Naunyn-Schmiedeberg's Archives of Pharmacology (2020) 393:445–455
https://doi.org/10.1007/s00210-019-01731-1

ORIGINAL ARTICLE

Effects of the solid lipid nanoparticle of carvacrol on rodents with lung injury from smoke inhalation

Fernanda O. Carvalho[1] · Érika R. Silva[1] · Paula S. Nunes[1] · Fernanda A. Felipe[1] · Karen P. P. Ramos[1] · Luiz Augusto S. Ferreira[1] · Viviane N. B. Lima[1] · Saravanan Shanmugam[1] · Alan Santos Oliveira[1] · Sílvia S. Guterres[2] · Enilton A. Camargo[1] · Tassia Virginia Cravalho Olivera[3] · Ricardo Luiz C. de Albuquerque Júnior[3] · Waldecy de Lucca Junior[1] · Lucindo José Quintans-Júnior[1] · Adriano A. S. Araújo[1]

Received: 3 May 2019 / Accepted: 10 September 2019 / Published online: 26 October 2019

Abstract

The aim of this present study was to evaluate the effect of solid lipid nanoparticles (SLN) containing carvacrol over the lung damage of airway smoke inhalation. The study was conducted with 30 rats subjected to smoke inhalation and divided into 5 groups such as, normal control, negative control, oxygen group, SLN alone, and SLN+CARV group. The animals were sacrificed 24 h after the induction of inhalation injury further, the tissues of larynx, trachea, and lungs were collected for the histological, hematological, myeloperoxidase, and malondialdehyde analysis. The obtained results showed that treatment with CARV+SLN minimized the inhalation injury, since it reduced malondialdehyde significantly, when compared to the negative control group and minimized the histological changes which proves the absence of pulmonary emphysema and exudate in laryngeal and tracheal lumen in the CARV+SLN-treated group. Meanwhile, the presence of lesion with chronic characteristics was observed in the negative control and oxygen groups. It is suggested that the SLN containing carvacrol minimized oxidative stress and histological damages generated from smoke inhalation in rodents.

Keywords Lung injury · Carvacrol · Natural product · Inhalation · Smoke · Solid lipid nanoparticle

Introduction

Inhalation injury is the leading cause of death in burn patients and usually initiated by uninhibited absorption of smoke, which causes extreme toxic condition into the respiratory system (Souza et al. 2004). The temperature, ventilation, and the type of material burned in the environment lead to the production of a lot of compounds and distinct mechanisms of injury, favoring the release of cytokines and other lipid mediators from inflammatory cells in lung airways and parenchyma (Ramírez Rivero et al. n.d.; Demling et al. 1995).

The search for products that act by inhibiting the development of the inflammatory process and enable an effective treatment, which can bring comfort and brief return to normality of the patient's life, is of great value (Boateng et al. 2008). Many natural products present a significant oxyreducing therapeutic property, such as the carvacrol (5-isopropyl-2-methylphenol), a natural monoterpenic phenolic compound abundantly present in many essential oils of Lamiaceae family, formerly known as Labiatae, which includes the genera Origanum and Thymus (Kirimer et al. 1995; Quintans-Júnior et al. 2002; Koparal and Zeytinoglu 2003; Baser 2008; Lima et al. 2013). Monoterpenes are the main chemical constituents of essential oils of plants like *Cymbopogon winterianus*, *C. citrates*, *Lavandula multifida*, and *Thymus pubescens*. They are obtained as a mixture of odoriferous components and can be extracted by steam distillation or solvent extraction from a wide variety of aromatic plants (Carlini 2003; Quintans-Júnior et al. 2008; Melo et al. 2010; Barreto et al. 2014).

✉ Paula S. Nunes
paulanunes_se@yahoo.com.br

✉ Saravanan Shanmugam
saranflora04@gmail.com

[1] Health Sciences Post-Graduate Program, Federal University of Sergipe, Claudio Batista St s/n, Aracaju, SE 49060-100, Brazil

[2] Post-Graduation Program in Pharmaceutical Sciences, Federal University of Rio Grande do Sul, Ipiranga Avenue 2752, Porto Alegre, RS 90610-000, Brazil

[3] Institute of Technology and Research, Tiradentes University, Murilo Dantas Avenue 300, Aracaju, SE 49032-490, Brazil

Carvacrol have many therapeutic properties that serve as a barrier of processes mediated by free radicals, such as irritation and inflammation, due to its antioxidant action (Aeschbach et al. 1994; Botelho et al. 2009; Miguel et al. 2010; Guimarães et al. 2010; Mahtaj et al. 2015). The carvacrol was described as a potentially effective agent against the inflammatory process (Wagner et al. 1986; Mastelić et al. 2008; Landa et al. 2009; Hotta et al. 2010; Seo et al. 2010a; Guimarães et al. 2012). However, carvacrol is a highly volatile and chemically labile component, which react to oxidation, chemical interactions, or volatilization (Gibbs et al. 1999) process. Furthermore, due to its low solubility in water, carvacrol limits its therapeutic and pharmacological effects (Trevaskis et al. 2008).

Encapsulation techniques have been widely used in recent decades, since the encapsulated material can be protected against moisture, heat, or extreme conditions, thus increasing its stability and maintaining the viability (Souto et al. 2011). Solid lipid nanoparticles (SLN) were developed in 1991, as an alternative system of encapsulation of active principles compared to traditional colloidal systems, such as emulsions, liposomes, and polymeric nanoparticles. The great differential of SLN is its excellent physicochemical stability, which provides greater protection against the degradation of labile drugs (Seo et al. 2010b; Shegokar et al. 2011).

Very few studies report the use of substances that minimize the inflammatory cascade after the smoke inhalation and subsequent oxidation and release of free radicals which affects all kinds of age group people. Having several adverse effects of the non-steroidal anti-inflammatory drugs, the carvacrol as a substance has a potential antioxidant effect and also has anti-inflammatory properties without any toxicity. Therefore, the present study aimed to investigate the beneficial effect of SLN+carvacrol on lung injury and airway inflammation induced by smoke inhalation.

Materials and methods

Animals

Female Wistar rats, approximately weighed 250 ± 50 g, were randomly housed in appropriate cages at 22 ± 2 °C on a 12-h light/dark cycle (lights on from 06:00 a.m. to 6:00 p.m.) with free access to water and food (Purina®, Brazil). All experiments were carried out between 09:00 a.m. and 2:00 p.m. All animal procedures were processed in accordance with the Normative Resolutions of the Conselho Nacional de Controle de Experimentação Animal - the Brazil's CONCEA (CEPA # 011014).

