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Research Article

Chemical Composition and Toxicity of *Ocimum sanctum* L. Var. *Cubensis* Essential Oil Up-Growing in the Eastern of Cuba

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ABSTRACT

Ocimum sanctum L. var. *cubensis* (OS) is a valuable medicinal plant. Some varieties have been reported and some of them remain almost unstudied. The aim of this study is therefore to evaluate the chemical composition and the *in vitro/in vivo* toxicity of the leaves essential oil from *O. sanctum*, up growing wild in the Eastern region of Cuba. The essential oil was extracted by in a Clevenger type apparatus and characterized by its chemical components helped by a Gas Chromatograph coupled to a Mass Spectrometer (GC/MS). For the evaluation of cytotoxicity, primary cultures of embryonic cardiac cells (ECC) were obtained from Swiss mice and purified, uninfected ECC cultures were exposed to compound studied at 37 ºC for 24, 48 and 72 h (up to 1200µg/mL). The cell death rates were measured by the PrestoBlue colorimetric assay. For the studies of Oral Acute Toxicity and Dermal Acute Toxicity, Sprague Dawley rats were used as biological models, fulfilling the guides 423 and 402 of the Organization for Economic Cooperation and Development and the Research Ethical Committee. The essential oil from the leaves of *O. sanctum* L. var. *cubensis* up growing wild in the eastern region of Cuba presented 20 compounds defined as the major components: Eugenol (21.96%), β-caryophyllene (20.79%) and Bicyclogermacrene (20.38%). At the maximum concentration the OS essential oil barely provokes the 5% of cell death, meaning that this substance does not result toxic for ECC at the concentration evaluated. In vivo studies also classified OS essential oil as not toxic do not showing any acute or oral toxicity (dose of 2000 mg/kg body weight). The obtained result indicates that the oil can be considered safe; harmless topically and orally showed no in vitro and in vivo toxicity studies.

Keywords: *Ocimum tenuiflorum*, Chemical Characterization, essential oil, Acute Oral Toxicity, Cytotoxicity assays, Acute Dermal Toxicity.

INTRODUCTION

The human development has a long history of using plants for food and medicinal purposes. Nowadays consumes a wide variety of fruits, vegetables, and plant food supplements or condiments, as well as plants for medicinal use[1,2]. Many of these plants used for medicinal purposes have proved their mechanisms of action, toxicity levels and active ingredients. Those aspects must be necessarily studied in the intent to obtain new principles with high bioenhancer capacity and effects less toxic[3].

The genus *Ocimum* (Lamiaceae) comprises more than 30 species, distributed in tropical and subtropical regions of Asia, Africa, Central, and South America. *Ocimum sanctum* (syn. *Ocimum tenuiflorum* L.f) is a plant with enormous properties for curing and preventing diseases. *Ocimum* species are popularly known as basilisks or basils [4], but clearly differs between there, not only

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morphologically, but also in their secondary metabolites and indeed in their pharmacological potentialities.

This plant is used as a home remedy for many ailments such as gastric and genitourinary disorders, respiratory and skin diseases, various forms of poisoning and psychosomatic stress disorders, arthritis, painful eye disease, chronic fever and insect sting[4,5,6]. Other properties reported are aromatic, diuretic and vermifuge[7], and for special seasoning in food[8].

Ocimum sanctum (OS) has a specific aromatic odour because of the presence of an essential or volatile oil, concentrated mainly in leaves. This aromatic volatile oil contains a variety of terpenes with phenols and aldehydes groups, differing their chemical composition according to studies in different parts of the world[9,10,11,12,13]. This looks logical, considering that essential oils can changes their composition according to the geographical region from which they comes, the time of collection of the plant and the vegetative state[14]. Regardless of where these plants come from, they usually have a high concentration of eugenol, which is a substance known for its insecticidal[15], bactericidal[16] and fungicidal activity[17]. Also is considered as safe being used as food additive, but when added in excess becomes a toxic substance[18].

