Revista Virtual de Química

ISSN 1984-6835

Artigo

Carvacryl Acetate: Synthesis and Toxicological and Pharmacological Activities

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Rev. Virtual Quim., **2020**, *12* (3), 554-568. Data de publicação na Web: 28 de Abril de 2020

http://rvq.sbq.org.br

Acetato de Carvacrila: Síntese e Atividades Toxicológicas e Farmacológicas

Resumo: Neste trabalho, relatamos uma via sintética para acetato de carvacrila. Além da síntese química, este estudo teve como objetivo avaliar a toxicidade aguda após a administração de acetato de carvacrila em doses de 1000 e 2000 mg/kg em camundongos machos e fêmeas suíços tratados por via oral e intraperitoneal. O acetato de carvacrila foi suspenso assepticamente em 0,05 % de Tween 80, dissolvido em solução salina a 0,9 % (veículo) e administrado por via oral (p.o.) e intraperitonealmente (i.p.) (0, 1000 e 2000 mg/kg). Além da toxicidade aguda, o presente estudo avaliou a capacidade antioxidante pela inibição da hemólise oxidativa e pelo uso de DPPH•, ABTS•+ e pelo potencial de redução [$Fe^{3+}(CN-)_6$ para $Fe^{2+}(CN-)_6$], ensaio *FRAP*. Foram observados sinais de toxicidade e mortalidade (1/5) em animais de ambos os sexos somente após administração intraperitoneal de acetato de carvacrila (2000 mg/kg). Além disso, nenhuma diferença significativa no peso corporal, ingestão de água, consumo de alimentos e produção de excreta de animais tratados foi detectada em comparação com o grupo controle (veículo). O estudo in vitro demonstrou capacidade antioxidante utilizando $DPPH^{\bullet}$ (EC_{50} = 6,1 ± 0,53 µg/mL), $ABTS^{\bullet+}$ (EC_{50} = 5,24 ± 0,38 µg/mL), potencial de redução (EC_{50} = 3,65 µg/mL) e por inibição de hemólise oxidativa induzida por peróxido de hidrogênio (EC_{50} = 2,58 ± 0,07 µg/mL).

Palavras-chave: Capacidade antioxidante; Toxicidade aguda; síntese eficiente; hemólise oxidativa.

Abstract

In this work, we report a synthetic route to carvacryl acetate. In addition to the chemical synthesis, this study aimed to evaluate the acute toxicity after administration of carvacryl acetate at doses of 1000 and 2000 mg/kg in male and female *Swiss* mice treated orally and intraperitoneally. Carvacryl acetate was aseptically suspended in 0.05 % Tween 80, dissolved in 0.9% saline (vehicle) and administered orally (p.o.) and intraperitoneally (i.p.) (0, 1000 and 2000 mg/kg). In addition to the acute toxicity, the present study evaluated the antioxidant capacity by inhibition of oxidative hemolysis and using $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and by reduction potential [$Fe^{3+}(CN-)_6$ to $Fe^{2+}(CN-)_6$], FRAP assay. Signs of toxicity and mortality (1/5) were observed in animals of both sexes only after intraperitoneal administration of carvacryl acetate (2000 mg/kg). Additionally, no significant difference in body weight, water intake, food consumption and excreta production of treated animals were detected in comparison with control group (vehicle). In *vitro* study demonstrated antioxidant capacity using $DPPH^{\bullet}$ (EC_{50} = 6.1 ± 0.53 µg/mL), $ABTS^{\bullet+}$ (EC_{50}=5.24 ± 0.38 µg/mL), reduction potential (EC_{50}=3.65 µg/mL) and by inhibition of oxidative hemolysis induced by hydrogen peroxide (EC_{50}= 2.58 ± 0.07 µg/mL).

Keywords: Antioxidant capacity; Acute toxicity; efficient synthesis; oxidative hemolysis.

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DOI: 10.21577/1984-6835.20200044

Volume 12, Número 3 Maio-Junho 2020

Revista Virtual de Química
ISSN 1984-6835

Carvacryl Acetate: Synthesis and Toxicological and Pharmacological Activities

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Recebido em 18 de Fevereiro de 2019. Aceito para publicação em 27 de Fevereiro de 2020

1. Introduction

The identification of new molecules with pharmacological properties and minimal toxic effects has been an ongoing challenge for the pharmaceutical industries. In this context, preclinical toxicology studies contribute to identify the most promising drugs through their results, which allows the exclusion of candidates before the consumption of financial resources.[1] The pharmacological identification of pharmaceutical products should be subjected to a series of preclinical toxicology studies to provide information about the safety of a new therapeutic option before the beginning of a variety of human clinical trials, which are required before a molecule becomes an approved drug. General studies begin with screening of acute toxicology and progresses through long periods of exposure.[2]

It is known that monoterpenes and synthetic derivatives have several pharmacological activities.[3,4] In this perspective, carvacryl acetate (5-isopropyl-2-methylphenyl acetate) is a monoterpene ester derived from the carvacrol (5-isopropyl-2-methylphenol), which presents important pharmacological properties *in vivo* such as anxiolytic,[5] anti-inflammatory,[6] anti-nociceptive,[6] anticonvulsant,[7] neuroprotector,[8] antibacterial,[9] and anti-nematode.[10] In addition to these studies, carvacryl acetate has shown a promising anthelmintic activity *in vitro* at low concentrations.[11]

Despite the promising pharmacological activity, there are very few studies on the toxicity of carvacryl acetate. Additionally, the development of an efficient synthesis for this monoterpene is still required to enable a thorough biological evaluation and thus has attracted the attention of organic chemists.[9] However, to the best of our knowledge, the synthesis of carvacryl acetate from carvacrol using acid catalysis has not been described in the literature.

In this work, based on our previous experience, we report a synthetic route to carvacryl acetate which is significantly easier and less expensive than previous syntheses.[5,6] In addition to the chemical synthesis, this study aimed to evaluate the acute toxicity after administration of carvacryl acetate at doses of 1000 and 2000 mg/kg in male and female *Swiss* mice treated orally and intraperitoneally. Additionally, the *in vitro* antioxidant capacity was assessed by reduction of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), as well as its ability to transfer electrons by reduction potential and by inhibition of oxidative hemolysis.

2. Materials and Methods

2.1 Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2' -azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), polyoxyethylenesorbitan monooleate (Tween 80), hydrogen peroxide (H_2O_2), trichloroacetic acid, ferric chloride, carvacrol, anhydrous sodium sulfate and potassium ferricyanide were from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals and solvents used were analytical grade and were obtained from Sigma–Aldrich.