Reagents

Carvacrol (Sigma-Aldrich, São Paulo - SP, Brazil) was encapsulated in SLN and for the preparation of SLN, we use cocoa butter (Sigma-Aldrich, São Paulo - SP, Brazil), 3,5-Di-*tert* 4-butylhydroxytoluene (BHT) (Sigma-Aldrich, São Paulo - SP, Brazil), polysorbate 80 (Tween 80®) (Sigma-Aldrich, São Paulo - SP, Brazil), ultra-pure water, and imidazolidinyl urea (Sigma-Aldrich, São Paulo - SP, Brazil). After the preparation, the SLN of carvacrol was administrated by nebulization and were dissolved in physiological saline. The oxygen cylinder (oxygen group) and compressed air were obtained from White Martins Praxair, Inc. (Aracaju, SE, Brazil).

Preparation and characterization of SLN of carvacrol

The preparation of carvacrol SLN was performed using a fusion-emulsification method in which the organic and the aqueous phase were separately heated to 40 °C, under stirring, and after total solubilization of the components, the organic phase was carefully poured into the aqueous phase. This mixture was kept under mechanical stirring in Ultra Turrax (IKA ®) for 2 min and, after this process, this mixture was transferred to the homogenizer at high pressure (HHP) (GEA Niro Soavi, SpA), as the HHP is characterized by applying the same shear stress throughout the specimen, due to the reduced dimensions of the homogenizer exit orifice (≤ 25–30 μm) (Bose et al. 1989; Zhu et al. 2012). The formulation of the composition for a final volume of 100 mL is shown in Fig. 1.

Characterization of carvacrol SLN was done by a laser diffraction method (Malvern Mastersizer® 2000) and the dynamic light scattering, electrophoretic mobility (Zetasizer ZS), and pH (B474 Micronal) were analyzed further. It was observed that the product had a polydispersity index (PDI = 0.126 ± 0.015 with an average size of 78.72 ± 0.85 nm) and particle diameter (D = 174 nm, d (0.5) = 140 nm, and span amounting to 1.70), being evidenced that the product was in the nanoscale, zeta potential (− 8.25 ± 0.19 mV), revealing that the product is stable, and pH = 5.26.

Experimental design

After three regular estrous cycles, only estrus phase female rats were selected for further experiment. All animals were randomized into five groups with six animals each (n = 6), in which group 1 treated as normal control group (only saline), group 2 received smoke inhalation plus isotonic saline (negative control group), group 3 received smoke inhalation plus oxygen therapy with 0.25 L/s (oxygen group), group 4 received SLN alone, and group 5 received the SLN+carvacrol (smoke inhalation plus SLN+carvacrol (240 μg/mL) treatment, CARV group). Thirty minutes after smoke inhalation,

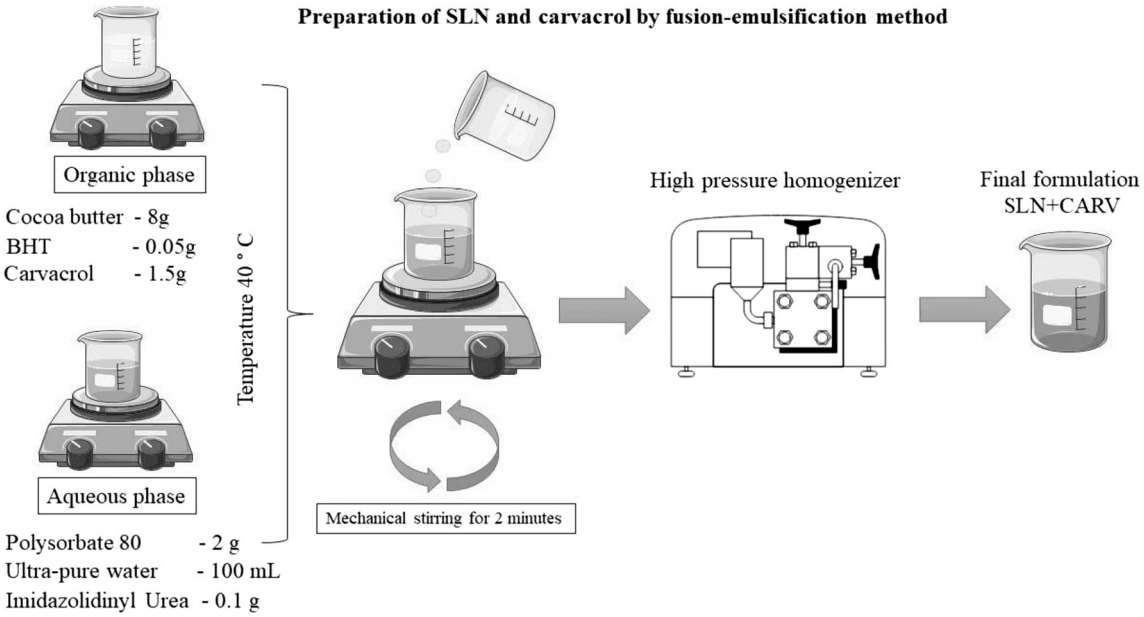

Fig. 1 Preparation of SLN and carvacrol by fusion-emulsification method

all animals were submitted to the nebulization. Twenty-four hours later, animals were euthanized and blood sample was collected for the biochemistry analysis. Furthermore, animals were sacrificed and the tissues of the larynx, trachea, and lungs were dissected for histology and BCG analysis respectively.

Smoke inhalation

Based on the model of Zhu et al. (2012) with some minor modification, we used the acrylic cage for the smoke chamber. The smoke chamber is made up of a fiberglass to monitor the animal situation. Animals were housed in acrylic cage (40.5 cm × 35 cm × 23 cm) and smoke was generated slowly by burned cotton (30 g/kg body weight) for 9-min periods of smoke separated into three times by 30-s exposure to ambient air.

Nebulization

The animals were anesthetized with ketamine (50 mg/kg) and xylasine (5 mg/kg) and submitted to nebulization with 3 mL of the solution by 10 min with an adapted mask.

Euthanasia

The animals were anesthetized with ketamine (100 mg/kg) and xylasine (10 mg/kg), their blood was collected from abdominal vein and, following, they were submitted to intracardiac perfusion with phosphate buffer (0.1 M) isotonic saline for 5 min followed by formalin (10%) buffered (0.1 M) for 40 min. After perfusion, the trachea and lung were collected, post-fixed for 2 h in the formalin (10%) buffered (0.1 M), paraffin-embedded, and stored in a refrigerator (4 °C) for histology processing.

Animal weight measurement

Animals were weighed initially before the smoke inhalation and 24 h after the smoke inhalation (at the end of exposure period); then, the difference between the two body weights was calculated.