OS has been evaluated in their mammal's toxic effects, but mainly for aqueous and alcoholic extracts. Bhargava and Singh (1981) informed a low toxicity for the ethanolic extract of OS in adult mice, setting the lethal concentration in LD_{50} of 4505 ± 80 mg/kg and 3241 ± 71 mg/kg by oral or intra-peritoneal routes respectively[19]. Using the same animal model, other studies confirms the ethanol extract LD_{50} at 4600 mg/kg, while aqueous extract were settle at 6200 mg/kg[5,20]. Recently, Gautam and Goel (2014) corroborates this low toxicity when in a acute and subacute toxicity test doses under 2000 mg/kg do not gives evidence of any hazardous symptoms or death in rats during the 28 days of the study[21]. Independently of such reports, some varieties of OS remain almost unstudied. This becomes true when considering *O. sanctum* L. var. *cubensis*, an OS variety that grown in all regions of Cuba with almost no studies about their leaves essential oil neither in their toxicity in mammals, but with a wide use by Cuban population as curative and/or spice [22]. Only one study of the essential oil chemical constituents for this variety is reported since 1998, but this study using all aerial parts, was conducted in the western side of Cuba and with cultivated plants[23].

The aim of this study is therefore to evaluate the chemical composition and the *in vitro/in vivo* toxicity of the leaves essential oil from *O. sanctum* L. var. *cubensis* upgrowing wild in the Eastern region of Cuba.

MATERIALS AND METHODS

Plant Material

Ocimum sanctum L. var. *cubensis* leaves were collected early morning (Before 9:00 AM) on December 2015, in the municipality of San Luis, Santiago de Cuba´s province during the flowering period. Plants were harvested from a population that grows up in a wild way and that was integrated for at least twenty individuals. Plants were taxonomically identified by specialists of "Centro Oriental de Ecosistemas y Biodiversidad (BIOECO)" from the Natural History Museum Tomas Romay - Santiago de Cuba City. A sample was deposited into the herbarium of the same institution under the registration number 3247.

Essential oil extraction

The essential oil extraction from *O. sanctum* L var. *cubensis* leaves was accomplished until exhaustion by hydrodistillation, helped by a conventional Clevenger apparatus. The essential oil was kept in amber flasks at 4 °C, protected of light until chemical and biological analysis.

Chemical Characterization

The essential oil chemical composition was determined in a Gas Chromatography Mega 2 series coupled to a mass spectrometer (GC/MS) Hewlett Packard model 5890 (USA). A VF-5MS capillary column (Agilent Technologies, USA) of 30 m × 0.32 m and 0.25 mm thick film was used. The program temperature condition was 60 °C (2 min), with an increment of 3 ºC/min until 110 ºC, of 15 ºC/min until 150 ºC and finally with an increment of 17 ºC/min until 290 ºC. The injection volume of the sample was 1µL with a split ratio of 100:1, using helium as the carrier gas at a flow rate of 0.5 mL per minute. Both, injector and detector temperature were maintained at 220 and 250 °C, respectively. A quadruple mass spectrometer analyzer by electron impact ionization at 70 eV was used to characterize the compounds, identifying them comparing their mass spectral data with the National Institute of Standards and Technology mass spectrometry library and according with their Kovats retention indexes.

Cytotoxicity assays

For the evaluation of cytotoxicity, primary cultures of embryonic cardiac cells (ECC) were obtained from Swiss mice and purified following the method previously described[24]. In order to rule out toxic effects upon mammalian host cells, uninfected ECC cultures were exposed to compound studied at 37 ºC for 24, 48 and 72 h (up to 1200 µg/mL). This was the highest concentration evaluated because it is the one that dissolves in 1% dimethylsulfoxide (DMSO) which was used solvent and above this concentration. DMSO is toxic to cardiac cells. Untreated cultures were used as control samples. The cell death rates were measured by the PrestoBlue colorimetric assay allowing the determination of LD_{50} values (compound concentration that reduces 50% of cellular viability)[25]. All cell cultures were maintained in an atmosphere of 5% CO_2 and air, and the assays were run at least three times in duplicates.

Animals and Ethical Considerations

All the animals included in the study (16 animals) received during their lifetime water and food ad libitum. They were maintained under favorable environmental conditions with a temperature of 25 °C, relative humidity between 40 and 70%, and cycles of light and darkness of 12/12 hours. Experiments were carried out following

ethical guidelines towards animal and on the established principles of Reduction and Refinement. Sprague Dawley rats aged from five to six week and weighing between 170 and 300 grams were used, provided by the National Center for Laboratory Animal Production (CENPALAB/Health Certificate number 08001414).

