon grass oil

Table 2: Effect of LGO and citral on body weight and body weight gain in female SD rats

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Dose (mg/kg)</th>
<th>Day 0 Body weight (g)</th>
<th>Day 7 Body weight (g)</th>
<th>Day 14 Body weight (g)</th>
<th>Days 0-7 Weight gain (%)</th>
<th>Days 7-14 Weight gain (%)</th>
<th>Days 0-14 Weight gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>2000</td>
<td>181.66±22.05</td>
<td>195±18.72</td>
<td>210.67±20.74</td>
<td>8.09±3.13</td>
<td>7.98±0.28</td>
<td>16.7±3.10</td>
</tr>
<tr>
<td>LGO</td>
<td>2000</td>
<td>180.33±8.90</td>
<td>200.33±9.00</td>
<td>217.83±9.84</td>
<td>11.34±2.56</td>
<td>8.77±1.57</td>
<td>21.23±4.17</td>
</tr>
<tr>
<td>Citral</td>
<td>2000</td>
<td>189.17±3.79</td>
<td>205.67±4.42</td>
<td>220.33±4.06</td>
<td>8.41±0.67</td>
<td>6.95±0.95</td>
<td>15.94±1.37</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM; n = 5; LGO: Lemon grass oil

Table 3: List of phytochemicals detected in LGO on GC-MS analysis

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Name of the Compound</th>
<th>Class of Compound</th>
<th>Molecular formula</th>
<th>Mol. Weight (g/mole)</th>
<th>RT (min)</th>
<th>Height %</th>
<th>Peak area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alpha-citral</td>
<td>Monoterpenoid</td>
<td>C_{10}H_{16}O</td>
<td>152.24</td>
<td>20.077</td>
<td>42.13</td>
<td>46.61</td>
</tr>
<tr>
<td>2</td>
<td>Beta-citral</td>
<td>Monoterpenoid</td>
<td>C_{10}H_{16}O</td>
<td>152.23</td>
<td>18.734</td>
<td>38.32</td>
<td>37.39</td>
</tr>
<tr>
<td>3</td>
<td>(z)-Geraniol</td>
<td>Monoterpenoid</td>
<td>C_{8}H_{16}O</td>
<td>136.23</td>
<td>19.324</td>
<td>5.99</td>
<td>5.79</td>
</tr>
<tr>
<td>4</td>
<td>(+)-Linalyl acetate</td>
<td>Monoterpenoid</td>
<td>C_{12}H_{20}O</td>
<td>196.29</td>
<td>24.815</td>
<td>3.78</td>
<td>2.99</td>
</tr>
<tr>
<td>5</td>
<td>(+)-Beta-caryophyllene</td>
<td>Bicyclic sesquiterpen</td>
<td>C_{3}H_{26}</td>
<td>204.36</td>
<td>26.263</td>
<td>2.52</td>
<td>1.88</td>
</tr>
<tr>
<td>6</td>
<td>Beta-copaene</td>
<td>Tricyclic sesquiterpen</td>
<td>C_{13}H_{26}</td>
<td>204.35</td>
<td>30.136</td>
<td>1.88</td>
<td>1.76</td>
</tr>
<tr>
<td>7</td>
<td>Bicycle [2.2.1] heptane, 2,2-dimethyl-3-methylene-, (1s)</td>
<td>Bicyclic Monoterpenoid</td>
<td>C_{10}H_{16}</td>
<td>136.23</td>
<td>6.828</td>
<td>2.08</td>
<td>1.38</td>
</tr>
<tr>
<td>8</td>
<td>4-Nonanone</td>
<td>Amyl Propyl Ketone</td>
<td>C_{9}H_{18}O</td>
<td>142.24</td>
<td>11.334</td>
<td>1.50</td>
<td>0.94</td>
</tr>
<tr>
<td>9</td>
<td>Beta-linalool</td>
<td>Monoterpenoid</td>
<td>C_{10}H_{18}O</td>
<td>154.52</td>
<td>12.494</td>
<td>1.18</td>
<td>0.87</td>
</tr>
<tr>
<td>10</td>
<td>D-Limonene</td>
<td>Monoterpenoid</td>
<td>C_{10}H_{18}O</td>
<td>136.23</td>
<td>9.585</td>
<td>0.61</td>
<td>0.39</td>
</tr>
</tbody>
</table>

LGO: Lemon Grass oil; GC-MS: Gas Chromatography-Mass Spectroscopy
DISCUSSION
Acute toxicity evaluation is imperative for recognising clinical signs/mortality provoked by the substance under investigation, as well as determining the dose range that might be utilised to assess the efficacy of the substances. Hence, an acute oral toxicity appraisal is warranted prior to any in vivo studies to confirm the safety aspect of the test compounds. Amongst the large array of EOs, LGO and its major component citral, though verified for antioxidant and hypolipidemic effects, their efficacy against fatty liver disease (FLD) has not been explored so far. There are many herbal drugs, plant extracts and plant secondary metabolites that can be proposed to effectively prevent and cure hepatic steatosis and associated degenerative changes [14,15]. Therefore, the present study was undertaken to establish preliminary safety of LGO and citral by acute oral toxicity evaluation prior to their efficacy evaluation against FLD.