2.2. General procedure for the synthesis of carvacryl acetate

The procedure employed was an adaptation of the methodology proposed by Ahluwalia *et al.*[12] for the acetylation of salicylic acid. The mixture was, 10 mL of acetic anhydride and 5.1 mL of carvacrol, followed by the addition of 10 drops of sulfuric acid. The system was heated in a water bath (55 °C) and maintained under agitation for 30 min. The reaction mixture was poured into the separatory funnel, 50 mL of distilled water was added and extracted three times with 50 mL of chloroform. The organic phase was collected, dried with anhydrous sodium sulfate and concentrated under reduced pressure in rotavapor.

The product was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and by ^{1}H– and ^{13}C–Nuclear Magnetic Resonance (NMR). The conditions of analysis by GC-MS were: injector temperature was fixed at 250 °C; transfer line at 100 °C, the temperature of the interface was maintained at 250 °C and columns programmed to operate at 60 °C with an elevation of temperature at the rate of 3 °C min^{-1} until the temperature of 240 °C, remaining for 10 min. The carrier gas used was helium

maintained at a constant flow rate of 1.0 mL per minute. The analysis with the mass detector was performed in scan mode with the acquisition time of 60.35 minutes and solvent cut was set at 3 min. The ionization electron energy was 70 eV and the mass range scanned was 40–600 Da in full scan acquisition mode. The ion source temperature of the mass spectrometer was 250°C. NMR spectra were obtained on a Varian INOVA spectrometer (Model 400) operating at 400 MHz for hydrogen and 100 MHz for carbon-13. The solvent used in the dissolution of the samples for obtaining the spectra was deuterated chloroform.

2.3. Evaluation of antioxidant capacity by DPPH$^{\bullet}$ method

For evaluation of antioxidant capacity against DPPH$^{\bullet}$ radical, the methodology employed was described by Oliveira *et al*.[13] In brief, a reaction mixture containing carvacryl acetate (0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL) and DPPH$^{\bullet}$ radical (40 µg/mL) was shaken vigorously and incubated at room temperature in the absence of light (in the dark) for 30 minutes. The antioxidant evaluation was performed in quintuplicate and the absorbance values (517 nm) were expressed as percentage of inhibition of DPPH$^{\bullet}$ radical in relation to the System (100 % of DPPH$^{\bullet}$ radical). The same experimental procedure was used with the positive control Trolox (140 µg/mL).

2.4. Evaluation of antioxidant capacity by ABTS$^{\bullet+}$ method

For evaluation of antioxidant capacity against ABTS$^{\bullet+}$ radical the methodology described by Re *et al.*[14] with some modifications was used. Initially formed the ABTS$^{\bullet+}$ radical cation from the reaction of 5 mL of a 7 mM ABTS solution with 88 µL of 2.45 mM potassium persulfate ($K_2S_2O_8$), incubated at room temperature and in the absence of light for 16 hours.[15] After this time, the ABTS$^{\bullet+}$ solution was diluted in ethanol to obtain a solution with absorbance of 1.00 (± 0.05), at 734 nm. In dark environment and at ambient temperature, a reaction mixture was made containing carvacryl acetate (0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL) and 1960 µL of ABTS$^{\bullet+}$ solution. The experiment was performed in quintuplicate and the absorbance values (734 nm) were expressed as percentage of inhibition of ABTS$^{\bullet+}$ radical in relation to the System (100 % of ABTS$^{\bullet+}$ radical). The same experimental procedure was used with the positive control Trolox (140 µg/mL).

2.5. Evaluation of reduction potential

In this study, the methodology described by Machado *et al*.[15] was used with modifications. Briefly a reaction mixture was prepared containing various concentrations of carvacryl acetate (0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL), 0.5 mL of 1 % potassium ferricyanide and 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6). The reaction mixture was incubated at 50 °C for 20 minutes, followed by addition of 0.5 mL of 10 % trichloroacetic acid, 0.5 mL of distilled water and 0.125 mL of 0.1 % ferric chloride ($FeCl_3$). The absorbance of the reaction mixture was measured at 700 nm against blank that contained only phosphate buffer. The same experimental procedure was used with the positive control Trolox (140 µg/mL).

2.6. Experiments with animals

Swiss mice were 2 months old of both sexes weighing 25–30 g (Central Animal Laboratory of the Federal University of Piauí). All animals were maintained under controlled temperature (26 ± 1 °C) and a 12-h light/dark cycle. Animals had free access to water and food. All behavioral tests were performed in quiet rooms under the same conditions mentioned above and isolated from external noise. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, Department of Health and Human Services, Washington DC, 1985. The project was approved by the Ethics Committee for Experimental Animals from the Federal University Piaui (ECEA/UFPI# 013/2011).

2.7. Evaluation of antioxidant capacity by inhibition of oxidative damage in erythrocyte membranes

For evaluation of antioxidant capacity by inhibition of mice erythrocyte hemolysis, the methodology described by Ugartondo *et al*.[16] with modifications was used. The 5 % erythrocyte suspension was prepared according to the procedure described by Oliveira *et al*.[17] Briefly,

various concentrations of carvacryl acetate (0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL) were mixed with 300 µL of erythrocytes (5 % v/v) in PBS (NaCl = 8 g/L, KCl = 0.2 g/L, Na_2HPO_4 = 1.44 g/L, and KH_2PO_4 = 0.24 g/L; pH 7.4) and added 300 µL of hydrogen peroxide (H_2O_2, 20 mM in PBS, pH 7.4). Simultaneously, a control was performed to detect spontaneous hemolysis in the absence of the oxidant (H_2O_2) or of carvacryl acetate. The reaction mixture was incubated at 37 °C for 30 minutes followed by centrifugation at 2500 rpm for 5 minutes. For 300 µL of supernatant obtained 2700 µL of PBS was added to determine hemolysis by measuring the absorbance at 540 nm. The results were expressed as percentage of inhibition of hemolysis induced by H_2O_2 (100 % of hemolysis). The same experimental procedure was used with the positive control, Trolox (140 µg/mL).

2.8. Acute toxicity study

The acute toxicity study was performed in accordance with Anvisa regulations.[18] Carvacryl acetate or vehicle was administered only once orally (p.o.) and intraperitoneally (i.p.) at dose levels of 0, 1000, 2000 mg/kg. Mice were divided into 12 groups of 5 animals matched by weight and size. Clinical signs of toxicity related to autonomic and neurobehavioral changes (Example: general appearance, ataxia, vocal fremitus, irritability, body tone, tremor, salivation, tearing, palpebral ptosis, seizures and abnormal movements) were monitored continuously for 1 h after dosing, periodically during the first 24 h (with particular attention during the first 3 h) and then, daily for a total of 14 days of observation.[3]

2.9. Behavioral toxicity

Effect of Carvacryl acetate (0, 1000, 2000 mg/kg) was also studied on muscular relaxation and locomotor activity using the rota rod test and open field test, respectively.[3] Parameters observed during open field test were the number of squares crossed (considering crossed when the four legs of each animal passed the previous square), the number of times the animal had a self-cleaning behavior (grooming) and the number of surveys (rearing). Parameters observed during rota rod test were the number of falls (up to three falls) and the time of permanence on the bar.