Acute Oral Toxicity Test

The Guidelines for Testing of Chemicals, Acute Oral Toxicity Acute Toxic Class Method 423 of the Organization for Economic Cooperation and Development (OECD), was used[26]. Substances ranges toxicity were settle in the followed classes: not classified, dangerous, toxic, very toxic, and highly toxic as shown in Table 1.

Twelve hours before starting the study food was suspended while the body weigh was monitored moments before the administration of the oil. Animals were randomly assigned in two groups of three female rats each one: a control group treated with physiological saline and the experimental group treated with the essential oil at dose of 2000 mg/kg at the rate of 2mL per 100g of body weight, using an orogastric tube. Clinical observations of animals were performed four times per day, paying attention to behavior, general physical condition, nasal mucosa, changes in skin and fur, respiratory frequency, somatomotor activity, and possible occurrence of signs such as tremors, convulsions, diarrhea, lethargy, drooling, low response to stimuli, sleep, photophobia, and coma. Palpation of the abdomen was carried out as well. After 48 hours of clinical observation without any signs of toxicity, the experimental group receives 2000 mg/kg of oil. Animals were weighed at day seven and fourteen in order to evaluate the weigh increment during the first and second week and to be able to accomplish statistical comparisons between groups. The statistical test applied was "t-Test for independent groups", implemented in the STATISTIC V. 7.0 for Windows; P values <0.05% were regarded as significant. The animals were humanely euthanized at the end of the study by administering an overdose of the anesthetic ketamine intraperitoneally. Internal organs were subsequently studied macroscopically.

Acute Dermal Toxicity Test

The Guidelines for Testing of Chemicals, Acute Dermal Toxicity 402 of the Organization for Economic Cooperation and Development (OECD), was used[27]. Animals (5 female and 5 male) were shaved 24 hours before the application of the oil uniformly on the back at both flanks (10% body surface). They were weighed just before the start of the study[28], thereby determining the volume to be applied using a dose of 2000 mg/kg body weight (dermal application). Patches with oil were applied on an area of about 6 cm 2 of one flank, and the other flank was used as a control. Test substance was held in contact with the skin using porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The test site was covered in a suitable manner to retain the gauze dressing and test substance, and to ensure that the animals cannot ingest the test substance. After this time, the skin was washed with sterile water leaving the animal other 24 hours under meticulous observation. Clinical observations include changes in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behavior pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. Behavior, general condition, posture and reflexes, attitude towards food, water, and hygiene were evaluated. Animal weights were recorded at 0, 7 and 14 days, and compared at the beginning and the end of the study, when test animals were weighed and then humanely euthanized by overdose of ketamine intraperitoneally. Statistical comparisons between groups were computed throughout "t-Test for independent groups", implemented in the STATISTIC V. 7.0 for Windows; P values <0.05 % were regarded as significant.

Necropsy of all animals was carried out and all gross pathological changes were recorded. If any macroscopic damage occurs (Table 2), was realized microscopic examination of organs. Dermal LD_{50} (median lethal dose), is a statistically derived single dose of a substance that cause death in the 50 % of treated animals. The LD_{50} value can be expressed in terms of weight of test substance per unit weight of test animal (mg/kg), and its values should always be considered in conjunction with the observed toxic effects as well as the necropsy findings.

RESULT AND DISCUSSION

Chemical Characterization of the essential oil

The Gas Chromatography/Mass Spectrometry (GC/MS) analysis allowed us to identify the chemical composition and relative abundance of the *O. sanctum* L var. *cubensis* essential oil constituents. The extraction resulted in a pale yellow essential oil with a yield of 0.5% (w/v). The oil of OS presented 20 compounds, of which eight have values exceeding 4%, representing more than 92% of the oil. Of them, three stand out by its high concentration, defined as the major components: Eugenol 21.96%, β-caryophyllene 20.79% and Bicyclogermacrene 20.38% (Table 3).

From a chemical point of view, most of the components are sesquiterpene type (16 compounds), which represent over 70% of the oil in terms of concentration. The monoterpenes (2 compounds) represent only 7.5 per cent while aromatic hydrocarbons (2 compounds) the 22%. Only the 31.88% of the compounds are oxygenated (9.92% excluding eugenol), with is present in relatively low concentration considering the wild condition of the plantation. For other or not specific varieties of OS, studies reveal an essential oil yielding from 0.4 up to 1.3% and comprising different concentration of components depending on the geo-agro-climatic conditions. This variability is also reported when considering the main constituents: Some paper point out eugenol as main compound (from 25.3 to 77.50%)[29,30] while others refers methyl-eugenol (from 37.95 to 76.27)[31].

