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Original Article

Volatile oil of *Croton zehntneri per oral* sub-acute treatment offers small toxicity: perspective of therapeutic use

Andrelina N. Coelho-de-Souza[a,*], Maria Valdenir A.P. Rocha[a], Keciany A. Oliveira[a], Yuri A.G. Vasconcelos[a], Edgleyson C. Santos[a], Kerly Shamyra Silva-Alves[a], Lúcio Ricardo L. Diniz[b], Francisco Walber Ferreira-da-Silva[c], Ariclécio C. Oliveira[a], Edson L. Ponte[d], Janaína Serra-Azul M. Evangelista[a], Ana Maria S. Assreuy[a], José Henrique Leal-Cardoso[a]

[a] *Instituto Superior de Ciências Biomédicas, Universidade Estadual do Ceará, Fortaleza, CE, Brazil*
[b] *Departamento de Morfologia, Universidade Federal de Aracaju, Aracaju, SE, Brazil*
[c] *Universidade Estadual do Vale do Acaraú, Sobral, CE, Brazil*
[d] *Centro Universitário Christus, Campus do Parque Ecológico, Fortaleza, CE, Brazil*

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ABSTRACT

Croton zehntneri Pax & K. Hoffm., Euphorbiaceae, or "canela-de-cunhé" is used in the Northeast Brazil to treat several diseases. Leaves and aerial parts of *C. zehntneri* are rich in volatile oil of high potential therapeutic. This study aimed to investigate volatile oil systemic toxicity after *per oral* treatment in rats. Volatile oil characterization (gas chromatography and mass spectrometry) showed 85.7% anethole and 4.8% estragole. Male Wistar rats (116–149 g) were treated with volatile oil (250 mg/kg *p.o.*) during ten weeks and evaluated for the following parameters: survival; food and water intake; body mass; absolute/relative organs weight; hemogram; plasma biochemical dosage; organs morphology. Volatile oil did not alter animal water and food consumption or the relative/absolute weight of most organs, but animals gained less weight. Volatile oil did not alter function biomarkers of pancreas, kidney, heart or liver, but increased plasma gamma-glutamyltranspeptidase (liver biomarker) and decreased uric acid (kidney biomarker). Although volatile oil had caused discrete morphological alterations in some organs, it did not induce architectural changes in these organs. In conclusion, the sub-acute *per oral* treatment with volatile oil no longer than ten weeks in rats offers small toxicity at doses below 250 mg/kg.

Introduction

Croton zehntneri Pax & K. Hoffm., Euphorbiaceae, is a plant native to Northeast Brazil, where it is popularly known as "canela-de-cunha". Its barks and leaves are widely used as infusions and teas to stimulate appetite and to treat inflammation, colic, diarrhea, nervous (anxiety, insomnia, irritability) and gastrointestinal disorders (Leal-Cardoso and Fonteles, 1999; Coelho-de-Souza et al., 2013). Popular sellers also prepare tea from *C. zehntneri* leaves to treat rheumatic, cardiac and respiratory diseases and other parts in decoctions and cataplasmas to treat inflammation and wound healing (Cavalcanti et al., 2012). Leaves and aerial parts of *C. zehntneri* are rich in volatile oil of pleasant aroma, resembling that of anise (*Illicium verum*) (Craveiro et al., 1977; Leal-Cardoso and Fonteles, 1999). The volatile oil of *C. zehntneri* (EOCz) is composed by several terpenes, terpenoids and phenylpropanoids, including trans-anethole (85.7%) and estragole (Craveiro et al., 1977).

Volatile oil has several biological and pharmacological effects, such as central nervous system depression (Batatinha et al., 1995; Lazarini et al., 2000), blockade of neuromuscular transmission (Albuquerque et al., 1995), antispasmodic (Coelho-de-Souza et al., 1997, 1998), antinociceptive (Oliveira et al., 2001), gastroprotective (Coelho-de-Souza et al., 2013), healing (Cavalcanti et al., 2012) and cardiovascular (de Siqueira et al., 2006). Other effects had also been described for EOCz, including anti-helmintic (Camurça-Vasconcelos et al., 2007), bactericidal (Andrade et al., 2015), antifungal (Fontenelle et al., 2008), hepatoprotective (Lima et al., 2008) and larvicidal (against *Aedes aegypti*) (Morais et al., 2006). Some of these effects have being assigned to trans-anethole, the main constituent of EOCz. Several studies demonstrated that

* Corresponding author.
E-mail: andrelina.noronha@uece.br (A.N. Coelho-de-Souza).