2.10. Analysis of physiological data

Physiological data were observed and recorded daily (weight, water consumption, food consumption and excreta production).

2.11. Macroscopic analysis and organ weights

After the end of treatment with carvacryl acetate, the remaining animals were anesthetized with xylazine-ketamine as described by Flecknell[19] and Wixson and Smiler[20] according to the protocol of Cornell University/Cornell Center for Animal Resources and Education. After euthanasia, a detailed necropsy was conducted on all animals and the criteria of gross pathological examination were based on position, shape, size, color, and consistency of the internal organs.[21] In addition, it was determined the absolute weight of the following organs: lungs, liver, heart, spleen and kidneys. Paired organs were weighed together.

2.12. Biochemical and hematological parameters

Blood samples were collected by cardiac puncture of the right ventricle of anesthetized animals and stored in tubes containing anticoagulant for determining the hematological parameters, and in tubes not containing anticoagulant to obtain serum for determination of biochemical parameters. Red blood cells (erythrocytes), white blood cells (leukocytes), platelets, hemoglobin, hematocrit and hematimetric indexes [mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)] were determined using automated Advia 120/hematology cell analyzer (Siemens). Differential leukocyte count was performed on areas stained with May-Grünwald-Giemsa. In each assay, 100 cells were analyzed and counted. Biochemical analysis was performed using Labmax 240 automatic device with LABTEST® commercial systems for determining concentrations of glucose, triglycerides, total cholesterol, creatinine, uric acid, aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

2.13. Statistical Analysis

The results were expressed as mean ± standard error of mean (S.E.M.). Statistical analysis was

performed using one-way ANOVA for multiple comparisons and followed by Student–Newman–Keuls as post hoc test by GraphPad Prism (version 6.0). Differences were considered statistically significant when $p<0.05$.

3. Results and Discussion

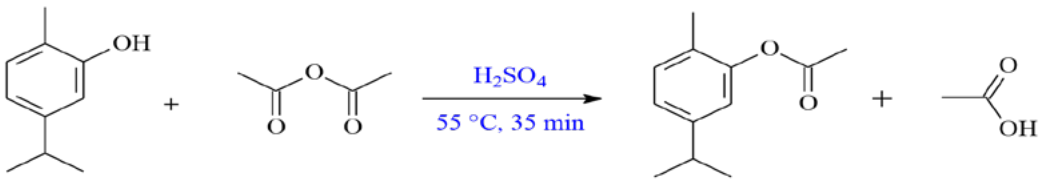
The proposed method according to Scheme 1 promoted the yield of 99.04 %, which is evident in the chromatogram of Figure 1A in which the signal in 23.2 min is relative to the acetylated derivative (99.04 % of the chromatogram). The signal at 20.07 min is relative to the residual carvacrol and corresponds to 0.96 % of the chromatogram (Figure 1A).

In the mass spectrum of carvacryl acetate (Figure 1B), it is possible to identify characteristic signs of acetylated derived, as the molecular ion $M^{\bullet+}$ 192 and the signal at *m/z* 177 corresponding to $[M-15]^+$. A proposal of fragmentation to some signs is diagrammed in Scheme 2.

The analysis of ^{1}H NMR (Figure 1S), ^{13}C NMR (Figure 2S) and ^{13}C NMR - DEPT 135 **(**Figure 3S, Supplementary material**)** show a concordance with the literature,[22] which can be observed in Table 1 in that the displacements (δ (ppm)) and coupling constants (*J*) are very close corroborating with the identity of the substance. The quality of the spectra also shows the purity of the sample confirming the high yield of the reaction and the efficiency of the acid catalysis in derivatization of this substrate.

3.1. Antioxidant capacity against the DPPH• radical

The values of the antioxidant capacity of carvacryl acetate against the DPPH• radical in



Scheme 1. Formation of carvacryl acetate using acid catalysis

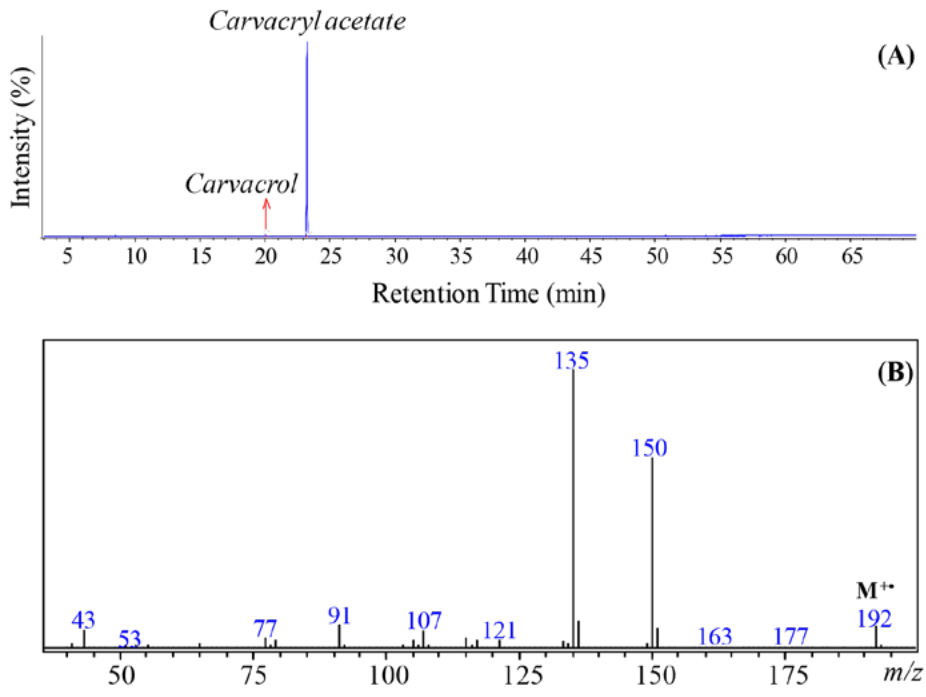
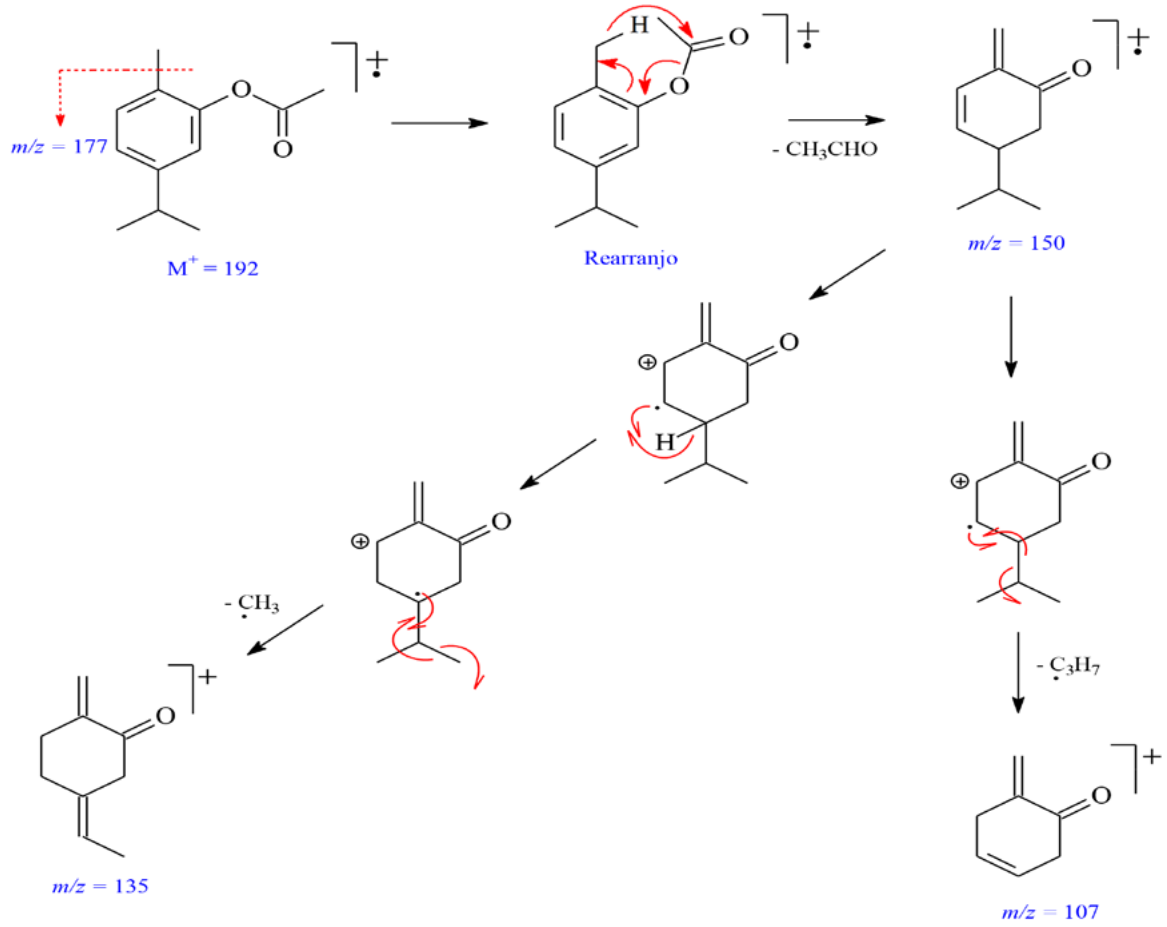