Table 1: Classification of substances according to the guideline 423 of the Organization for Economic Cooperation and Development.

DL 50 ranges (mg/kg)	ATC	Classification
DL 50 > 2000mg/kg	ATC 5	Not classified
300 < DL 50 ≤ 2000mg/kg	ATC 4	Dangerous
	ATC 3	Toxic
50 < DL 50 ≤ 300mg/kg	ATC 2	Very toxic
5 < DL 50 ≤ 50mg/kg	ATC 1	Highly toxic
DL 50 < 5mg/kg		

Table 2: Graduation of skin reactions according to the guideline 402 of the Organization for Economic Cooperation and Development.

Erythema and bedsore	Edema formation	Grade
No erythema	No edema	0
Very slight erythema	Very slight edema. (Barely perceptible)	1
Well defined erythema	Slight edema (Application area´s edges well definite by an elevate central area)	2
Moderate to severe erythema	Moderate edema (raising approximately 1 mm)	3
Erythema to severe bedsore	Severe edema (raising more than 1 mm and extended beyond the site of exposure	4

Regarding the previous work conducted in the western region of Cuba, some common points are evident. In both studies, Eugenol and β-caryophyllene are the major compounds, even when the plant that grows in the Western part show highest levels of Eugenol. In addition, 11 compounds are reported in both essential oils but with high fluctuations in their concentrations (Linalool 0.2% vs 7.13%, γ-Muurolene traces vs 5.82 %, β- bisabolene 1.1% vs 4.12%, Caryophyllene oxide with 3.8% vs 1.18%. The most important differences are in the third main compounds that in our case is Bicyclogermacrene (20.37%) which is absent in the previous work and β-elemene with an abundance of 18.0% in the Western plantation but detected only in a 1.52% in this investigation[23]. All this provide evidences once again that the chemical composition of essential oil can be highly variable due to genetic diversity, geographical region, habitat and cultural practices[14,32].

Evaluation of cytotoxicity activity

The results presented in Table 4 reflect the mean and standard deviation of three individual tests (performed in triplicate). This analysis of toxicity on primary culture of cardiomyocytes evaluates the essential oil toxicity of *O. sanctum* var. *cubensis* identifying damage to mitochondrial level through electron transport system (removal of oxygen and replaced by hydrogen) and cytochromes[33]. The data sets show no toxicity to morphological, physiological and cell density changes, so well as mitochondrial fractions and viability tested at 24, 48 and 72h of incubation. At the maximum concentration (1200 µg/mL) the OS essential oil barely provokes the 5% of cell death, meaning that this substance does not result toxic for those kinds of cell at the concentration evaluated. *Acute Oral Toxicity Test*

The behavior of the body weight of the study animals was not affected after administration of the essential oil of *O. sanctum* var. *cubensis* (2000 mg/kg), demonstrating a normal increased (Figure 1), without significant differences between the averages of two samples for a confidence level of 95% (P-valor = 0.13337). This corresponds with the arguments presented by the reference standards for the use and care of laboratory animals, in relation to the species used[34,35,36]. An important indicator for determining the toxicity of a substance is the evaluation of clinical manifestations, since it is possible to know damage associated with injuries to organs and systems. No behavioral change were observed during the 14 days of the experiment, neither changes in skin and fur; nor diarrhea, lethargy, drooling, low response to stimuli, sleep alteration, nor photophobia. In addition, no pathological changes were found without animal organs and organ systems when were examined in the Pathology Laboratory. Those observations allow us to propose that the *O. sanctum* var. *cubensis* essential oil administered orally at single dose do not show any acute oral toxicity, therefore qualified as "Not Classified" as specify the Directive 423 of the Organization for Economic Cooperation and Development (OECD).

Many investigations refers that eugenol (the main compound in essential oil of *O. sanctum* var. *cubensis*) may be dangerous, particularly if more than the recommended dosage is taken. In other cases, it may cause convulsions, nausea, rapid heartbeat, and dizziness[37]. No studies were found about the toxicity in vitro nor in vivo of the second and the third majoritarian compounds (β-Caryophyllene and Bicyclogermacrene).