Accordingly, an acute oral toxicity study was conducted as per OECD test guidelines TG no. 420 (Fixed Dose Procedure, OECD, 2002) to estimate the preliminary safety specifics. Though several internationally accepted protocols for acute oral toxicity testing were available, the fixed dose procedure was chosen as it uses fewer animals, uses clear signs of toxicity rather than mortality as an endpoint and causes less suffering than traditional methods of acute toxicity testing. In view of that, a single dose of LGO and citral at 2000 mg/kg body weight dose level (limit dose) as well as the vehicle (0.1% tween 80) were given orally in the sighting study and main study.

Any change in the animal’s physical appearance, clinical and behavioural symptoms immediately after administration of the test substance and until the end of the research period indicates an alteration in normal state of the animal due to the test substance. After administration of the test substances, the animals were observed for changes in skin/fur, eyes and mucous membranes. Alterations in the respiratory, circulatory, autonomic and central nervous systems, as well as somatoform activity and behaviour patterns were also assessed [16]. The animals in the experiment did not show any mortality or adverse clinical signs during the entire 14 days of observation period.

One of the most sensitive markers that an animal’s condition deteriorating is the significant body weight loss, especially when body weight is reduced by more than 20% compared to control animals, or when bodyweight is reduced by more than 25% over a period of 7 days or more [16]. Here, there was an increase in body weight after LGO and citral administration, which was comparable to the vehicle treated group. Thus, it revealed the lack of adverse effect of LGO and citral on body weight of the animals.

The gross pathology and histopathology revealed normal appearance and architecture of liver in all the experimental animals. Altogether, these observations indicated the preliminary safety profile of the LGO and citral. Costa et al [17], found that there was no alteration grossly and histologically in liver of male Swiss mice treated with LGO. Similarly, Lutalek et al [18], performed acute and subacute toxicity study of essential oil from Cymbopogon spp. in Swiss albino mice and found no histopathological alterations and reported that it was relatively safe and nontoxic, which was in corroboration with the present findings.

The acute oral toxicity assessment warrants the profiling of the test substance using the global harmonised system (GHS) for chemical classification [19]. The chemicals are harmonized and labelled in accordance with GHS and thereby the chemicals have been organised into five categories based on its risk and safety measures. The oral LD₅₀ of the chemical from categories 1 to 5 has been reported respectively as ≤ 5mg/kg (category 1), between 5 and 50 mg/kg (category 2), between 50 and 300 mg/kg (category 3), between 300 and 2000 mg/kg (category 4) and between 2000 mg/kg and 5000 mg/kg (category 5). In the present study, as there was no evidence of clinical or behavioural changes after acute oral administration of LGO and citral even at the limit dose level of 2000 mg/kg, these compounds can be grouped into category 5/unclassified in the hazard category with respect to GHS classification.

The pharmacological activities of a compound are largely attributed by its constituents. Lemon grass (Cymbopogon spp.) is an aromatic medicinal plant having many therapeutic potentials and the LGO is derived from the steam distillation of the freshly or partly dried leaves extracted from this plant and its chemical constituents have been established via GC-MS analysis. The present study revealed ten compounds from LGO derived from Cymbopogon spp. and most of them were found to be of terpenoid class. The two major phytochemical components were alpha citral (46.61%) and beta citral (37.39%) and thus 84% of the total composition was contributed by citral. The minor components detected were geraniol (5.79%), linalyl acetate (2.99%), beta Caryophyllene (1.88%), beta copea (1.76%), bicycle heptane dimethyl methylene (1.38%), Nona none (0.94%), beta linalool (0.87%) and limonene (0.39%).

Ali et al [19], revealed presence of 43 compounds in the LGO derived from Cymbopogon citratus and the major phytochemical compounds reported were citral (34.8%), neral (30.72%), b-myrcene (11.28%), geraniol (5.54%) and citronellol (1.34%), whereas other significant compounds were geranyl acetate (0.57%), cycloheptane dimethyl carboxaldehyde (0.23%) and limonene (0.03%). Moreover, the quality and therapeutic activities of LGO is mainly affected by the amount of citral present in the essential oil. The GC – MS evaluation of LGO by Boukhatem et al [14], also unveiled almost 23 chemical constituents, among which geranal (42.2%), neral (31.5%), b-myrcene (7.5%) were the major ones and geranyl acetate (4.3%) and isoeugenol (1.4%) were few of minor ones.

Terpenes, ketones, aldehydes, flavonoids, alcohols, phenols and esters are the main classes of compounds found in essential oils, which are responsible for its biological actions. Citral, a monoterpenoid accounts the major share of phytochemical component of LGO and its pharmacological actions [20]. However, geographical region of plants is one of the major factors, that determines its chemical composition [21].

CONCLUSION
The major chemical constituents of LGO as detected by GC-MS analysis was found to be alpha citral and beta citral, which comprised of 84 per cent out of all the phytochemicals obtained. Based on the toxicity findings of the present study, it can be concluded that LGO and citral would be safe even at the limit dose level of 2000 mg/kg body weight after single dose oral administration to female Sprague-Dawley rats and hence the LD₅₀ could be proclaimed as more than 2000 mg/kg, enabling to be labelled as category 5/unclassified in hazard category of Globally Harmonized System for classification of chemical. However, further comprehensive study is indeed required for detailed toxicity assessment of these compounds.

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Conflict of Interest
None declared.

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