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trans-anethole exhibits antioxidant (Galicka et al., 2014), antibacterial (Zahid et al., 2015), anticancer (Chen and de Graffenried, 2012; Choo et al., 2011), antigenotoxic (Newberne et al., 1999), antiplatelet (Tognolini et al., 2007) and anti-inflammatory properties (Ponte et al., 2012; Ritter et al., 2017).

Acute studies have shown that EOCz (Oliveira et al., 2001) and *trans*-anethole (Newberne et al., 1999; Ponte et al., 2012) exhibit low toxicity. EOCz administered by oral route presents wide therapeutic window (LD_{50} > 2.5 g/kg) (Oliveira et al., 2001), since most of its pharmacological effects are manifested at doses between 0.03 and 0.3 g/kg. Moreover, according to Food and Drug Administration, *trans*-anethole is considered safe and devoid of genotoxic and carcinogenic effects (Newberne et al., 1999). However, toxicity studies performed for longer treatment periods (10-week period) are necessary to validate the consumption of both *C. zehntneri* and EOCz.

Based in the popular use of *C. zehntneri* and in the pharmacological effects of EOCz, this study aimed to investigate EOCz toxicity after *per oral* treatment in rats along ten weeks.

Material and methods

Plant material, extraction and EOCz chemical analysis

Croton zehntneri Pax & K. Hoffm., Euphorbiaceae, leaves were collected in the small village of Cocalzinho, near to the city of Viçosa do Ceará (Ceara State, Brazil) and its identification was confirmed by Dr. F.J. Abreu Matos (Laboratory of Natural Products, Federal University of Ceara-UFC, Fortaleza, CE, Brazil). A voucher specimen (no. 277477) has been deposited at the herbarium Prisco Viana of the Federal University of Ceara (UFC). EOCz was obtained by steam distillation and its chemical constituents (*trans*-anethole: 85.7%, estragole: 4.8%, 1,8-cineole: 2.95%, β-myrcene: 2.2%, anisaldehyde: 1.22%, *trans*-caryophyllene: 0.9%, unidentified: 2.23%) were determined by gas chromatography coupled to mass spectrometry in the Technological Development Park of the Federal University of Ceará – PADETEC (Technological Development Park)/UFC. The analysis conditions were as following: Hewlett-Packard 6971 (Palo alto, CA, USA); column of dimethylpolysiloxane DB-1 fused silica capillary column (30 m × 0.25 mm; 0.1 μm); helium (1 ml/min) as carrier gas; injector temperature: 250 °C; detector temperature: 200 °C; column temperature: 35–180 °C at 4 °C/min and 180–250 °C at 10 °C/min; and mass spectra: electronic impact 70 eV. The compounds were identified using mass spectral library search.

Animals

Male Wistar rats (n = 30) of the Central Animal House/UFC, presenting initial body mass that ranged from 116–149 g, were randomly distributed to collective cages (five per cage). Rats were chosen for this study, since they are considered the most conservative model to evaluate hepatotoxicity of terpenes in humans (Newberne et al., 1999). Before treatment, animals were maintained for 14 days in the Experimental Physiology Laboratory-UECE at 23 ± 2 °C, 12-h light/dark cycle. Standard rodent chow (BioBaseBio-TEC) and water were available *ad libitum*. The general animal appearance was observed daily for disease detection, and weekly for the analysis of parasites in feces samples.

Experimental design

Volatile oil or *trans*-anethole (1-methoxy-4-[(*E*)-prop-1-enyl] benzene) (Sigma–Aldrich, St. Louis, MO, EUA) was mixed with water daily prior to animal administration. Rats (n = 10/group) received 250 mg/kg EOCz (*p.o.*) or *trans*-anethole (used as the reference drug) for 10 weeks. The effective dose of 250 mg/kg (body mass) was selected since it is lower than 10% of EOCz LD50 (Oliveira et al., 2001). The control group received water by gavage in equivalent volume. During treatment, animals were weighted weekly and the food and water intake were daily measured by subtracting the amount measured from the amount offered at a given cage, after 24 h of consumption, and dividing the result by the number of animals in each cage (five animals).