Figure 1. Total Ion Chromatogram (TIC) of reaction product (A) and mass spectrum of carvacryl acetate (B)



Scheme 2. Proposal of fragmentation to the carvacryl acetate

concentrations of 0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL were 27.74 ± 0.29, 32.79 ± 3.03, 39.70 ± 3.16, 46.48 ± 2.13 and 53.42 ± 0.58 %, respectively, in which significantly reduced (p<0.05) the DPPH$^{\bullet}$ radical in relation to System (Figure 2). The Trolox (140 µg/mL) also significantly reduced (p<0.05) the DPPH$^{\bullet}$ radical showing 88.56 ± 0.72 % of antioxidant capacity (Figure 2). According to the results of antioxidant capacity in the inhibition of DPPH$^{\bullet}$ radical, the EC_{50} value was 6.1 ± 0.53 µg/mL with the 95 % confidence interval ranging from 4.87 ± 0.33 to 12.43 ± 1.31 µg/mL.

3.2. Antioxidant capacity against the ABTS$^{\bullet+}$ radical

The values of the antioxidant capacity of carvacryl acetate in concentrations of 0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL were 18.34 ± 1.14, 29.92 ± 0.72, 34.91 ± 1.35, 48.36 ± 2.35 and 63.35 ± 1.76 %, respectively, in which significantly reduced (p<0.05) the ABTS$^{\bullet+}$ radical in relation to System (Figure 3). The Trolox (140 µg/mL) also significantly reduced (p<0.05) the ABTS$^{\bullet+}$ radical presenting 91.09 ± 3.00 % of antioxidant capacity (Figure 3). According to the results of antioxidant capacity in the inhibition of ABTS$^{\bullet+}$ radical, the EC_{50} value was of 5.24 ± 0.38 µg/mL with the 95 % confidence interval ranging from 3.84 ± 0.08 to 6.45 ± 0.02 µg/mL.

3.3 Reduction potential

The increase of absorbance at 700 nm in the concentrations of 0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL shows the reduction potential of carvacryl acetate (p<0.05) when compared to control (Figure 4). The Trolox (140 µg/mL) also presented reduction potential statistically significant (p<0.05) when compared with the control (Figure 4). According to these results, the EC_{50} value of carvacryl acetate necessary to reduce the potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) in 50 % of their initial absorbance was 3.65 µg/mL with the 95 % confidence interval ranging from 3.33 to 3.98 µg/mL.

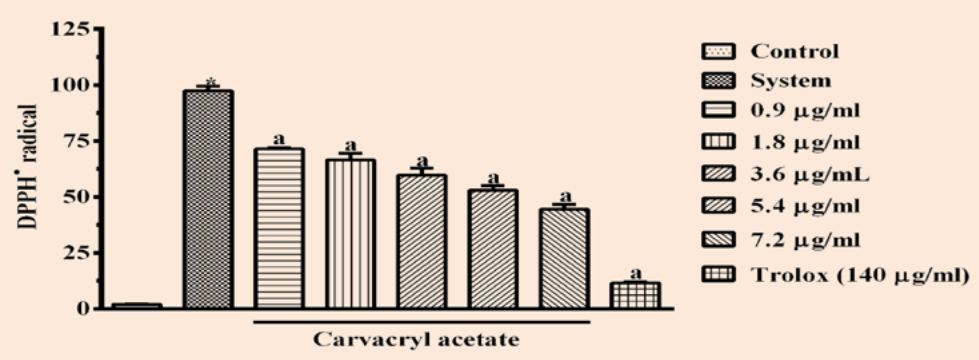


Figure 2. Antioxidant capacity of carvacryl acetate in different concentrations (0.9-7.2 µg/mL) against the DPPH• radical. The results represent the mean ± S.E.M. of the values of *in vitro* inhibition, n=5, of the experiments in duplicate. The Trolox (140 µg/mL) was used as standard antioxidant. *p<0.05 versus control (0.05 % Tween 80 dissolved in 0.9 % saline) (ANOVA and Newman-Keuls as post hoc test); [a] p<0.05 *versus* System (100 % of DPPH• radical) (ANOVA and Newman-Keuls as post hoc test)

