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Cholesterol reduction and lack of genotoxic or toxic effects in mice after repeated 21-day oral intake of lemongrass (*Cymbopogon citratus*) essential oil

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ABSTRACT

Cymbopogon citratus (lemongrass) is currently used in traditional folk medicine. Although this species presents widespread use, there are no scientific data on its efficacy or safety after repeated treatments. Therefore, this work investigated the toxicity and genotoxicity of this lemongrass's essential oil (EO) in male Swiss mice. The single LD_{50} based on a 24 h acute oral toxicity study was found to be around 3500 mg/kg. In a repeated-dose 21-day oral toxicity study, mice were randomly assigned to two control groups, saline- or Tween 80 0.01%-treated groups, or one of the three experimental groups receiving lemongrass EO (1, 10 or 100 mg/kg). No significant changes in gross pathology, body weight, absolute or relative organ weights, histology (brain, heart, kidneys, liver, lungs, stomach, spleen and urinary bladder), urinalysis or clinical biochemistry were observed in EO-treated mice relative to the control groups. Additionally, blood cholesterol was reduced after EO-treatment at the highest dose tested. Similarly, data from the comet assay in peripheral blood cells showed no genotoxic effect from the EO. In conclusion, our findings verified the safety of lemongrass intake at the doses used in folk medicine and indicated the beneficial effect of reducing the blood cholesterol level.

1. Introduction

The use of medicinal plants in bactericidal, antiparasitical, insecticidal, medicinal and cosmetic applications has increased extensively, especially by pharmaceutical companies, since most of the essential oils obtained from such plants are known to be non-toxic (Bakkali et al., 2008; Fandohan et al., 2008). Essential oils are very complex natural mixtures formed as secondary metabolites by aromatic plants. Usually, these oils are devoid of potential genotoxic activity and some of them present antimutagenic action that may be related to anticarcinogenic activity (Bakkali et al., 2008).

Cymbopogon citratus (DC) Stapf (Poaceae), commonly known as West Indian lemongrass, is a widely cultivated aromatic plant used as infusion or decoct in traditional medicine. In contrast to the other chemotype of lemongrass (East Indian lemongrass – *Cymbopogon flexuosus*, whose essential oil consists of equal amounts of myrcene and citral), more than 70% of the essential oil from West Indian lemongrass is composed of citral (Sousa et al., 1991). In Brazil, *C. citratus* is mostly used for treating nervous excitement and gastrointestinal disturbances (Blanco et al., 2009; Carlini et al., 1986; Negrelle and Gomes, 2007), but it has been used worldwide in the treatment of these and other diseases. The Cuban population has employed the species as an antihypertensive and anti-inflammatory drug (Carbajal et al., 1989). In eastern Nigeria, this plant has been utilized for treating diabetes, obesity and coronary disease (Adeneye and Agbaje, 2007). Additionally, various studies have shown antimutagenic/anticarcinogenic and antioxidant properties of lemongrass extracts or their majority compounds (citral, β-myrcene and geraniol) in distinct *in vitro* and *in vivo* systems (Cheel et al., 2005; Choi et al., 2000; Mitić-Ćulafić et al., 2009; Melo et al., 2001; Pereira et al., 2009; Rabbani et al., 2006; Suaeyun et al., 1997; Tapia et al., 2007).

Recently, we demonstrated that lemongrass essential oil protected DNA against chemically-induced damage and also exhibited anticarcinogenic activity against chemically induced mammary carcinogenesis in female Balb/C mice (Bidinotto et al., 2010). Furthermore, our research group demonstrated an anxiolytic-like effect of *C. citratus* essential oil which might be mediated by the GABAergic system (Costa et al., 2009). In fact, it has been speculated that secondary metabolites of herbal medicines may serve as alternatives to synthetic chemical products (Bakkali et al.,

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2008) thus denoting the importance of studying its potential toxicity. Data from an investigation of acute toxicity indicate safety after its oral and dermal administration, with mild to moderate skin-irritating potential (Opdyke, 1976). Despite the widespread folk use of lemongrass, there are few controlled toxicological studies confirming its efficacy and safety in a long-term treatment, since sub-acute toxicity data are required to verify these two objectives (WHO, 2000). Therefore, the aim of the current study was to investigate the toxicological profile of *C. citratus* essential oil after acute or repeated oral treatment in male Swiss mice, focusing on biochemical and histopathological endpoints, and its genotoxicity by means of the comet assay.