Protocols were approved by the Ethical Committee (CEUA-UECE no. 06379067-0).

After treatment, animals were anesthetized with xylazine (10 mg/kg) and ketamine (75 mg/kg), and blood was collected for hematological and clinical chemistry analysis. Blood samples were collected in tubes containing potassium EDTA for hematological evaluation (automated equipment Pentra 60®) of the following parameters: erythrocyte, leukocyte and platelet counts; hematocrit (Ht); hemoglobin (Hb); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); hemoglobin mean concentration (MCHC) and red distribution width (RDW). The morphological analysis of blood cells and the differential leukocyte counts were performed on smears stained with fast panoptic dye (Laborclin®). For the clinical chemistry analysis, blood samples were collected in tubes containing separator gel and left to clot retraction. Samples were centrifuged and the serum analyzed (Diagnostic Labtest S.A., 2009) in automated equipment (Labmax 240®) for the following parameters: amylase, lipase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), gamma-glutamyltranspeptidase (GAMA-GT), total creatine kinase (CK-NAC), creatine kinase isoenzyme MB (CK-MB), total bilirubin (TB), direct and indirect bilirubin (DB, IB), total proteins (TP), albumin and globulin.

Animals were euthanized for histological analysis of liver, lung, adrenal gland, spleen, stomach, duodenum, cerebral cortex and heart. The organs were removed, weighed, fixed during 24 h in 10% buffered formaldehyde, transferred to 70% ethanol, trimmed using an automated histotechnique (Leica®), embedded in paraffin, sectioned to a thickness of 3–5 μm and stained with hematoxilin and eosin (H&E). The analysis was performed in a double-blind fashion manner. The photomicrographs were performed by the use of Microscope Nikon Eclipse Nis and Software Nis 4.0.

Statistical analysis

The results were presented as mean ± S.E.M. and analyzed by one-way ANOVA followed by Bonferroni or Student–Newman–Keuls Method. $p \leq 0.05$ was considered significant.

Results

Survival, body mass, absolute and relative organs weight and food and water intake of rats treated with EOCz

Similar to *trans*-anethole, rats treated with EOCz (250 mg/kg; *p.o.*) during ten weeks survived until the end of the experiment (Table 1). Besides, the treatment did not alter the animal water consumption, except at the fifth week, in which EOCz induced a discrete increase (Fig. 1A), but gained less weight (15–16%) compared to control (Table 1). Accordingly, the treatment with EOCz, as with anethole, exhibited small, but significant reduction in food intake from week 8 to week 10, reducing by 12% at week 10 (EOCz: 187.00 ± 1.90 g *vs.* control: 210.0 ± 3.98 g) (Fig. 1B). In

Table 1
Survival and body mass of rats after 10 week-treatment with volatile oil of *Croton zehntneri* or anethole.

Treatment	Survival	Body mass (g)			Final body mass gain (% of control)
		Initial	Final	Body mass gain	
Control	10/10	[b]163.2 ± 4.97	384.8 ± 9.48	221.6 ± 5.85	100.00
[a]EOCZ	10/10	175.6 ± 4.66	364.0 ± 8.00	188.4 ± 8.11[c]	94.59
Anethole	10/10	177.1 ± 2.97	362.5 ± 8.71	185.4 ± 8.03[c]	94.20

[a] EOCz, volatile oil of *C. zethneri*.
[b] Mean ± S.E.M.
[c] $p \leq 0.05$ *vs.* control (ANOVA and Student's *t*-test).