Histological procedures

The surgical specimens were taken from each left upper lung lobe and the trachea, and were fixed with 10% formalin and embedded in paraffin according to the routine laboratorial techniques. Subsequently, serial 5-μm-thick sections were obtained and stained in hematoxylin-eosin and picrosirius.

Biochemical assays

In determining the thiobarbituric acid reactive substances (TBARS), lung sample was weighed and homogenized in potassium phosphate buffer (50 mM, pH 7.4) containing butylated hydroxytoluene (12.6 mM). Then, aliquots of the homogenate (in duplicate) were incubated (90 °C, 45 min) with thiobarbituric acid (0.37%) in an acidic solution (trichloroacetic acid at 15% and hydrochloric acid at 0.25 N). At the end of incubation, the homogenates were centrifuged (5 min, 8000×*g*), and aliquots from the supernatants were extracted

with *n*-butanol, followed by stirring in a vortex for 30 s and further centrifugation (2 min, 8000×*g*). The supernatant absorbance was measured at 535 nm in a microplate reader (corrected by the values of absorbance at 572 nm). The results were calculated using a molar extinction coefficient of $1.55 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nanomoles of malondialdehyde (MDA) formed per milligram of tissue (Bradley et al. 1982; Santana et al. 2012).

For myeloperoxidase (MPO) determination, lung sample was collected, weighed, and homogenized with potassium phosphate buffer (50 mM, pH 6.0 containing 0.5% of hexadecyltrimethylammonium bromide), and 1 mL aliquot of the homogenate was centrifuged (2 min, 8000×*g*, 4 °C). In a 96- well plate, aliquots of supernatant were incubated with a solution of *o*-dianisidine hydrochloride (0.167 mg/mL containing 0.005% H_2O_2). The MPO activity was measured kinetically in a microliter plate scanner (Labsystem Multiskan) at 460 nm and intervals of 15 s over a period of 5 min. Results were expressed as units of MPO per milligram tissue (UMPO/mg tissue). A UMPO was considered as the amount of enzyme that degrades 1 mmol of hydrogen peroxide/min (Bradley et al. 1982; Santana et al. 2012).

Hematological analysis

After the 24 h of post-smoke inhalation, blood sample was collected and examined in the method described previously by Lima et al. (Lima et al. 2014).

Statistical analysis

Data obtained from MDA, MPO, and BCG were expressed as the mean ± standard error of the mean, and the differences were evaluated through one-way ANOVA with repeated measures with Tukey post hoc test. Repeated measures *t* test was used to analyze the animal body weight. Also, ImageJ program was used to analyze the quantification of the histological parameters and further, significant differences were evaluated through ANOVA with repeated measures with Tukey post hoc test and p less than 0.05 were considered as significant value.

Results

Effect of the SLN of carvacrol on the weight of animals subjected to smoke exposure

There was no statistically significant difference in body weight between the groups when compared to the initial body weight and 24 h after the smoke inhalation (Table 1).

Action of the SLN of carvacrol on the histopathological changes induced by smoke inhalation in the tissue of the larynx, trachea, and lung of rats

The main histopathological changes were observed in the tissue of the upper respiratory tract of the larynx (Fig. 2). When compared to normal animal, negative control group was observed with intense inflammatory infiltration in the conjunctive tissue of the larynx composed of neutrophils and occasional lymphocytes. Also, extended areas of ulcer and the presence of serofibrinous exudate in the interior light cavity of the larynx, also rich in neutrophils, was observed. The oxygen group was characterized by intense neutrophilic infiltration and formation of ulceration, as well as the presence of luminal serofibrinous exudate rich in neutrophils. SLN+CARV group showed inflammatory reaction mainly composed of lymphocytes and occasional neutrophils, ulceration areas were unusual, while superficial ulcerative changes (sloughing areas) were seen more frequently. It is important to note that the conjunctive tissue of all the groups showed remarkable interstitial edema and occasional forming of discreet granulation reaction. The quantification results from the graphical illustration revealed the inflammation affected area of the animals. The smoke-induced animals provide maximum results of inflammation affected area when compared to the normal control group. Meanwhile, the oxygen and SLN+CARV group animals show the positive significant ($p < 0.001$) results which reduce the inflammation affected area when compared to the negative control group.

Table 1 Effects of the SLN of carvacrol on the weight of animals before and 24 h post-smoke inhalation

	Before smoke inhalation	24 h post-smoke inhalation	*p*
Normal control	226.0000 ± 13. 3562	226.0210 ± 14.6506	0.0511
Negative group	236.0000 ± 24.7386	234.3333 ± 22.1420	0.3452
Oxygen group	232.3333 ± 13.5450	236.5000 ± 15.0566	0.8927
SLN alone	231.6754 ± 21.2645	231.1874 ± 12.3241	0.4623
SLN+CARV	228.2000 ± 16.4378	222.0000 ± 21.4476	0.0767

Data are presented as mean ± SEM ($n = 6$ in each group)