2. Material and methods

2.1. Plant material, essential oil (EO) extraction and phytochemical analysis

Plant seedlings of specimens, from the garden of medicinal plants (Lageado Farm, UNESP), were cultivated at the Botucatu Biosciences Institute. The plant was identified in the BOTU Herbarium, Department of Botany, UNESP, where a voucher specimen (# 23031) was deposited. Leaves of *C. citratus* were collected between May and June 2008, and immediately processed to obtain the essential oil. Fresh leaves were submitted to a Clevenger apparatus and the EO was obtained by hydrodistillation (yield: 0.46% v/w), protected against light and heat, and stored at 4 °C up to the moment of use. The profile of EO compounds was analyzed by gas chromatography coupled with mass spectrometry as previously described (Blanco et al., 2009). EO was dissolved in Tween (polyoxyethylenesorbitan monooleate – Tween 80® – 0.01% v/v in saline, Sigma–Aldrich, USA). All samples were freshly prepared at the moment of use.

2.2. Animals

All experiments were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee for Animal Research of the Botucatu Biosciences Institute. Adult male Swiss mice (30 days old) from the colony of the UNESP Central Animal House were used in all experiments after a 1 week-adaptation period at the Animal House of the Department of Pharmacology. The animals were maintained under controlled environmental conditions of temperature (21 ± 2 °C) and light (12/12 light/dark cycle) with food and water *ad libitum* until 2 h before experimental procedures.

2.3. Toxicity evaluation of acute and repeated dosages

In our first approach to evaluating signs of acute toxicity, single doses of EO (5–4000 mg/kg b.w.) were given to groups of mice (7–8 animals per group) by gavage (p.o.). Clinical signals of toxicity, motor activity and posture were monitored throughout a 24-h period. The deaths that had occurred were registered and these data were used to determine the LD_{50}, using the probit test (Litchfield and Wilcoxon, 1949).

Additionally, in a repeated dose 21-day acute toxicity study, mice were treated with EO (1, 10 or 100 mg/kg b.w., p.o.), saline 0.9% or Tween 0.01% (5–7 animals per group) for 21 days, between 01:00 p.m. and 03:00 p.m. At the 21st day, the animals were euthanized by decapitation and blood samples were collected for serum biochemical analyses. The brain, heart, kidneys, liver, lungs, stomach, spleen and urinary bladder were removed, rinsed in saline 0.9%, and weighed. Animal body weight was measured every 5 days. Relative body weight was calculated as: [absolute body weight after a time interval divided by initial body weight of each mouse] × 100. The relative organ weights were calculated based on the organ-to-body weight ratio multiplied by 100 (Michael et al., 2007).

Tissue fragments of brain, heart, kidneys, liver, lungs, stomach, spleen, and urinary bladder were fixed in 10% buffered formalin, dehydrated with successive concentrations of ethanol (70–100%), cleared in xylene, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological analysis. The urinary bladder was inflated with fixative and processed. Degenerative or proliferative lesions associated with drug toxicity were evaluated using criteria published by the Society of Toxicologic Pathology (SSNDC, 2006).

2.4. Serum and biochemical analyses

Peripheral blood samples were collected in tubes without anticoagulant, kept at room temperature for 30 min and centrifuged at 4000 rpm for 20 min. Serum samples were aspirated off and stored at −80 °C until analysis in a Cobas Mira Plus® Chemistry Analyzer (Roche Diagnostic Systems, USA). The following clinical biochemistry parameters were measured: aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase (GGT), urea, albumin, creatinine, total protein, cholesterol and triglycerides.