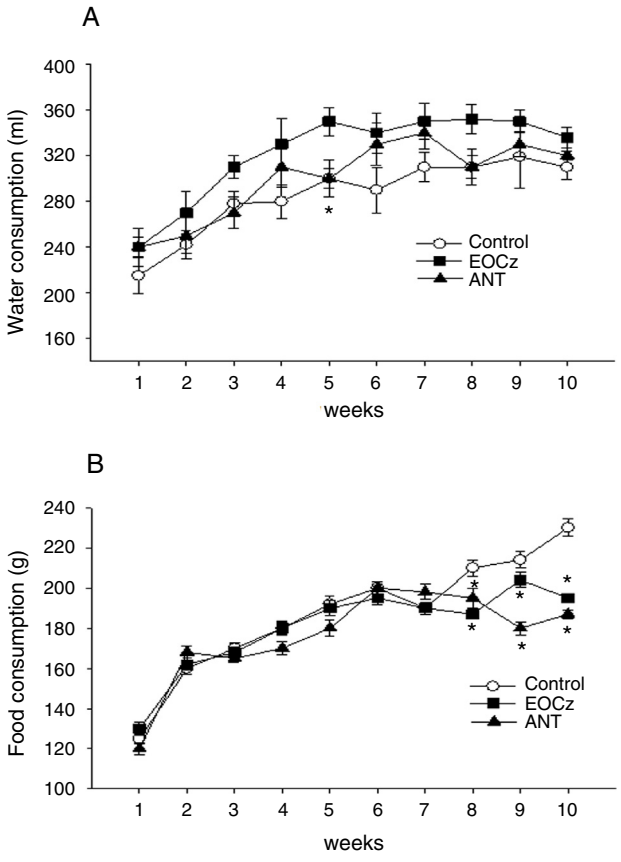

Fig. 1. Water (A) and food (B) consumption of rats treated with volatile oil of *Croton zehntneri*. Animals received water, anethole or volatile oil at 250 mg/kg b.w., *p.o.* during 10 weeks. EOCz: volatile oil of *C. zehntneri*. ANT: anethole. Mean ± S.E.M. ANOVA and followed by Bonferroni (*$p < 0.05$ *vs.* control).

addition EOCz and *trans*-anethole, did not alter either the relative or the absolute weight of most of the organs analyzed, except for the relative weight of kidney, which was increased by 11% (Table 2). Clinical abnormalities that would suggest toxicity were not observed, such as external and eye lesions, changes in hair color and distribution (data not shown).

Biochemical and hematological parameters of rats treated with EOCz

The treatment with EOCz, as with trans-anethole, did not induce alterations in the function biomarkers of pancreas (α-amylase and lipase), kidney (urea and creatinine), heart (CK and CK-MB) and liver (ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; AP, alkaline phosphatase; TB, total bilirubin; DB, direct bilirubin; IB, indirect bilirubin; TP, total protein). However, EOCz and trans-anethole increased in 7 and 4 fold, respectively, the serum biomarker of liver function (gamma glutamyl transpeptidase (GAMA-GT), but only EOCz, decreased uric acid, the serum biomarker of kidney function (Table 3). The hematological parameters were unaltered by EOCz or *trans*-anethole (Table 4), being the erythrocytes normocytic and normochromic (data not shown).

Table 2
Absolute and relative organs weight of rats after 10 week-treatment with volatile oil of *Croton zehntneri* or anethole.