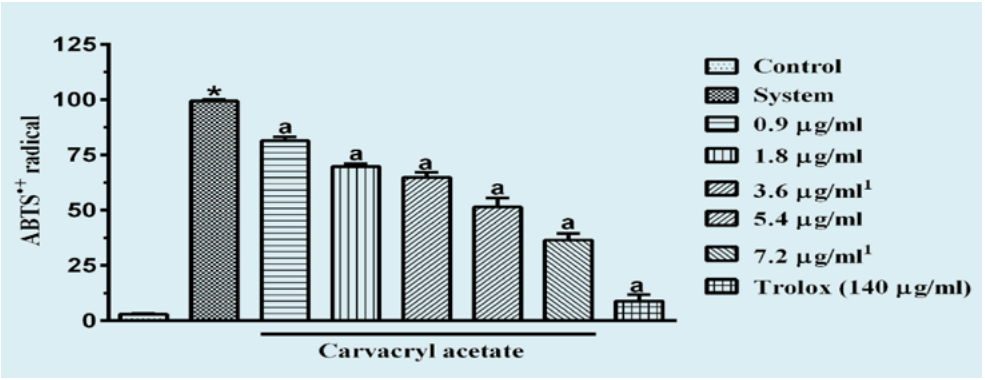


Figure 3. Antioxidant capacity of carvacryl acetate in different concentrations (0.9-7.2 µg/mL) against the ABTS•+ radical. The results represent the mean ± S.E.M. of the values of *in vitro* inhibition, n=5, of the experiments in duplicate. The Trolox (140 µg/mL) was used as standard antioxidant. *p<0.05 versus control (0.05 % Tween 80 dissolved in 0.9 % saline) (ANOVA and Newman-Keuls as post hoc test); [a] p<0.05 versus System (100 % of ABTS•+ radical) (ANOVA and Newman-Keuls as post hoc test)

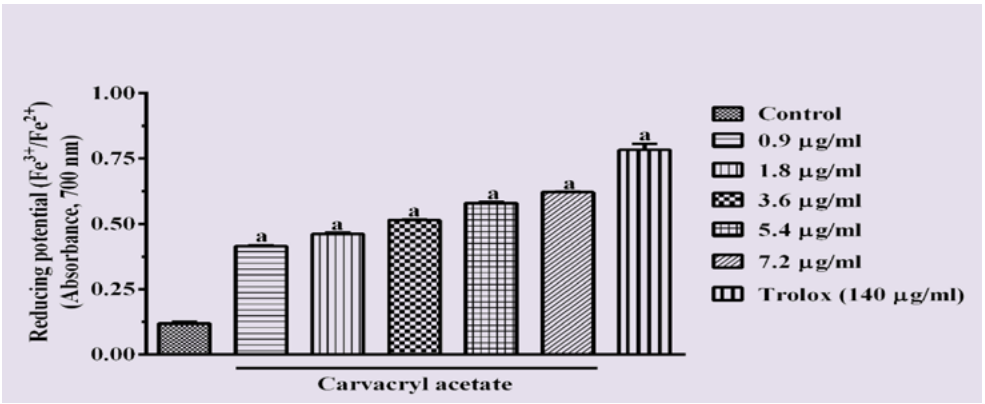


Figure 4. Reduction potential (Fe^{3}+/Fe^{2+}) of carvacryl acetate. The results represent the mean ± S.E.M. of the values of *in vitro* inhibition, n=5, of the experiments in duplicate. The Trolox (140 µg/mL) was used as standard antioxidant. *p<0.05 versus control (without carvacryl acetate) (ANOVA and Newman-Keuls as post hoc test)

3.4. Antioxidant capacity against the oxidative hemolysis

The values of antioxidant capacity for the carvacryl acetate in the concentrations of 0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL were 27.61 ± 0.79, 37.85 ± 0.66, 54.54 ± 3.20, 68.14 ± 2.66 e 77.21 ± 2.55 %, in which significantly reduced (p<0.05) the hemolysis rate when compared only with System (100 % hemolysis) (Figure 5). The Trolox (140 µg/mL) showed 81.57 ± 0.08 % of antioxidant capacity and also significantly reduced (p<0.05) the hemolysis rate when compared with the System (Figure 5). According to the results of antioxidant capacity by inhibition of erythrocyte hemolysis, the EC_{50} value was 2.58 ± 0.07 µg/mL with the 95 % confidence interval ranging from 1.87 ± 0.21 to 3.53 ± 0.45 µg/mL.

3.5. Study of acute toxicity

The results showed that carvacryl acetate produced no toxic effects as evidenced by the absence of signs of toxicity or mortality in the animals after oral administration (Table 2). However, signs of toxicity and mortality were observed in animals of both sexes after intraperitoneal administration of carvacryl acetate (Table 2). Additionally, no significant difference (p>0.05) in body weight (Figure 4S, Supplementary material), water intake, food consumption and excreta production of treated animals were detected in comparison with control group (Table 1S, Supplementary material). In addition, necropsy revealed no macroscopic changes in the organs (position, shape, size, color, and consistency) of treated animals. There were no treatment-related changes in organ weights (lungs, liver, heart, spleen and kidneys) (Table 2S, Supplementary material).

3.6. Behavioral toxicity

Animals treated with carvacryl acetateat doses of 1000 and 2000 mg/kg (p.o. and i.p.) showed no changes in locomotor activity (Table 3S, Supplementary material) and motor coordination (Table 4S, Supplementary material) as shown by open field test and rota rod test, respectively, when compared with the control group.

3.7. Biochemical and hematological parameters

Some parameters such as glucose, urea, uric acid, ALT and AST had no significant differences between the animals treated with carvacryl acetate and control groups (p>0.05) (Table 3). Lower values of triglycerides were identified in male and female animals treated orally and intraperitoneally with a dose of 2000 mg/kg, as well as female animals treated orally and

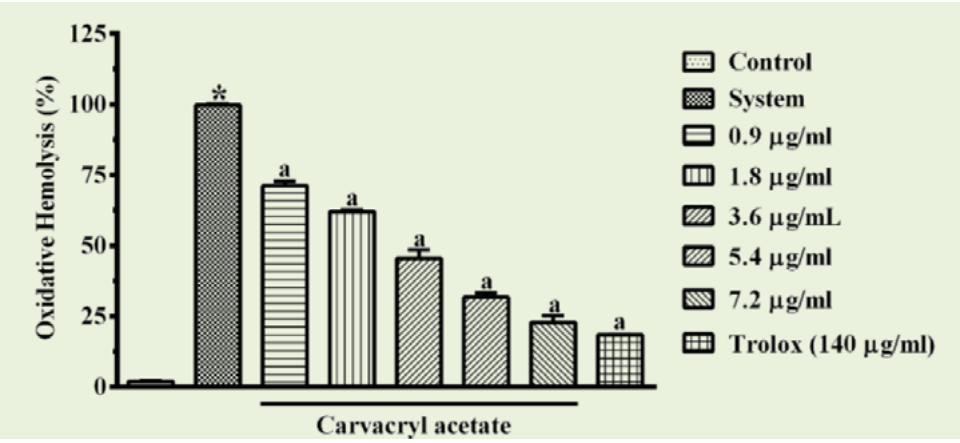


Figure 5. Antioxidant capacity of carvacryl acetate at different concentrations (0.9-7.2 µg/mL) by inhibition of erythrocyte hemolysis induced by H_2O_2. The results represent the mean ± S.E.M. of the values of *in vitro* inhibition, n=5, of the experiments in duplicate. The Trolox (140 µg/mL) was used as standard antioxidant. *p<0.05 versus control (PBS, pH 7.4) (ANOVA and Newman-Keuls as post hoc test). [a]p<0.05 versus total hemolysis induced by System (H_2O_2) (100 % of hemolysis) (ANOVA and Newman-Keuls as post hoc test)