2.5. Comet assay

In order to test the genotoxicity produced by long-term EO administration, animals were treated with EO at the dose of 100 mg/kg. Negative control animals received only saline solution or TW (vehicle control). Additionally, to evaluate the antigenotoxic potential of EO at low doses, animals were treated with 1 or 10 mg/kg of EO for 21 days; prior to blood collection the genotoxic agent MNU (30 mg/kg, i.p.) was administered. Blood samples were collected 4 h after MNU treatment for the comet assay, which was performed under alkaline conditions according to a previously described protocol (Tice et al., 2000). Briefly, 5 μl of blood was mixed with 120 μl of 0.5% low-melting-point agarose at 37 °C and layered onto conventional microscope slides, precoated with 1.5% normal-melting-point agarose (Invitrogen). The slides were left overnight in a cold freshly-prepared lysing solution (1% Triton X-100, 2.5 mM NaCl, 0.1 mM Na_2EDTA, 10 mM Tris with 10% dimethylsufoxide, pH 10.0) and then inserted into a horizontal electrophoresis apparatus containing alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13) at 4 °C for 20 min. Using the same buffer, electrophoresis was performed at 25 V and 300 mA for 20 min. After electrophoresis, the slides were washed twice in neutralizing buffer (0.4 M Tris–HCl, pH 7.5), fixed in absolute alcohol, air-dried, and stored at room temperature. The slides were stained with 50 μl of ethidium bromide (20 μg/ml) and immediately examined at 400× magnification in an epi-illumination fluorescence microscope connected to an image analysis system (Comet II; Perspective Instruments, Suffolk, UK). Coded slides were scored blindly and 50 nucleoids from each animal were randomly analyzed (25 cells per slide from two slides per animal). Tail intensity (the quantity of DNA in the comet tail) was used to measure the extent of DNA damage. Comet images with a "cloudy" appearance or with a very small head and balloon-like tail were excluded from the analysis (Hartmann and Speit, 1997).

2.6. Statistical analysis

Body weight evolution was analyzed by a repeated-measures analysis of variance with treatment as the main factor and days of treatment as the within-subject variable; contrasts were made by the Fisher LSD test. Data on relative organ weights after 21-day treatment as well as the biochemical and comet assays were analyzed by the one-way ANOVA followed by Dunnett's Multiple Comparisons Test, when applicable. For all tests, the level of significance was set at $p \leqslant 0.05$.

3. Results

3.1. EO composition

The chromatogram of *C. citratus* EO, obtained by gas chromatography coupled with mass spectrometry as previously described (Bidinotto et al., 2010), indicated the monoterpene citral, a mixture of the stereoisomers geranial (51.46%) and neral (19.83%), as well as beta-myrcene (16.5%) and geraniol (1.28%) as its main compounds.

3.2. Acute toxicity study

Male mice exposed to a single dose (5–1500 mg/kg b.w.) of *C. citratus* EO presented no alterations in general behavior when compared to the controls. There was only one death in the group treated with lemongrass EO at 2000 mg/kg dose. The animals that received doses higher than 3000 mg/kg b.w. showed abnormalities such as torpor and cyanosis, while those exposed to 4000 mg/kg b.w. died within 24 h after the oil administration. Therefore, the lethal dose, calculated by the probit test, was around 3500 mg/kg.

3.3. 21-day acute toxicity

3.3.1. Relative body and organ weights

Fig. 1 shows the evolution of the body weight in relation to prior treatment on day 0. Animals treated with *C. citratus* EO did not differ from controls ($F_{(4,28)} = 1.13$, $p = 0.363$), and body weight increased ($F_{(3,84)} = 73.78$, $p < 0.001$) throughout the duration of treatment without interaction between factors ($F_{(12,84)} = 1.41$,

$p = 0.18$). There were no significant changes in the absolute (data not shown) or relative organ weight of the EO-treated mice in relation to control groups (Table 1).

Histological examination of the brain, heart, kidneys, liver, lungs, stomach, spleen and urinary bladder revealed no changes that might suggest *C. citratus* EO toxicity (1, 10 or 100 mg/kg b.w.).

3.3.2. Biochemical analysis

The 21-day treatment with lemongrass EO did not induce abnormalities in serum levels of aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase (GGT), urea, albumin, creatinine, total protein or triglycerides (Table 2). Relative to the saline control group, there was a significant reduction in serum total cholesterol ($F_{(4,27)} = 3.061$, $p < 0.05$) after the administration of the highest EO dose (100 mg/kg).

3.3.3. Comet assay

Data from the lemongrass EO genotoxicity and antigenotoxicity tests are shown in Fig. 2. EO at 100 mg/kg did not increase ($F_{(2,15)} = 0.05813$, $p = 0.9437$) DNA damage in mouse blood cells. Similarly, low EO doses (1 and 10 mg/kg) did not protect ($F_{(4,27)} = 15.172$, $p < 0.0001$) against the MNU-induced DNA damage in peripheral leukocytes.