Parameters	Treatment		
	Control	EOCz	Anethole
Body mass(g)	[a]379 ± 6.6	364 ± 7.97	371 ± 10.14
Liver			
Absolute (g)	12.356 ± 0.486	12.923 ± 0.512	12.661 ± 0.371
Relative (mg/g)	32.540 ± 0.144	35.590 ± 0.145	34.190 ± 0.050
Heart			
Absolute (g)	1.051 ± 0.046	1.016 ± 0.036	1.054 ± 0.045
Relative (mg/g)	2.770 ± 0.011	2.810 ± 0.013	2.850 ± 0.012
Ileum			
Absolute (g)	9.147 ± 0.203	8.481 ± 0.325	8.924 ± 0.480
Relative (mg/g)	24.180 ± 0.075	23.340 ± 0.088	23.790 ± 0.105
Empty stomach			
Absolute (g)	1.762 ± 0.055	1.799 ± 0.050	1.797 ± 0.045
Relative (mg/g)	4.660 ± 0.018	5.030 ± 0.013	4.870 ± 0.014
Full stomach			
Absolute (g)	4.539 ± 0.467	4.773 ± 0.656	4.435 ± 0.473
Relative (mg/g)	11.000 ± 0.089	13.210 ± 0.195	11.900 ± 0.113
Kidney			
Absolute (g)	1.101 ± 0.055	1.165 ± 0.022	1.218 ± 0.039
Relative (mg/g)	2.890 ± 0.015	3.210 ± 0.005[b]	3.320 ± 0.016[b]
Pancreas			
Absolute (g)	1.366 ± 0.089	1.364 ± 0.085	1.380 ± 0.098
Relative (mg/g)	3.600 ± 0.023	3.730 ± 0.019	3.770 ± 0.031
Spleen			
Absolute (g)	0.755 ± 0.029	0.735 ± 0.050	0.719 ± 0.022
Relative (mg/g)	1.990 ± 0.008	2.000 ± 0.023	1.950 ± 0.006
Lung			
Absolute (g)	1.522 ± 0.083	1.517 ± 0.054	1.522 ± 0.054
Relative (mg/g)	3.850 ± 0.014	4.180 ± 0.017	4.120 ± 0.015
Adrenal gland			
Absolute (g)	0.163 ± 0.022	0.173 ± 0.066	0.122 ± 0.019
Relative(mg/g)	0.427 ± 0.006	0.482 ± 0.0184	0.551 ± 0.016
Brain			
Absolute (g)	1.651 ± 0.074	1.740 ± 0.066	1.730 ± 0.045
Relative (mg/g)	4.360 ± 0.021	4.790 ± 0.018	4.700 ± 0.017

[a] Mean ± S.E.M. ($n = 10$).
[b] $p \leq 0.05$ *vs.* control (ANOVA and Student's *t*-test).

Histological pattern of organs from rats treated with EOCz

Some histological alterations were observed in the following organs: liver – discrete hydroponic degeneration and vascular congestion; lung, stomach and duodenum - discrete inflammatory infiltration (laminas not shown); adrenal gland cortex-discrete

Table 3
Biochemical blood parameters of rats after 10 week-treatment with volatile oil of *Croton zehntneri* or anethole.

Parameters	Treatment		
	Control	EOCz	Anethole
ALT (U/l)	62.00 ± 3.26	66.44 ± 6.15	70.89 ± 4.23
AST (U/l)	190.00 ± 18.00	230.00 ± 42.00	230.00 ± 35.00
LDH (U/l)	4034.00 ± 185.00	4097.00 ± 179.00	4010.00 ± 236.00
AP (U/l)	225.00 ± 15.00	204.00 ± 10.00	192.00 ± 14.00
GAMA-GT (U/l)	0.30 ± 0.15	2.10 ± 0.27[b]	1.33 ± 0.16[a]
TB (mg/dl)	0.33 ± 0.06	0.24 ± 0.04	0.25 ± 0.03
DB (mg/dl)	0.03 ± 0.01	0.03 ± 0.004	0.03 ± 0.005
IB (mg/dl)	0.27 ± 0.06	0.30 ± 0.08	0.22 ± 0.03
TP (mg/dl)	6.56 ± 0.14	6.67 ± 0.11	6.62 ± 0.13
Albumin (mg/dl)	3.15 ± 0.05	3.30 ± 0.06	3.31 ± 0.05
Globulin (mg/dl)	3.41 ± 0.10	3.30 ± 0.06	3.31 ± 0.05
Urea (mg/dl)	54.00 ± 2.86	49.00 ± 1.22	53.00 ± 53.00
Creatinine (mg/dl)	0.44 ± 0.04	0.40 ± 0.02	0.45 ± 0.02
Uric acid (mg/dl)	1.17 ± 0.19	0.73 ± 0.05[a]	0.91 ± 0.08
CK-total (mg/dl)	1820.00 ± 319.00	1956.00 ± 361.00	2501.00 ± 502.00
CK-MB (mg/dl)	1890.00 ± 262.00	1915.00 ± 150.00	1795.00 ± 140.00
Amylase (U/l)	1206.00 ± 62.00	1227.00 ± 47.00	1185.00 ± 39.00
Lipase (U/l)	11.00 ± 0.31	12.00 ± 1.41	11.00 ± 0.39

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; AP, alcaline phosphatase; GAMMA-GT, gamma glutamyl transpeptidase; TB, total bilirubin; DB, directbilirubin; IB, indirect bilirubin; TP, total protein: CK-Total, total creatine phosphokinase; CK-MB, creatine phosphokinase MB fraction. Mean ± S.E.M.