Table 1. ^{1}H and ^{13}C NMR Spectral Data for carvacryl acetate

Carbon	Multiplicity	δC (ppm)		δH (ppm)	
		Pires *et al.*[5]	Analysis	Ben Arfa *et al.*[22]	Analysis
1	C	149.1	149.4	-	-
2	C	127.0	127.2	-	-
3	CH	130.7	131.0	7.14 (d, *J* = 7.80 Hz, 1H)	7.16 (d, *J* = 7.80 Hz,1H)
4	CH	124.0	124.3	7.01 (dd, *J* = 7.80 Hz, 1H)	7.03 (dd, *J* = 8.00 Hz, 1H)
5	C	147.9	148.2	-	-
6	CH	119.6	119.9	6.85 (s, *J* = 1.5, 1H)	6.88 (d, *J* = 1.6 Hz, 1H)
7	CH_3	20.6	20.9	2.13 (s, 3H)	2.16 (s, 3H)
8	CH	15.6	15.8	2.87 (sept., *J* = 6.9 Hz, 1H)	2.93 – 2.86 (sept., *J* = 6.93 Hz, 1H)
9	CH_3	23.7	24.0	1.23 (d, *J* = 6.90 Hz, 6H)	1.25 (d, *J* = 7.20 Hz, 6H)
10	CH_3				
11	C	169.1	169.4	-	-
12	CH_3	33.4	33.6	2.31 (s, 3H)	2.33

Table 2. Effects of carvacryl acetate (CA) after acute administration

Dose (mg/kg)	Sex	D/T	Latency to Mortality (min)	Symptoms of toxicity
Oral				
Control	M	0/5	–	None
	F	0/5	–	None
1000 mg/kg	M	0/5	–	None
	F	0/5	–	None
2000 mg/kg	M	0/5	–	None
	F	0/5	–	None
Intraperitoneal				
Control	M	0/5	–	None
	F	0/5	–	None
1000 mg/kg	M	0/5	–	None
	F	0/5	–	None
2000 mg/kg	M	1/5	>144 and<168	Hypoactivity, asthenia
	F	1/5	> 120 and<144	Hypoactivity, asthenia

Males (M), Females (F). Deaths (D), Treated (T)

intraperitoneally with a dose of 1000 mg/kg, both related to control groups (p<0.05). In the total cholesterol values, male mice treated with a dose of 1000 and 2000 mg/kg in both routes, showed lower values when compared to control groups (p<0.05). When significant differences were observed, there was no association with possible pathological conditions. Changes in levels of hepatic transaminases (ALT and AST), urea and creatinine used as indicators of hepatic and renal function were not identified.

The values of erythrocytes, hemoglobin, hematocrit, MCV, MCH, platelets, leukocytes, neutrophils and lymphocytes showed no significant difference when compared to control groups (p>0.05) (Table 4).

Table 3. Effects of carvacryl acetate on biochemical parameters of male and female mice after treatment of 14 days

Parameters	Oral			Intraperitoneal		
	Control (n=5)	1000 mg/kg (n=5)	2000 mg/kg (n=5)	Control (n=5)	1000 mg/kg (n=5)	2000 mg/kg (n=4)
Males						
Glucose (mg dL^{-1})	130.60 ± 15.45	134.30 ± 15.07	131.30 ± 15.82	117.80 ± 16.36	118.30 ± 4.70	120.7 ± 0.03
Urea (mg dL^{-1})	60.85 ± 6.82	61.20 ± 12.88	61.80 ± 8.58	50.25 ± 1.25	51.24 ± 4.90	51.00 ± 0.04
Creatinine (mg dL^{-1})	0.47 ± 0.02	0.46 ± 0.04	0.45 ± 0.06	0.44 ± 0.02	0.43 ± 0.01	0.44 ± 0.05
Uricacid (mg dL^{-1})	1.95 ± 0.35	1.94 ± 0.22	1.97 ± 0.52	1.98 ± 0.85	1.94 ± 0.26	1.99 ± 0.03
Triglycerides (mg dL^{-1})	92.68 ± 6.22	93.00 ± 4.96	71.50 ± 10.78*	96.75 ± 4.76	93.60 ± 6.82	75.00 ± 0.01*
Total cholesterol (mg dL^{-1})	103.60 ± 7.85	75.01 ± 7.07*	65.28 ± 4.09*	94.23 ± 5.64	79.50 ± 4.17*	61.00 ± 0.04*
ALT (U mL^{-1})	49.40 ± 10.46	48.03 ± 7.04	49.29 ± 9.37	50.70 ± 3.48	50.20 ± 1.10	50.01 ± 0.11
AST (U mL^{-1})	166.10 ± 18.27	165.20 ± 3.61	166.3 ± 51.47	155.46 ± 4.92	154.03 ± 3.46	153.02 ± 0.21
Females						
Glucose (mg dL^{-1})	90.01 ± 38.81	89.33 ± 31.86	90.81 ± 31.91	90.67 ± 17.23	91.02 ± 1.55	92.00 ± 0.21
Urea (mg dL^{-1})	31.01 ± 11.08	32.10 ± 8.40	31.03 ± 4.02	32.67 ± 5.45	31.01 ± 5.77	33.09 ± 0.02
Creatinine (mg dL^{-1})	0.43 ± 0.04	0.44 ± 0.12	0.42 ± 0.10	0.43 ± 0.03	0.44 ± 0.13	0.42 ± 0.23
Uricacid (mg dL^{-1})	3.05 ± 0.22	3.08 ± 0.57	3.13 ± 0.08	2.13 ± 0.03	2.16 ± 0.05	2.15 ± 0.02
Triglycerides (mg dL^{-1})	106.00 ± 5.77	88.20 ± 30.01*	72.3 ± 9.91*	100.30 ± 8.99	68.26 ± 6.89*	58.00 ± 0.05*
Total cholesterol (mg dL^{-1})	70.80 ± 10.93	70.67 ± 6.98	69.52 ± 3.82	71.10 ± 2.64	70.64 ± 4.37	71.00 ± 0.03
ALT (U mL^{-1})	46.60 ± 5.06	47.00 ± 3.22	47.78 ± 8.77	46.50 ± 11.77	47.25 ± 4.15	45.00 ± 0.23
AST (U mL^{-1})	166.30 ± 5.77	166.60 ± 3.26	165.20 ± 20.91	166.50 ± 26.26	164.83 ± 11.34	165.00 ± 0.01