SLN+CARV group carvacrol group

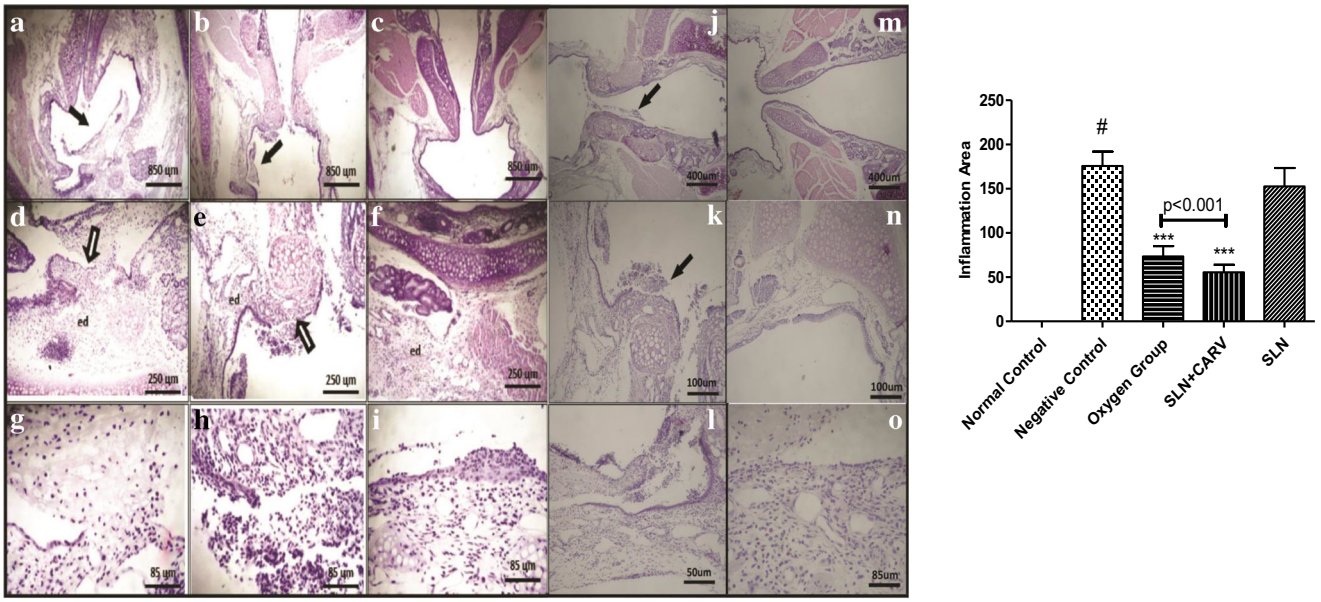

Fig. 2 Histological analysis of the larynx colored in HE. Rich serofibrinous exudate in the interior of the larynx is present (dark arrows) in **a** negative control group and **b** oxygen group but absent in **c** SLN+CARV group (× 40) and **j** normal control group. Intense interstitial edema in every group and extensive areas of ulceration in the epithelial tissue (clear arrows) in **d** negative control group and **e** oxygen group, but in **f** SLN+CARV group and **k** normal control animals these areas of ulceration were uncommon, being sloughing areas seen with higher frequency (× 100). Intense inflammatory infiltration in the connective tissue composed of neutrophils and occasional lymphocytes in **g** negative control group and **h** oxygen group and rich in lymphocytes in **i** SLN+CARV group (× 400), **l** normal control animals and **o** SLN group. The graph shows the affected area by the inflammation cells including neutrophils and occasional lymphocytes. $^{\#}p < 0.001$ negatively significant compared to normal group $^{***}p < 0.001$. Statistical differences between negative control group vs oxygen and SLN+CARV group

As demonstrated in Fig. 2, negative, SLN, and oxygen groups, tracheal connective tissues exhibited intense interstitial edema and highly vascular granulation tissue. Sloughing and squamous metaplasia of the epithelial lining were also observed. Meanwhile, SLN+CARV group presented granulation tissue and focal sloughing, but there is no remarkable pathological change was observed in the other group animals. The quantification result shows that oxygen and SLN+CARV group animals significantly ($p < 0.001$) control the disease condition. Same time, the SLN alone slightly increases the inflammation condition (Fig. 3).

Regarding the lung tissues, strong inflammatory infiltration, composed of neutrophils and lymphocytes, association of interstitial edema and hyperemia, was observed in all the studied groups (Fig. 4). Alveolar spaces were often filled with serofibrinous exudate and leucocytes karyorrhexis-derived nuclear cells. Enlargement of alveolar spaces with loss of alveolar walls, consistent with emphysematous changes, were found in negative, SLN, and oxygen groups, but not in the normal control group and SLN+CARV group. None of the groups showed histological signs of desmoplastic alterations. Based on the cell infiltration, neutrophils, edema, hyperemia, and lymphocyte cell quantification the SLN+CARV group and the oxygen groups significantly ($p < 0.001$) maintain the regular cellular architecture, when compared to the negative control group animal (Table 2).

Effect of the SLN of carvacrol on MDA and MPO

There was a statistically significant difference ($p < 0.05$) relative to MDA between SLN+CARV group when compared to negative control group (smoke exposure). Between the oxygen group and the CARV group, there was no statistically significant difference (Fig. 5). When compared to normal control group animal, the negative control group (smoke exposure) animals have significant increases in the MDA and UMPO levels.

In the results of MPO activity, there was no statistically significant difference between all the groups (Fig. 6).

Action of the SLN of carvacrol on the hematological changes in rats induced by smoke inhalation

There was a statistically significant difference ($p < 0.05$) of the mean corpuscular volume (MCV) between the SLN+CARV group and the negative control group. The other blood markers did not show statistically significant difference.

Discussion

The present study demonstrated that the treatment with SLN of carvacrol attenuated the histopathological changes of the