[a] $p \leq 0.05$ *vs.* control (ANOVA and Student's *t*-test).

[b] $p \leq 0.001$ *vs.* control (ANOVA and Student's *t*-test).

Table 4
Hematological parameters of rats after 10 week-treatment with volatile oil of *Croton zehntneri* or anethole.

Parameters	Treatment		
	Control	EOCz	Anethole
Erythrocytes $\times 10^6$	8.40 ± 0.14	8.39 ± 0.32	8.24 ± 0.28
Hgb (g/dl)	15.97 ± 0.33	16.01 ± 0.79	16.02 ± 0.51
HCT (%)	50.24 ± 1.03	50.16 ± 2.21	50.37 ± 1.76
MCV (μm^3)	59.78 ± 0.28	59.78 ± 0.40	59.78 ± 0.52
MCH (pg)	19.04 ± 0.09	19.02 ± 0.29	19.09 ± 0.18
MCHC (g/dl)	31.84 ± 0.10	31.89 ± 0.37	31.89 ± 0.20
RDW (%)	14.00 ± 0.19	14.60 ± 0.21	14.79 ± 0.26
Leukocytes/mm^3	7.68 ± 0.62	8.31 ± 0.53	8.24 ± 0.75
Neutrophils (%)	11.09 ± 0.78	11.12 ± 0.85	9.58 ± 0.71
Lymphocytes (%)	86.74 ± 0.99	86.18 ± 1.29	88.27 ± 1.12
Monocytes (%)	0.80 ± 0.19	0.70 ± 0.11	0.54 ± 0.09
Eosinophils (%)	0.80 ± 0.15	0.73 ± 0.23	0.99 ± 0.59
Basophils (%)	0.57 ± 0.06	0.90 ± 0.30	0.62 ± 0.12
Platelets $\times 10^3$ mm^3	596.00 ± 45.16	541.11 ± 62.02	519.00 ± 51.78

Hgb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width. Mean ± S.E.M.

[a] $p \leq 0.05$ *vs.* control (ANOVA and Student–Newman–Keuls Method).

vascular congestion; spleen – discrete hemorrhage and inflammatory cell infiltrate; cerebral cortex – moderate hemorrhage and vascular congestion; heart – moderate lymphohistiocytic inflammatory cell infiltrate (Fig. 2).

Discussion

The investigation of EOCz (*p.o.*) systemic toxicity in rats, assessed by several parameters (survival, water and food consumption, body mass, absolute and relative organs weight, blood biochemical dosage, hemogram and organs morphology), demonstrated that all animals survived and no major toxicity was observed in most of the organs analyzed.