AspartateAminotransferase (AST), AlanineAminotransferase (ALT). The values represent the mean ± S.E.M. The differences in the experimental groups were determined by Analysis of Variance (ANOVA), followed by t-Student–Newman–Keuls's as *post-hoc* test. *p<0.05 compared to the control group

4. Discussion

In the literature review it was observed that all the works employ basic catalysis for the acetylation of carvacrol, which involves reflux in an inert atmosphere, a reaction time of 24h with yields less than 80 %.[6] Some studies use the reaction time of 15 min, such as the study by Halim *et al*.[23] However, the yield was less than 70 %, and chromatographic methods were necessary for the purification of the product. The method using acid catalysis is a viable alternative for the acetylation of carvacrol, since it presents several benefits such as a drastic reduction of the reaction time, it does not require the use of reflux and inert atmosphere, use of low temperatures and promote the yield of 99.04 % to the detriment of the various requirements of acetylation using basic catalysis. This is the first time that the acetylation reaction of carvacrol is described in the literature by acid catalysis.

This study is relevant, since carvacryl acetate is a potential product for the development of new pharmaceutical formulations in need to have their safety initially investigated by non-clinical studies. The difficulty to find data in the literature to evaluate and discuss the acute toxicity of carvacryl acetate reinforces the need for this kind of study to provide support for non-clinical studies in order to ensure the safe use of this compound for humans and animals. This study showed that doses up to 2000 mg/kg have no mortality or signs of toxicity after oral administration of carvacryl acetate. This dose can be considered as the non-observed adverse effect dose. The oral LD_{50} is greater than 2000 mg/kg in mice,

Table 4. Effects of carvacryl acetate on hematological parameters of male and female mice after treatment of 14 days

Parameters	Oral			Intraperitoneal		
	Control (n=5)	1000 mg/kg (n=5)	2000 mg/kg (n=5)	Control (n=5)	1000 mg/kg (n=5)	2000 mg/kg (n=4)
Males						
Erythrocytes (mm^3)	8.04 ± 0.82	8.07 ± 0.46	8.17 ± 0.59	8.13 ± 0.76	8.41 ± 0.57	8.45 ± 0.01
Hemoglobin (g dL^{-1})	13.22 ± 0.44	13.58 ± 0.43	13.33 ± 1.03	13.13 ± 0.57	13.08 ± 1.39	13.60 ± 0.01
Hematocrit (%)	44.66 ± 0.81	45.22 ± 1.40	44.29 ± 3.07	46.95 ± 0.85	43.23 ± 2.21	47.40 ± 0.03
MCV (fL)	52.40 ± 0.80	52.20 ± 1.73	52.27 ± 1.79	53.20 ± 1.00	52.80 ± 2.32	52.00 ± 0.02
MCH (pg)	16.24 ± 0.86	16.84 ± 0.48	16.30 ± 0.48	15.75 ± 0.45	16.28 ± 2.02	16.60 ± 0.01
MCHC (g dL^{-3})	27.64 ± 0.74	28.38 ± 0.69	27.78 ± 3.68	27.60 ± 0.30	27.23 ± 1.68	27.10 ± 0.04
Platelets (mm^3)	308.80 ± 46.92	303.80 ± 59.99	308.00 ± 58.89	305.50 ± 32.50	306.50 ± 43.40	307.0 ± 0.05
Total Leukocytes (mm^3)	8.04 ± 0.19	7.96 ± 0.43	8.07 ± 1.21	7.95 ± 0.95	7.90 ± 0.34	7.91 ± 0.03
Neutrophils (%)	8.01 ± 2.07	8.04 ± 2.29	8.05 ± 1.37	8.02 ± 0.50	8.06 ± 2.53	8.01 ± 0.04
Lymphocytes (%)	74.80 ± 3.30	74.80 ± 2.15	73.19 ± 2.21	73.50 ± 1.50	74.50 ± 4.40	73.00 ± 0.01
Females						
Erythrocytes (mm^3)	8.16 ± 0.78	8.06 ± 0.42	8.01 ± 0.59	8.19 ± 0.20	8.23 ± 0.23	8.08 ± 0.01
Hemoglobin (g dL^{-1})	15.48 ± 0.99	14.79 ± 0.37	15.48 ± 0.35	15.98 ± 0.30	15.82 ± 0.54	15.71 ± 0.03
Hematocrit (%)	48.82 ± 2.63	45.14 ± 1.08	49.85 ± 3.67	48.23 ± 2.19	47.84 ± 1.41	48.72 ± 0.02
MCV (fL)	52.12 ± 1.93	52.36 ± 0.83	52.00 ± 1.87	53.05 ± 1.12	53.22 ± 0.44	52.03 ± 0.01
MCH (pg)	17.66 ± 0.63	16.12 ± 0.75	17.18 ± 0.32	17.53 ± 0.34	17.08 ± 0.62	17.01 ± 0.03
MCHC (g dL^{-3})	29.00 ± 0.75	29.48 ± 1.62	28.58 ± 1.82	28.53 ± 0.33	29.40 ± 1.74	29.23 ± 0.02
Platelets (mm^3)	309.80 ± 57.82	307.60 ± 50.58	306.30 ± 39.46	309.00 ± 40.22	308.00 ± 30.22	309.01 ± 0.04
Total Leukocytes (mm^3)	6.79 ± 0.17	6.72 ± 0.53	6.76 ± 1.53	6.67 ± 0.49	6.72 ± 0.47	6.72 ± 0.05
Neutrophils (%)	6.80 ± 1.02	6.73 ± 2.42	6.75 ± 1.03	6.78 ± 1.47	6.49 ± 0.66	6.52 ± 0.03
Lymphocytes (%)	72.80 ± 1.31	74.80 ± 2.37	72.25 ± 3.09	76.50 ± 1.55	76.20 ± 1.53	75.01 ± 0.01

Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC). The values represent the mean ± S.E.M. The differences in the experimental groups were determined byAnalysis of Variance (ANOVA), followed by t-Student–Newman–Keuls's as post-hoc test

which is inserted in the category 5 of substances with relatively low acute toxicity in the Globally Harmonized System.[24] In addition, the guidelines for acute toxicity of the Organization for Economic Cooperation and Development discourage tests with animals in the category 5 (2000-5000 mg/kg).[25] Despite of this, mortality and signs of toxicity such as hypoactivity and asthenia were observed in mice that received the carvacryl acetate at the dose of 2000 mg/kg (LD_{50} = 2287 mg/kg, i.p.).