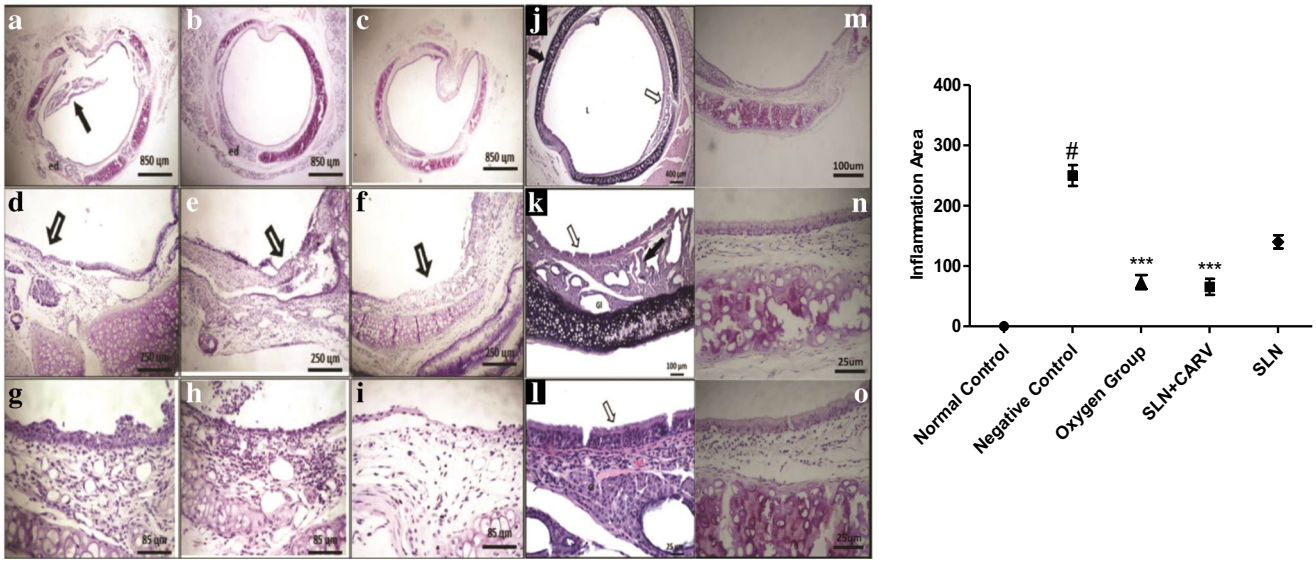

Fig. 3 Histological analysis of the trachea colored in HE. Interstitial edema of connective tissue (ed) in **a** negative control group and **b** oxygen group and exudate in the interior of the cavity of light of the larynx (dark arrow) in **a**, found absent in **c** SLN+CARV group (× 40) and **j** normal control animals. Epithelial thinning areas (clear arrows) in every group (**d**–**f**, **k**, **n**) (× 100). Intense inflammatory infiltration rich in neutrophils in the connective tissue and formation of the granulation tissue rich in small caliber blood vessels, occasionally congested **g** negative control group and **h** oxygen group, but absent in **i** SLN+CARV group (× 400), **l** normal control animals and **o** SLN group. The graph displays the intense inflammation cells in negative control group which reduced in oxygen and SLN+CARV group. $^{\#}p < 0.001$ negatively significant compared to normal group $^{***}p < 0.001$. Statistical differences between negative control group vs oxygen and SLN+CARV group

lung tissue and decreased the MDA level. But there was no statistically significant difference in the body weight of the animal, MPO, and even the hematological values. As per the previous study results, a dose of 240 μg carvacrol effectively reduced the systemic inflammation in guinea pig model of chronic obstructive pulmonary disease (COPD) induced by cigarette smoke exposure (Mahtaj et al. 2015). This report authors used three different doses (60, 120, and 240 μg/mL) of pure carvacrol in which the higher dose 240 μg/mL indicated a preventive effect on total and differential WBC, serum levels of MDA and IL-8, weight changes of COPD guinea pigs which was equal or even more potent than the effect of dexamethasone at used concentrations. Therefore, in our present study, we used that same 240 μg/mL higher dose with the preparation of solid lipid nanoparticle to evaluate the smoke inhalation effect on rat model.

Our results indicate that carvacrol has a preventive effect on oxidative stress by decreasing the MDA marker when compared to all other groups. The oxygen group also presented a statistically significant difference when compared to the negative control group, but the oxygen group and the SLN+CARV group did not present any significant difference. MDA is a polyunsaturated fatty acid and is hydrolyzed into biologically active aldehydes and carbonyl compounds produced during lipid peroxidation process. The disintegration of cell membrane integrity inactivates the membrane proteins and receptors, as well as their bound enzymes which develops the lipid peroxidation reaction and causes the oxidative damages to the cells (Rajpura 2002; Jayakumar et al. 2012). Furthermore, it has already been proved that the antioxidant effect of carvacrol (Peters 1981; Dubick et al. 2002; Cox et al. 2005; Wang et al. 2010; Ramos et al. 2013; Mahtaj et al. 2015) was maintained even when it was administered via SLN. According to Jayakumar et al. (2012), carvacrol is able to prevent lipid peroxidation and hepatocellular lesion, protecting the antioxidant system. Therefore, the obtained results of decreasing MDA might be because of the encapsulation of the carvacrol compounds with the SLN which improves the solubility and oral bioavailability of the carvacrol compound.

The major histological changes occurred in larynges likely to be the most proximal region of the respiratory tract, which resulted in more extensive tissue damage promoted by smoke and high temperature. Supporting our findings, Souza et al. (2004) have reported that supralaryngeal regions have large heat exchange capacity, since the mucous membranes have higher relative content of water, which facilitates the exchange of heat with smoke. Therefore, the deleterious effects induced by the high temperature of the inhaled smoke are supposed to be rarely observed in the anatomical regions below the larynx. The histological changes of the sublaryngeal respiratory tract were consistent with those changes in both tracheal (Demling et al. 1994, 1996) and pulmonary tissues (Nguyen et al. 1995; Abali et al. 2013; Li et al. 2013; Guimarães et al. 2014; Qiu et al. 2012). Furthermore, such changes were less expressive than in larynx. In fact, Dubick et al. (2002) have previously

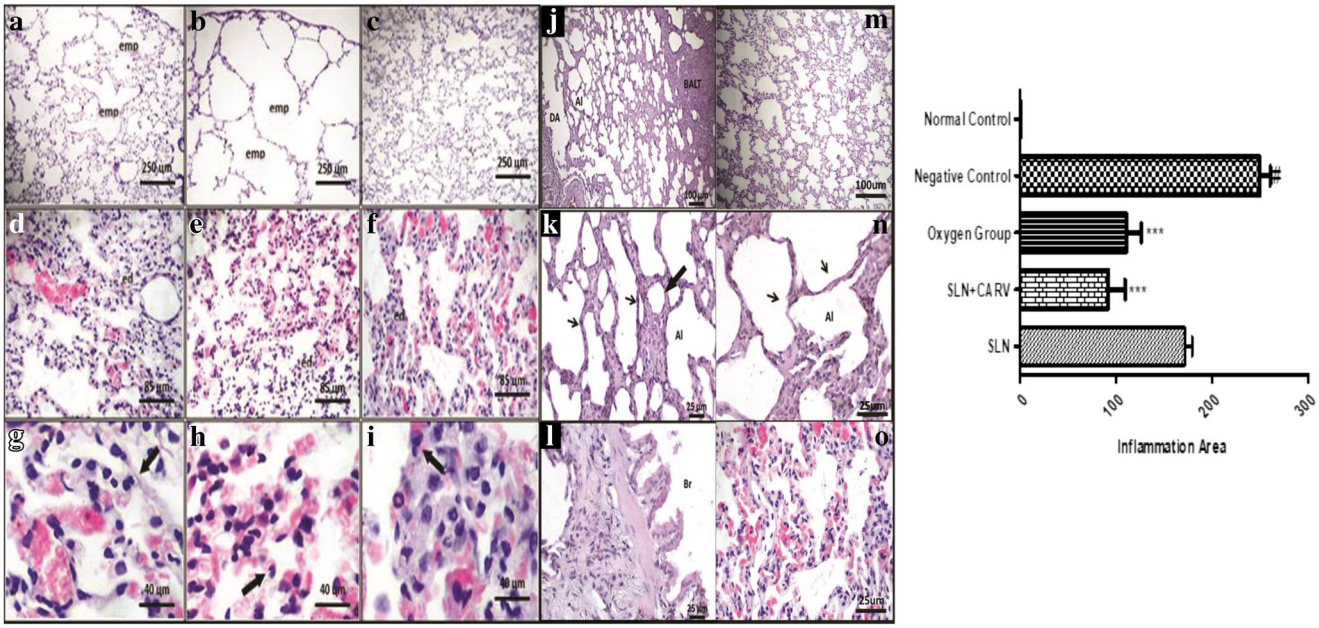

Fig. 4 Histological analysis of lung colored in HE. Increase in alveolar diameter, suggestive of emphysema (emp) in **a** negative control group and **b** oxygen group, but absent in **c** SLN+CARV, **j** normal control groups, and **m** SLN group (× 100). Edema, hyperemia, and hemorrhage, with presence of fibrin-hemorrhagic exudate in every group (**d**–**f**, **k**, **n**) (× 400). Inflammatory exudate rich in neutrophils (dark arrows) in every group (**g**–**i**, **l**, **o**) (× 800). The graph illustrates the fibrin-hemorrhagic exudate in all groups comparable with normal group. $^{\#}p < 0.001$ negatively significant compared to normal group $^{***}p < 0.001$. Statistical differences between negative control group vs oxygen and SLN+CARV group