4. Discussion

The majority of preclinical toxicity studies assess the effect of plant extracts or essential oils after a single acute treatment. However, the utilization of medicinal plants by traditional medicine has relied largely on long-term clinical experience with little or no scientific data on their efficacy or safety after long-term treatment (Ernst, 2004; Zhu et al., 2002). As is the case in relation to many other medicinal plant species, there are few toxicological/toxicogenetic studies on the safety of *Cymbopogon citratus*.

Our data showed that EO did not present an extensive toxic effect in rodents, since the LD_{50} in mice was around 3.5 g/kg, which is a much higher dose than those usually taken as an infusion by humans. Moreover, it is 350 times greater than the dose that presented anxiolytic-like effect (Costa et al., 2009). Our result was similar to that obtained by Fandohan and colleagues (2008) which demonstrated that LD_{50} in rats was 3.25 g/kg b.w. Administration of citral and β-myrcene, the major compounds of EO, has been reported to cause an embryofetal toxic effect only at high doses in pregnant Wistar rats (Delgado et al., 1993; Nogueira et al., 1995). The LD_{50} of geraniol (the other major constituent) was shown to be around 4.8 g/kg (Lapczynski et al., 2008). These results confirm and partially explain the low toxicity of the acute EO treatment. We did not observe toxic signs such as death occurrence, piloerection, abdominal contortions, locomotion, muscle tone or convulsions in the animals treated with doses from 5 to 1500 mg/kg b.w.

During the 21-day acute toxicity treatment, a slight weight loss was observed among the mice treated with EO at 1 or 100 mg/kg, starting after 16 days of treatment. The results were similar to those obtained by Adeneye and Agbaje (2007). The amount of weight lost by the animals may be directly related to a reduction in food ingestion (not measured in this study). This fact could be due to the existence of an endogenous ligand of central-type benzodiazepine receptors known as endozepine octadecaneuropeptide (ODN), which are inhibitors of food intake in rodents (Do Rego et al., 2007). Such an association makes sense given that *C. citratus* EO act on benzodiazepine receptors, as we described previously (Costa et al., 2009).

Histopathological examination of the tissues showed no difference and no apparent abnormalities. Microscopic analysis of the organs revealed that their architectural and cellular appearances were normal and there was no significant difference in the body or organ weights between experimental groups.

After 21 days of EO treatment there were no signs of important changes in serum biochemical parameters. The only modified parameter was the cholesterol level, which was reduced in the group treated with 100 mg/kg/day. This effect was previously described in both rats (Adeneye and Agbaje, 2007) and humans (Elson et al., 1989). In a study of 22 human hypercholesterolemic subjects who took a daily capsule containing 140 mg of lemongrass EO, Elson and colleagues (1989) showed evidence that the constituents of lemongrass oil effectively lowered the cholesterol levels among this hypercholesterolemic subset. Moreover, Adeneye and

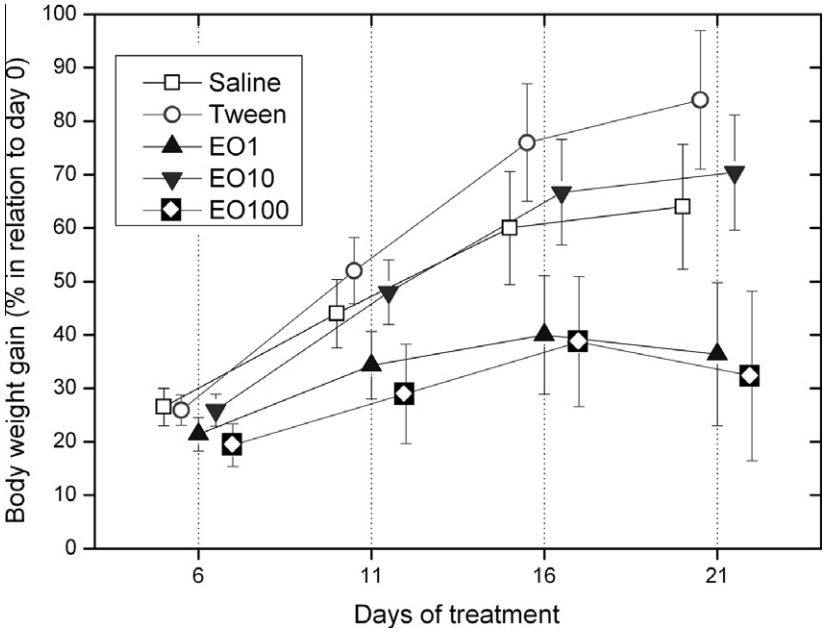

Fig. 1. Body weight gain, in percentage, of mice treated with *C. citratus* essential oil (EO) at 1, 10 or 100 mg/kg (p.o.) for 21 days. Each point represents the mean ± SEM of each group.