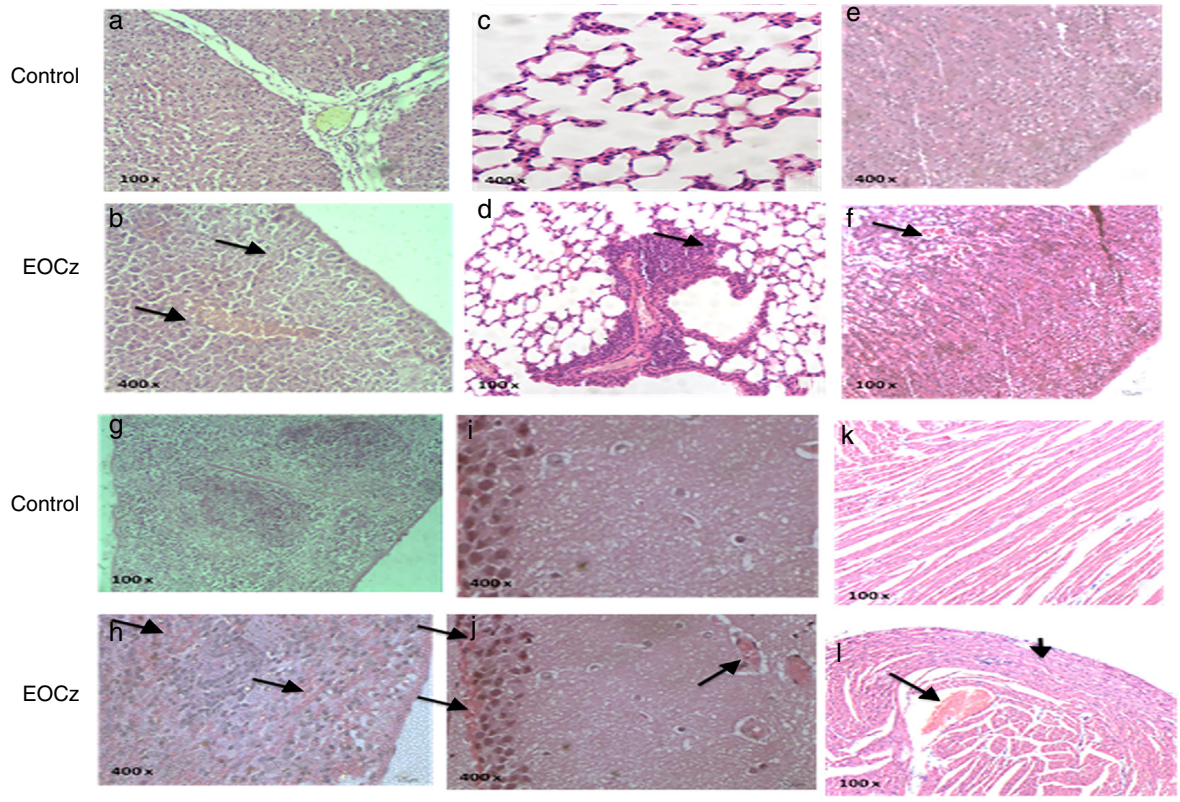

Fig. 2. Histological pattern of organs from rats treated with volatile oil of *Croton zehntneri*. Liver (a, b): discrete hydroponic degeneration and vascular congestion; lung (c, d): discrete inflammatory infiltration; adrenal cortex (e, f): discrete vascular congestion; spleen (g, h): discrete hemorrhage and inflammatory infiltration; cerebral cortex (i, j): moderate hemorrhage and vascular congestion; heart (k, l): moderate lymphohistiocytic inflammatory infiltration and discrete vascular congestion. H&E, Microscope Nikon Eclipse Nis, Nis Software 4.0Â®.

Volatile oil reduced the animal weight gain and food intake after eight weeks of treatment, an effect that was also elicited by the reference drug *trans*-anethole, the main constituent of EOCz (Le Bourhis and Soenen, 1973). In accordance, other studies revealed that *trans*-anethole (600–1500 mg/kg) reduces the animal weight gain (Newberne et al., 1999). This data suggest that the reduction in the animal weight gain and in food intake caused by EOCz could be assigned to trans-anethole.

Treatment with EOCz or *trans*-anethole did not change pancreatic or cardiac function, since no alterations were observed in the serum concentration of α-amylase, lipase, CK or CK-MB. Although it is difficult to correlate enzyme activity with cardiac injury, as most enzymes are not confined into specific organs, CK and CK-MB are very useful to indicate heart damage. In respect to kidney, EOCz and *trans*-anethole did not alter plasma levels of urea and creatinine. However, EOCz caused significant reduction in uric acid, the main product of purine metabolism, that is excreted in the urine and its increase in plasma concentrations is usually associated with renal dysfunctions. Moreover, the hematological profile was unaltered by EOCz, but it was by *trans*-anethole, that caused slight increase in RDW (red distribution width), a parameter that numerically represents variations of the volume of red blood cells. High RDW values may indicate changes in erythrocyte maturation, however, this alteration isolated, not associated to other hematological changes, does not indicate toxic effect, although may be a potential prognostic marker in patients with cancer (Hu et al., 2017), as observed in this study.