demonstrated that more distal regions of the respiratory tract, such as tracheal and pulmonary tissues, are more susceptible to the chemical action of the smoke constituents than to the physical effects caused by high temperature.

In laryngeal and tracheal tissue, the inflammatory infiltration of SLN+CARV group was predominantly composed of lymphocytes and macrophages which is opposite to the neutrophil-rich infiltration seen in negative control and oxygen group animals. These data seem to suggest that carvacrol might have played a modulatory role over the inflammatory response. Supporting that hypothesis, carvacrol has been demonstrated to be a potent suppressor of cyclooxygenase-2, downregulating the release of prostaglandin E2 (PGE2), an important pro-inflammatory mediator (Ciolino and Levine 1997; Esechie et al. 2008). In addition, carvacrol also increases IL-10 and IL-10 mRNA, an anti-inflammatory cytokine, as well as decreases the release of pro-inflammatory IL-8, TNF-alpha, and nitric oxide (NO) level, which seems to attest the anti-inflammatory properties of this compound Guimarães et al. (2014) and Mahtaj et al. (2015). However, further studies are still required in order to clarify the precise mechanisms underlying the modulatory effect of carvacrol on the inflammatory infiltration of the current experimental model.

Histological changes in the lungs of rats that undergone smoke exposure without treatment were characterized by mild

Table 2 Effects of the SLN of carvacrol on the hematological changes of the animals 24 h after smoke inhalation

	Normal control	Negative control group	Oxygen group	SLN alone	SLN+CARV	*p*
Erythrocytes	7.0120 ± 0.1221	7.0167 ± 0.4021	7.3500 ± 0.2588	6.7461 ± 0.7854	7.0667 ± 0.4320	0.3109
Hemoglobin	13.2165 ± 0.8732	14.5500 ± 0.8735	15.0000 ± 0.4940	12.2715 ± 0.1248	14.3167 ± 1.0187	0.3337
Hematocrit	43.2731 ± 0.2901	42.1667 ± 2.4014	43.6667 ± 1.2111	41.2924 ± 4.2151	40.3333 ± 3.4448	0.1039
MCV	56.4312 ± 0.3261	60.6550 ± 1.2535*	59.2083 ± 0.9992	61.5443 ± 2.2598	57.0733 ± 2.8102$^{\#}$	< 0.05*
MCHC	33.7807 ± 0.0754	34.5417 ± 0.2245	34.3917 ± 0.5123	36.3496 ± 3.8547	35.5900 ± 1.5247	0.0555
Plasma protein	7.3482 ± 0.3125	8.0333 ± 0.5125	7.8000 ± 0.4561	8.9879 ± 1.6139	7.7000 ± 0.4517	0.5623
Leukocytes	7.6791 ± 0.9831	7.4833 ± 2.1757	6.3833 ± 0.7985	8.1298 ± 1.5127	11.0667 ± 8.3479	0.6184
Thrombocytes	301.2387 ± 72.4637	433.1667 ± 300.9494	291.8333 ± 61.2190	361.8317 ± 89.6839	266.1667 ± 85.9288	0.2636

Statistical differences only between negative control group vs CARV group in MCV: $^{*}p < 0.05$

MCV mean corpuscular volume, *MCHC* mean of corpuscular hemoglobin concentration

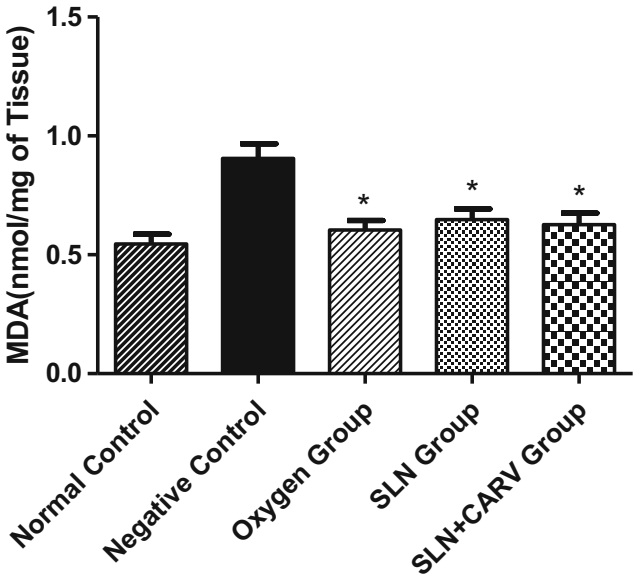

Fig. 5 Effect of inhaled SLN of carvacrol or oxygen group (O_2) on TBARS formation in the model of smoke-induced lung injury in female rats at 24 h post-smoke inhalation. Statistical differences between negative control group vs oxygen group: $*p < 0.05$. Statistical differences between negative control group vs SLN+CARV group: $*p < 0.05$

to moderate inflammatory infiltration and emphysema. Previous studies by Ramos et al. (2013), Li et al. (2013), Abali et al. (2013) and Wang et al. (2010) have reported similar findings. We found that treatment with carvacrol, but not with oxygen, promoted a remarkable reduction of emphysematous changes. It is possible that such biological effect may be related to the antioxidant properties of carvacrol. It has been demonstrated that lung exposure to reactive oxygen species (ROS), responsible for oxidative stress, is a consistent observation after smoke inhalation injury (Hogg and Senior 2002; Di Petta and Di Petta 2010; Alam et al. 2013). Enhanced ROS generation may lead to a status of dysfunctional cellular metabolism (Di Petta and Di Petta 2010), resulting in an imbalance of protease-antiprotease system (Alam et al. 2013). This pathophysiological process promotes the destruction of the alveolar wall, as a result of the action of active proteolytic enzymes that degrade the extracellular matrix (ECM) and affect the integrity of its components, particularly collagen and elastic fibers (Cox et al. 2009; Hashemipour et al. 2014). Surprisingly, we found no significant difference in MDA production between groups treated with carvacrol and oxygen. MDA is formed during oxidative degeneration as a product of free oxygen radicals, regarded as one of the end products in the lipid peroxidation process. Although lipid is a major component of cell membrane and thus its peroxidation almost directly correlates with peroxidative damage of cell in vivo, there are other methods related to evaluation of antioxidant activity, including assessment of catalase (CAT) activity and glutathione peroxidase (GSHPx) estimation (Alam et al. 2013). Therefore, other investigations, using additional methods, are necessary to determine whether the decrease in the extent of pulmonary oxidative damages might be related to the prevention of the emphysematous changes.