Table 1
Relative organ weight (mean ± SD) of mice after 21 days treatment with *Cymbopogon citratus* essential oil (EO).

Treatment	*n*	Relative organ weight (g%)							
		Heart	Lung	Spleen	Kidney R	Kidney L	Liver	Brain	Stomach
Saline	7	0.41 ± 0.07	0.60 ± 0.15	0.38 ± 0.11	0.60 ± 0.07	0.61 ± 0.11	5.33 ± 0.76	1.11 ± 0.11	0.80 ± 0.05
Tween 0.01%	5	0.44 ± 0.06	0.64 ± 0.08	0.35 ± 0.05	0.57 ± 0.08	0.58 ± 0.13	5.24 ± 0.60	1.12 ± 0.11	0.80 ± 0.07
MNU	7	0.43 ± 0.06	0.60 ± 0.15	0.30 ± 0.11	0.59 ± 0.04	0.56 ± 0.07	5.22 ± 0.66	1.06 ± 0.11	0.75 ± 0.13
EO 1 mg/kg	7	0.50 ± 0.08	0.67 ± 0.08	0.31 ± 0.07	0.61 ± 0.08	0.60 ± 0.06	5.18 ± 0.47	1.26 ± 0.28	0.84 ± 0.10
EO 10 mg/kg	7	0.46 ± 0.08	0.63 ± 0.07	0.34 ± 0.05	0.68 ± 0.13	0.65 ± 0.13	5.41 ± 0.46	1.14 ± 0.12	0.86 ± 0.11
EO 100 mg/kg	7	0.51 ± 0.23	0.78 ± 0.22	0.41 ± 0.16	0.61 ± 0.11	0.63 ± 0.14	5.18 ± 0.80	1.29 ± 0.40	0.90 ± 0.20

MNU = N-methyl-N-nitrosourea; R and L = Right and Left, respectively. One-way ANOVA followed by the Dunnett's Multiple Comparisons Test.

Table 2
Biochemical parameters (mean ± SD) evaluated after 21 days treatment with *Cymbopogon citratus* essential oil (EO).

Treatment	*n*	Biochemical parameters								
		UR (mg/dL)	CR (mg/dL)	TPRO (g/dL)	ALB (g/dL)	AST (UI/L)	AP (UI/L)	GGT (UI/L)	CHOL (mg/dL)	TRGL (mg/dL)
Saline	7	55.3 ± 9.2	0.33 ± 0.05	6.68 ± 0.56	3.03 ± 0.40	246.57 ± 66.31	270.57 ± 74.48	2.64 ± 1.25	167.43 ± 22.55	247.43 ± 88.42
Tween 0,01%	5	47.5 ± 4.7	0.30 ± 0.00	6.46 ± 0.62	2.78 ± 0.13	235.40 ± 73.61	175.60 ± 100.57	2.20 ± 1.30	152.40 ± 30.70	215.60 ± 95.00
EO 1 mg/kg	7	45.8 ± 12.9	0.31 ± 0.04	6.37 ± 0.48	2.74 ± 0.26	225.43 ± 63.12	214.43 ± 89.30	1.86 ± 0.38	143.43 ± 29.02	213.43 ± 77.11
EO 10 mg/kg	7	51.0 ± 5.7	0.31 ± 0.04	6.21 ± 0.33	2.83 ± 0.11	262.14 ± 30.83	296.86 ± 91.98	3.14 ± 1.34	145.71 ± 31.51	201.43 ± 83.96
EO 100 mg/kg	7	49.0 ± 5.9	0.30 ± 0.00	6.38 ± 0.54	2.88 ± 0.07	284.00 ± 99.45	299.43 ± 76.74	2.14 ± 0.70	125.71 ± 24.57*	234.86 ± 62.67

UR (urea), CR (creatinine), TPRO (total protein), ALB (albumin), AST (aspartate aminotransferase), AP (alkaline phosphatase), GGT (gamma-glutamyl transferase), CHOL (cholesterol), TRGL (triglycerides).
* $p \leqslant 0.05$ in relation to control group saline (one-way ANOVA followed by the Dunnett's Multiple Comparisons Test).