In mammals, the major constituent of EOCz, *trans*-anethole, is completely metabolized in the liver, conjugated and excreted in the urine. Its detoxification occurs *via* three main routes (ω-oxidation, *o*-demethylation and epoxidation) that vary according to specie, sex, dose and duration. Epoxidation, the minority route (3–8.8%) provides the stable metabolite *trans*-anethole epoxide (AE), by which the daily production of AE during a period longer than 90 days and doses higher than 120 mg/kg induces hepatotoxic effects, such as cellular hypertrophy, inflammatory infiltrate, sinusoidal dilation, hyperplasia and carcinoma (Newberne et al., 1999). Despite of the dose used in this study (250 mg/kg), either greater than or at the higher end of the range for effective pharmacological activity, we believe that very likely no functional important hepatotoxic effect was observed, as we discuss below, suggesting no liver accumulation of AE metabolite. In the present study both EOCz and *trans*-anethole did not alter hepatic function markers (ALT, AST, alkaline phosphatase, lactate dehydrogenase-LDH, total protein) or caused important alteration in the liver histology. In addition, no changes in the main bile pigment (total, direct and indirect bilirubin) were observed, indicating that neither EOCz nor *trans*-anethole interfere with the conjugation of bile salts or with their elimination by the biliary ducts.

Dealing more specifically with the subject of hepatic toxicity, it is important to consider and to analyze the fact that the animals treated with EOCz or *trans*-anethole exhibited increased levels of GAMA-GT. This enzyme is of clinical importance and considered a biomarker of high sensitivity to hepatobiliary injury, being associated with liver and bile ducts diseases (Pratt and Kaplan, 2000; Dufour et al., 2000). However, the increased levels of GAMA-GT here observed, as related to hepatic toxicity, should be interpreted with caution. First, the range of Gamma-GT normal values is very large (in humans is ≤38 for women and ≤55 for men) and the value obtained in presence of anethole and EOCz is very close to control in other experimental studies in rats (Abdelhalim et al., 2018; Salama et al., 2018). Second, as mentioned above, other blood biochemical biomarkers of liver toxicity were not affected. Third, an increase in GAMA-GT might reflect a minor toxicity of other organ, in our study the kidney, perhaps, which is increased in weight (see Ward, 1975; Kwiatkowska et al., 2014). Fourth, the literature describes that both *trans*-anethole and EOCz exert hepatoprotective effect in rats *via* reduction of ALT and AST in the model of acute hepatotoxicity induced by acetaminophen (Lima et al., 2008; da Rocha et al., 2016). Fifth, GAMA-GT has low specificity since it can be altered by the use of drugs and alcohol or by various pathological conditions, such as cardiovascular diseases, fatty liver (Yokoyama et al., 2000; Nomura et al., 1986), obesity and diabetes type 2 (Perry et al., 1998; Nakanishi et al., 2004). Thus, despite the fact that the increase in GAMMA-GT unauthorizes ruling out OECz and anethole hepatotoxicity, the chance of occurrence or, should it occur, of functional significance of this toxicity is likely to be minimum, since hepatocellular injuries induced by certain drugs may be translated to an increase in serum concentrations of AST, ALT and other signs of hepatic injury (Farghali et al., 2016), which were not observed in this study. Although EOCz had caused discrete morphological alterations in some organs and blood biochemical modifications, it did not induce major morphological and functional changes. Accordingly, the discrete vascular congestion observed in the liver may be analyzed following this line of reasoning.

In conclusion, the sub-acute *per oral* treatment with EOCz and anethole, no longer than ten weeks in rats offers small toxicity at doses below 250 mg/kg, and have a potentiality for therapeutic use, provided that the analysis risk/benefit endorse that.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors contributions

MVAPR, KAO, YAGV, ECS and ELP conducted animal experiments; KSSA conducted the EOCz extraction; MVAPR and ACO performed the hematologic and biochemical analysis; JSAME performed morphological analysis; ANCS, LRLD and JHLC supplied critical input to experimental design and data interpretation; and FWFS provided statistical analysis and interpretation; ANCS, AMSA and JHLC were responsible for writing the manuscript. All authors have read and approved the submission of the manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

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