Is it known that the mammalian toxicity of essentials oils (EOs) is low and they are well studied experimentally and clinically because of their use as medicinal products. The majority of EOs, including chamomile, citronella, lavender, clove, eucalyptus, anise, and marjoram have an oral LD_{50} value ranging from 2000 mg/kg to 5000 mg/kg in rats.

Acute Dermal Toxic Test

The administered dose (2000 mg/kg body weight) in the experimental group did not cause significant changes in clinical signs of the rats during the first 24 hours. Once this time has elapsed, removed the plasters with care to not injure the skin, and washed the application area, no apparent changes were found in the lateral skin where the animals were treated. Further strict observation and clinical evaluation throughout the experimental period (14 days) were accomplished without any symptom reported. The Pathological Anatomy Laboratory did also not report any abnormality within the macromorphological study in hearts, lungs, kidneys,

Table 3: Chemical compounds identified by gas chromatography coupled to a mass spectrometer (GC/MS) in the *Ocimum sanctum* L. var. *cubensis* (Lamiaceae) essential oil.

No	KI(a)	Constituents	RI(b)	% area
1	960	Benzaldehyde	962	0.44
2	1031	1.8 Cineole	1031	0.13
3	1096	Linalool	1101	7.13
4	1359	Eugenol	1357	21.96
5	1388	β-Bourbonene	1375	1.25
6	1388	β-Cubebene	1386	0.14
7	1390	β-Elemene	1388	1.52
8	1419	β-Caryophyllene	1419	20.79
9	1434	α-trans Bergamotene	1432	0.29
10	1454	α-humulene	1454	4.20
11	1460	alloaromadendrene	1459	1.16
12	1479	γ-Muurolene	1480	5.82
13	1490	β-Selinene	1488	7.69
14	1500	Bicyclogermacrene	1496	20.38
15	1505	β- Bisabolene	1510	4.12
16	1522	δ-Cadinene	1524	0.30
17	1522	β- Sesquiphellandrene	1531	0.20
18	1561	β- Germacrene	1569	0.26
19	1582	Caryophyllene oxide	1585	1.18
20	1590	Globulol	1588	1.05
(a)	Kovats retention index reported by NIST (National Institute for Standard and Technology)			
(b)	Kovats retention index calculated.			

Table 4: *Ocimum sanctum* var. *cubensis* essential oil toxicity on primary culture of cardiomyocytes.

Substance	24 h	48 h	72 h
Control	0.0000 ± 0.00	0.0000 ± 0.00	0.0000 ± 0.00
1200 µg/mL	5.2195 ± 0.03	5.0973 ± 0.33	4.8448 ± 3.65
600 µg/mL	0.5601 ± 0.01	0.4940 ± 0.49	0.3096 ± 0.09
300 µg/mL	0.0514 ± 0.04	0.0000 ± 0.00	0.0000 ± 0.00
150 µg/mL	0.0000 ± 0.00	0.0617 ± 0.09	0.0000 ± 0.00
DMSO	6.8291 ± 0.05	2.0360 ± 1.18	5.6845 ± 0.00

livers, stomachs and spleens. Regarding the body weight, a significant increase after administration of the test substance (Figure 2) was observed. Such significant differences (p> 0.05%) were established between body weight variances between day 0, 7 and 14 for males (P-valor = 0.1244); however, in females the only significant differences were observed between days 0 and 14. Nevertheless, these results are consistent with the issues raised by the reference standards for the use and care of laboratory animals, in relation to the species used[35,36,37].

With some essential oils or at least with the monoterpenes constituting them, dermal toxicity was observed, among them are the clove, eucalyptus, wintergreen, which are known for their irritability[38]. Bergamot and angelica essential oils cause photosensitivity[39]; D-limonene produces further irritating transdermal absorption 40; and another that tea-tree oil can cause skin allergies[41,42].

According to the data obtained in the Acute Dermal Toxicity test conducted according to Directive No. 402 of the Organization for Economic Cooperation and Development (OECD), the animals treated with the essential oil of *O. sanctum* var. *cubensis*, classified as not toxic for skin after topical administration in a single dose in Sprague-Dawley line. The obtained result indicates that the oil can be considered safe and harmless to topically not toxic to skin contact.

Numbers on the horizontal lines represent weight gain in milligrams (mg) from day 0 until the seventh and final day of the trial.