The tested substance showed significant preclinical safety when compared to its precursor carvacrol (LD_{50} = 919 mg/kg),[10] and with other monoterpenes including; *m*-cresol (LD_{50} = 450 mg/kg),[26] *o*-cresol (LD_{50} = 350 mg/kg),[26] *p*-cresol (LD_{50} = 150 mg/kg),[26] and thymol (LD_{50} = 1134.03 mg/kg).[27] A possible explanation for the reduction of toxicity of carvacryl acetate in relation to carvacrol can be in the replacement of the hydroxyl group by ester group. In addition, acetylation of carvacrol has also been proposed as a way to increase their pharmacological potential.[6,10]

There were no significant changes (p>0.05) in consumption of water and food by mice of both sexes treated with carvacryl acetate (1000 and 2000 mg/kg) in a period of 14 days compared to control groups. The magnitude of reduction in body weight of groups of both sexes treated with carvacryl acetate did not exceed 5 % compared to control groups. Similar results were demonstrated for the average weight of main organs (spleen, heart, liver, lungs and kidneys) of mice in both sexes. These

results are in agreement with the study of Lima,[28] which observed that mice of both sexes treated with carvacrol in the dose of 250 mg/kg showed no significant changes in food consumption, as well as changes in the absolute weight of spleen, heart, liver, lungs and kidneys. Moreover, in this study, a macroscopic analysis of the internal organs (spleen, liver, kidneys, heart and lungs) showed no changes in position, size, color, shape and consistency when compared to the control groups of both sexes.

As part of the toxicity study, an evaluation of motor activity in mice treated with carvacryl acetate was done to demonstrate possible changes in normal central nervous system function. Open field test is used to evaluate the exploratory activity of the animals, as emotionality measure in rodents. Literature data show that the reduction in spontaneous exploratory activity gives an indication of the excitation level of the CNS,[3] and this reduction may be related to depression resulting from CNS sedation. Carvacryl acetate at the doses studied did not affect the psychomotor activity, suggesting that carvacryl acetate is not able to produce changes in the CNS, which supports the safety of this compound for non-clinical studies. In rota rod test, the difference in the length of stay and number of falls between carvacryl acetate-treated and vehicle-treated groups was used as a muscle relaxation index. Thus, carvacryl acetate at the tested doses showed no psychomotor changes, suggesting potential safety of this substance, and providing further information on the toxicity of this natural compound. The absence of effects on motor activity of mice treated with carvacryl acetate was demonstrated by study of Pires *et al*.[5]

Hematopoietic system is very susceptible to compounds with toxic properties and, therefore, is as an important indicator of the physiological and pathological state of the animals in toxicological studies. In this study, after treatment with carvacryl acetate at several doses, there were no changes in hematological parameters, that could indicate toxicity, compared to control groups. In addition to hematological assessment, serum biochemical parameters that could show potential toxicological effects in different organs, such as kidney and liver, were analyzed. Therefore, indicators of change in hepatic (ALT and AST) and renal (urea and creatinine) function, as well as metabolic indicators (glucose, triglycerides and total cholesterol) were assessed. The results provided in Table 3 for carvacryl acetate showed that variations in all biochemical parameters did not indicate toxicity compared to control group, mainly suggesting absence of adverse effects, such as hepatotoxicity and nephrotoxicity.

Highlighting a pharmacological potential, the antioxidant capacity of carvacryl acetate was evaluated in several *in vitro* methods. The method that involves the scavenging of the DPPH$^{\bullet}$ radical is one of the most used as it is fast and easy to perform. In this method, the antioxidant capacity was determined by analysis of the decrease in the absorbance of DPPH$^{\bullet}$ solution at 517 nm in a spectrophotometer, in which the DPPH$^{\bullet}$ radical of purple color is reduced to form DPPH.[13] Based on this principle, the result obtained in this study clearly demonstrated that the carvacryl acetate is a powerful antioxidant (EC_{50} = 6.1 ± 0.53 µg/mL). According to the spectrophotometric method described, the antioxidant action of carvacryl acetate against DPPH$^{\bullet}$ radical occurs by transfer of hydrogen atom to generate a sTable molecule of DPPH. The elimination of ABTS$^{\bullet+}$ radical is a conventional antioxidant method, simple and extensively used along with the DPPH$^{\bullet}$ radical to evaluate the antioxidant capacity.[13] In this method, the ABTS$^{\bullet+}$ radical of blue/green coloration is produced by the oxidation of the ABTS solution by potassium persulphate and the reaction with an antioxidant compound is monitored by decrease in absorbance of the reaction at 734 nm. In this way, and similarly to the results obtained in DPPH$^{\bullet}$ method, the present study demonstrated that the carvacryl acetatein concentrations of 0.9, 1.8, 3.6, 5.4 and 7.2 µg/mL is a potent antioxidant by the inhibition of the ABTS$^{\bullet+}$ radical.

Processes such as free radical scavenging and/or inhibition of lipid peroxidation is mediated by redox reaction and in agreement with the result obtained in the present study, the carvacryl acetate presented high reduction potential according to the increase in concentration. In addition to the *in vitro* studies, the antioxidant capacity can be analyzed more accurately by the inhibition of oxidation in biological fluids. This way, the easy accessibility and simplicity of erythrocytes makes them an attractive model to study oxidative stress *ex vivo*. In this study, the inhibition of oxidative damage induced by hydrogen peroxide in erythrocyte suspensions of rats was performed and in agreement with the results obtained, the carvacryl acetate managed to protect the erythrocytes proportionally to the

increase of concentration in comparison with the damage induced by hydrogen peroxide.

The antioxidant result obtained in this study for the carvacryl acetate is in accordance with the work of Pires *et al*.,[8] which demonstrated that the semisynthetic derivative presents high antioxidant capacity *in vivo*. In addition to this result, Pires *et al*.[8] showed *in vitro* that the carvacryl acetate (0.9-7.2 µg/mL) is a potent antioxidant compound by reduction lipid peroxidation (EC_{50} = 0.1785 µg/mL), nitrite production (EC_{50} = 0.1940 µg/mL) and hydroxyl radical (EC_{50} = 0.1397 µg/mL) formation.

5. Conclusions

In conclusion, the results of this study provide an initial understanding regarding the toxicity profile of carvacryl acetate. Based on the results of this acute toxicity study performed according to Anvisa guidelines, carvacryl acetate could be classified as a compound with toxicity at doses higher than 2000 mg/kg body weight. In addition, carvacryl acetate showed significant antioxidant capacity *in vitro* and protected against oxidative damage induced by hydrogen peroxide in erythrocytes.

Acknowledgements

The authors are grateful to the researchers of the Laboratory of Organic Geochemistry (LAGO/UFPI), Nuclear Magnetic Resonance Laboratory (UFPI), FAPEPI and CAPES for materials, equipments, scholarships and collaborations.

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