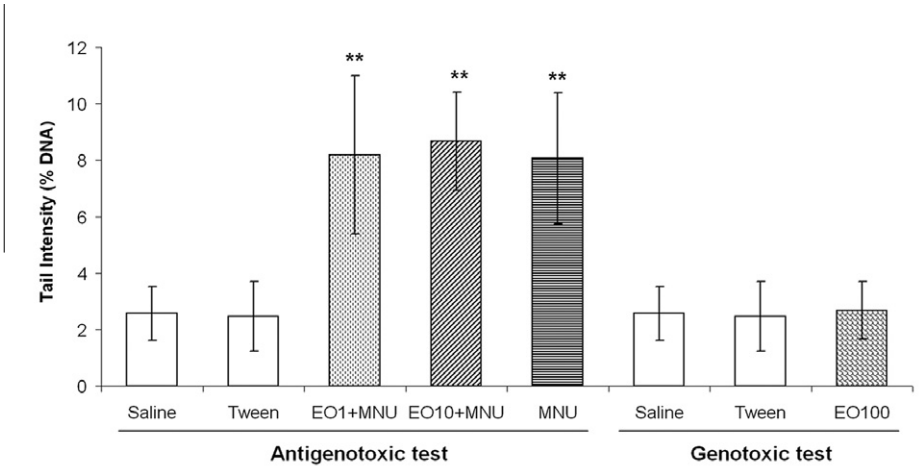

Fig. 2. DNA damage (tail intensity) in peripheral blood leukocytes 4 h after the last treatment (n = 5–7 animals per group). **$p \leqslant 0.01$ in relation to control group (one-way ANOVA followed by Dunnett's Multiple Comparisons Test).

Agbaje (2007) showed dose-dependent effects of weight loss, hypoglycemia and hypolipidemia in normal Wistar rats after a single, daily oral dosing (125–500 mg/kg) of fresh aqueous leaf extract of *C. citratus* for 42 days. These findings also suggest putative beneficial effects in type 2 diabetes patients, corroborating its folkloric use in Nigeria. But according to the authors, the mechanisms that underlie such activity must be investigated more thoroughly (Adeneye and Agbaje, 2007).

In a previous study (Bidinotto et al., 2010), we found that a long-term administration of EO at a high dose mitigated the genotoxic effects of MNU. Therefore, we decided to evaluate whether such genotoxicity is provoked even at small doses. Our data showed that the administration of low EO doses did not ease these effects. Considering that our relevant findings in the serum (cholesterol reduction) were produced by the highest EO dose (100 mg/kg), we decided to assess its genotoxicity but found no increase of DNA damage in peripheral blood leukocytes.

It is recognized that essential oils contain various compounds and that the synergistic or additive effect of multiple constituents as assessed by toxicological tests remains unknown. Therefore, the present study provides new information on the use of the complete oil. In a review of the literature, some authors have presented effects of the two main compounds of the *C. citratus* EO. According to Carnesecchi et al. (2004) geraniol inhibits colon cancer-cell proliferation by inducing membrane depolarization and interfering with ionic canals and signaling pathways. Furthermore, the group demonstrated that geraniol inhibits DNA synthesis and reduces the volume of colon tumors. While evaluating the major compound citral (500–4000 ppm), by *in vitro* and *in vivo* tests for genotoxicity in a two-year feed study of F344/N rats and B6C3F1 mice, the group found no evidence of toxicological or carcinogenic activity (National Toxicology Program, USA, 2003).

The present evaluation of toxicological biomarkers and genotoxicity shows that *C. citratus* EO presents low toxicity and can

be considered relatively safe in a long-term treatment at doses up to 100 mg/kg. Accordingly, we did not detect any deleterious effect on the liver or kidney functions which remained normal throughout the experiment at all doses tested. Moreover, we showed that the 21-day acute toxicity treatment with lemongrass EO reduces cholesterol levels. These results corroborate its long-term medicinal use by different traditional populations over the years. However, other pharmacological studies are required to elucidate the action mechanism by which this EO reduces serum cholesterol and body weight.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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