CONCLUSIONS

The essential oil from the leaves of *Ocimum sanctum* L. var. *cubensis* cultivated in the eastern region of Cuba presented 20 compounds defined as the major components: Eugenol, β-caryophyllene and Bicyclogermacrene. This essential oil does not result toxic in the "*in vitro*" test on primary culture of cardiomyocytes at 1200 µg/mL, neither on "*in vivo*" when the acute oral toxicity at 2000 mg/kg was tested. In addition, the essential oil of *Ocimum sanctum* L. var. *cubensis* showed no Acute Dermal Toxicity classifying this test substance as NO TOXIC according to Directive No. 402 of the Organization for Economic Cooperation and Development (OECD).

The obtained result indicates that the oil could be considered safe, harmless topically and oral, showing no in vitro and in vivo toxicity.

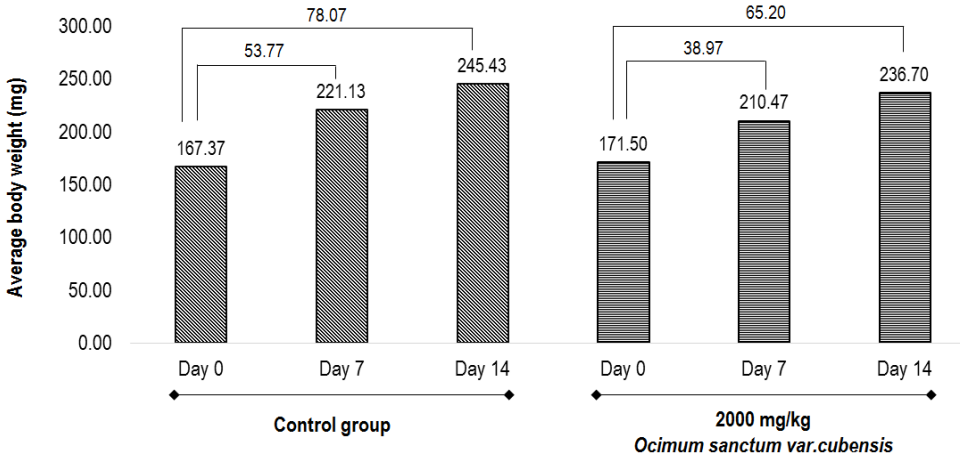

Figure 1: Behavior of body weight of female rats in the Acute Oral Toxicity test of the essential oil of *Ocimum sanctum* var. *cubensis*. Numbers on the horizontal lines represent weight gain in milligrams (mg) from day 0 until the seventh and final day of the trial.

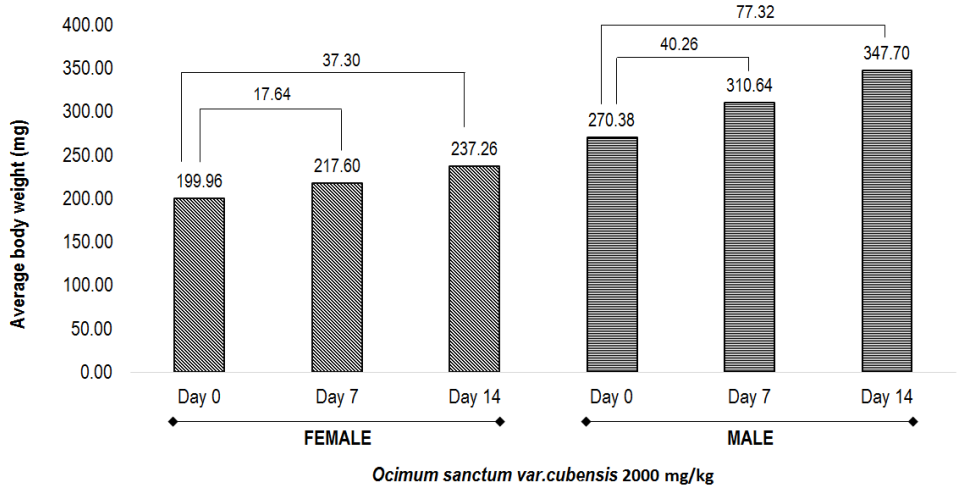

Figure 2: Behavior of body weight of male and female rats in the Acute Dermal Toxicity test of the essential oil of *Ocimum sanctum* var. *cubensis*.

CONFLICT OF INTERESTS

The authors declare that they have no financial and commercial interests. No conflict of interests has been declared.

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