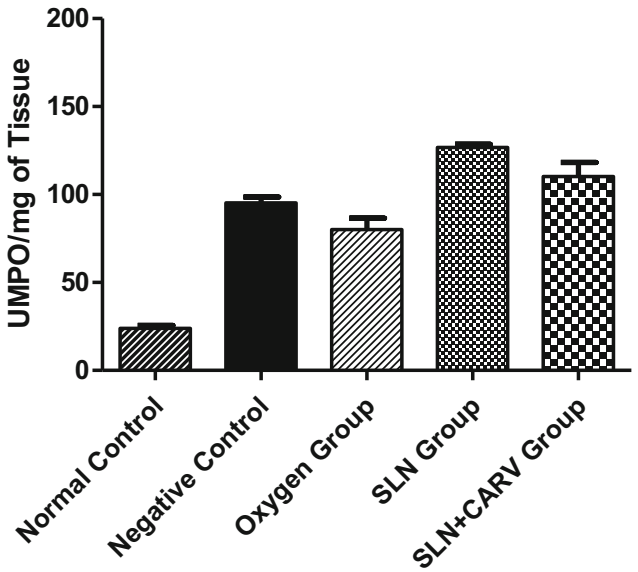

Fig. 6 Lack of effect of inhaled SLN of carvacrol or O_2 on lung MPO activity in the model of smoke-induced lung injury in female rats at 24 h post-smoke inhalation

In the current study, infiltration of macrophages and polymorphonuclear (PMN) were observed in pulmonary tissues 24 h after injury, as previously reported by Cox et al. (2009). However, no difference was seen between the morphological features of the inflammatory response pattern of the studied groups.

These findings were unexpected as long as carvacrol was able to modulate the inflammatory response in upper regions of the respiratory tract. The reason for such controversial results is still unclear, but we hypothesized that it might be related to the milder intensity of the inflammatory response occurring in the lower respiratory tract. Thus, the inflammation had already low intensity in pulmonary tissues; it would be expectable that the modulatory effects of carvacrol, which had been observed in the upper airways, were not apparent in trachea and larynx.

Observing the body weight variation of the rats, there was no influence of the SLN of carvacrol, probably because it was an acute lesion relative to the weight of the animal, there were no statistically significant differences in the intergroup and even intragroup. These findings do not corroborate with the ones from Hashemipour et al. (2014), which showed that carvacrol has no effect on food consumption but leads to a weight increase, and from Mahtaj et al. (2015), which performed a study with guinea pig model in a COPD model exposed to cigarette smoke and verified that the animals treated with carvacrol had a weight increase, but in this study, the animals were weighed before the smoke exposure and 3 months after.

The same happens when we correlate the time with hematological analysis, where red blood cell (RBC) and white blood cell (WBC) values lie within the normal range, having, just, an increase of the mean corpuscular volume (MCV), which measures the size of red blood cells. The measure is attained by multiplying a volume of blood by the proportion of blood that is cellular (the hematocrit) and dividing that product by the number of erythrocytes (red blood cells) in that volume. The mean corpuscular volume is a part of a standard complete blood count. Presenting an increase in 3 groups when comparing with the value range within normal (45.00–56.70 fl), according to Lima et al. (2014). There was a statistically significant difference ($p < 0.05$) of MCV between the SLN+CARV group and the negative control group, where the negative control group showed a larger increase of MCV, suggesting macrocytic anemia.

According to Furman et al. (2014), such anemia can be a result of, among other factors, hemolytic anemia or myelodysplastic syndrome. Besides the change in MCV, all 3 groups presented thrombocytopenia when comparing with value ranging within normal ($760–1313 \cdot 10^3/\mu L$) from Lima et al. (Lima et al. 2014). Das et al. (2011) reveal that the etiology of myelodysplastic syndrome is largely unknown, but authors correlated myelodysplastic syndrome and thrombocytopenia with cigarette smoke inhalation in guinea pigs, during 21 days of exposure. The same reason for these findings that happened in our model still is somewhat unclear, but there is a possibility that the smoke exposure in our study was more intense and the components of cotton combustion may have caused myelodysplastic syndrome and thrombocytopenia.

According to Fein et al. (1980), the smoke from the wood, cotton, and paper may result in the formation of numerous organic acids, aldehydes, and other harmful gases, including sulfuric oxide and nitrogen. Aldehydes, especially acrolein, can cause irritation of the exposed mucous membranes and pulmonary edema. Coleman (Diana Lewis 1981) adds, relating that irritating gases, like ammonia and sulfuric oxide, can be absorbed by particulate material, being transported to the alveolar space where it will cause more damages, such as mucosal edema, bronchorrhea, and bronchial obstruction that increases airway resistance which produces the lung inflammation and produces the toxic substances in neutrophil phagolysosomes. Therefore, we analyzed the myeloperoxidase (MPO); it was observed that there was no statistically significant difference between the groups. MPO is an oxidative enzyme which uses H_2O_2 to produce hypochloric acid and other toxic substances in neutrophil phagolysosomes which also plays an important role in the chronic stage of various diseases including neurodegenerative diseases, inflammatory, and atherosclerosis. The MPO can be used as an indicator of neutrophils in the lung (Diana Lewis 1981) and these findings resemble to the one found in the histological analysis, inflammatory infiltration in every group, rich in neutrophils.

Conclusion

In general, our results demonstrated that the carvacrol SLN complex treatment minimized the inhalation injury by improving the antioxidant action and lowering the oxidative stress marker. Also, the histopathological changes are as follows: absence of exudate laryngeal, tracheal lumen, and pulmonary emphysema. Furthermore, the hematological results also confirm that the carvacrol SLN complex maintains the biochemical properties of the blood serum. All these results prove the anti-inflammatory activity of the SLN encapsulated carvacrol compound. Further, mechanistic studies are needed for this SLN encapsulated carvacrol compound to develop the pharmaceutical product for the treatment of airway lung diseases.

Authors' contributions FOC, ÉRS, FAF, KPPR, LASF, and VNBL conducted all the experimental procedures. FOC and SS wrote the whole manuscript. TVCO conducted the quantification of histological results. ADCS and SSG conducted the in vivo antioxidant studies. EAC provided the laboratory facility for the biochemical analysis. RLCAJ did the histopathological analysis. PSN, WLJ, LJQJ, and AASA conceived and designed the research.

Funding information This study received financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico/CNPq/Brazil (Pronex 2014-Fapergs/CNPq) and Fundação de Amparo à Pesquisa do Estado de Sergipe/FAPITEC-SE.

Compliance with ethical standards All animal procedures were processed in accordance with the Normative Resolutions of the Conselho Nacional de Controle de Experimentação Animal - the Brazil's CONCEA (CEPA # 011014).

Conflict of interest The authors declare that there is no conflict of interest.

References

Abali AES, Karakayali H, Ozdemir BH, Bayraktar N, Abbas OL, Haberal M (2013) Destructive pulmonary effects of smoke inhalation and simultaneous alterations in circulating IL-6, TNF-α, and IFN-γ levels at different burn depths. J Burn Care Res 34:334–341. https://doi.org/10.1097/BCR.0b013e3182644e9b

Aeschbach R, Löliger J, Scott BC, Murcia A, Butler J, Halliwell B, Aruoma OI (1994) Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food Chem Toxicol 32:31–36

Alam MN, Bristi NJ, Rafiquzzaman M (2013) Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharm J 21:143–152. https://doi.org/10.1016/j.jsps.2012.05.002

Barreto RSS, Albuquerque-Júnior RLC, Araújo AS et al (2014) A systematic review of the wound-healing effects of monoterpenes and iridoid derivatives. Molecules 19:846–862. https://doi.org/10.3390/molecules19010846

Baser KHC (2008) Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Curr Pharm Des 14:3106–3119

Boateng JS, Matthews KH, Stevens HNE, Eccleston GM (2008) Wound healing dressings and drug delivery systems: a review. J Pharm Sci 97:2892–2923

Bose R, Sutherland GR, Pinsky C (1989) Biological and methodological implications of prostaglandin involvement in mouse brain lipid peroxidation measurements. Neurochem Res 14:217–220

Botelho MA, Martins JG, Ruela RS, I R, Santos JA, Soares JB, França MC, Montenegro D, Ruela WS, Barros LP, Queiroz DB, Araujo RS, Sampio FC (2009) Protective effect of locally applied carvacrol gel on ligature-induced periodontitis in rats: a tapping mode AFM study. Phyther Res 23:1439–1448. https://doi.org/10.1002/ptr.2798

Bradley PP, Priebat DA, Christensen RD, Rothstein G (1982) Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol 78:206–209. https://doi.org/10.1111/1523-1747.EP12506462

Carlini EA (2003) Plants and the central nervous system. Pharmacol Biochem Behav 75:501–512

Ciolino HP, Levine RL (1997) Modification of proteins in endothelial cell death during oxidative stress. Free Radic Biol Med 22:1277–1282

Cox RA, Burke AS, Oliveras G, Enkhbaatar P, Traber LD, Zwischenberger JB, Jeschke MG, Schmalstieg FC, Herndon DN, Traber DL, Hawkins HK (2005) Acute bronchial obstruction in sheep: histopathology and gland cytokine expression. Exp Lung Res 31:819–837. https://doi.org/10.1080/01902140600574967

Cox RA, Burke AS, Jacob S, Oliveras G, Murakami K, Shimoda K, Enkhbaatar P, Traber LD, Herndon DN, Traber DL, Hawkins HK (2009) Activated nuclear factor kappa B and airway inflammation after smoke inhalation and burn injury in Sheep. J Burn Care Res 30: 489–498. https://doi.org/10.1097/BCR.0b013e3181a28e13

Das A, Dey N, Ghosh A, Das T, Chatterjee IB (2011) NAD(P)H: quinone oxidoreductase 1 deficiency conjoint with marginal vitamin C deficiency causes cigarette smoke induced myelodysplastic syndromes. PLoS One 6:e20590. https://doi.org/10.1371/journal.pone.0020590

Demling R, Lalonde C, Picard L, Blanchard J (1994) Changes in lung and systemic oxidant and antioxidant activity after smoke inhalation. Shock 1:101–107

Demling R, Lalonde C, Youn YK, Picard L (1995) Effect of graded increases in smoke inhalation injury on the early systemic response to a body burn. Crit Care Med 23:171–178

Demling R, LaLonde C, Ikegami K (1996) Fluid resuscitation with deferoxamine hetastarch complex attenuates the lung and systemic response to smoke inhalation. Surgery 119:340–348

Di Petta A, Di Petta A (2010) Pathogenesis of pulmonary emphysema – cellular and molecular events. Einstein (São Paulo) 8:248–251. https://doi.org/10.1590/s1679-45082010rb1480

Diana Lewis C (1981) Smoke inhalation - medical staff conference. West J Med 135:300–309

Dubick MA, Carden SC, Jordan BS et al (2002) Indices of antioxidant status in rats subjected to wood smoke inhalation and / or thermal injury. Toxicology 176:145–157

Esechie A, Kiss L, Olah G, Horváth EM, Hawkins H, Szabo C, Traber DL (2008) Protective effect of hydrogen sulfide in a murine model of acute lung injury induced by combined burn and smoke inhalation. Clin Sci 115:91–97. https://doi.org/10.1042/CS20080021

Fein A, Leff A, Hopewell PC (1980) Pathophysiology and management of the complications resulting from fire and the inhaled products of combustion: review of the literature. Crit Care Med 8:94–98

Furman E, Leidinger E, Hooijberg EH, Bauer N, Beddies G, Moritz A (2014) A retrospective study of 1,098 blood samples with anemia from adult cats: frequency, classification, and association with serum creatinine concentration. J Vet Intern Med 28:1391–1397. https://doi.org/10.1111/jvim.12422

Gibbs BF, Kermasha S, Alli I, Mulligan CN (1999) Encapsulation in the food industry: a review. Int J Food Sci Nutr 50:213–224

Guimarães AG, Oliveira GF, Melo MS, Cavalcanti SCH, Antoniolli AR, Bonjardim LR, Silva FA, Santos JPA, Rocha RF, Moreira JCF, Araújo AAS, Gelain DP, Quintans-Júnior LJ (2010) Bioassay-guided evaluation of antioxidant and antinociceptive activities of carvacrol. Basic Clin Pharmacol Toxicol 107:949–957. https://doi.org/10.1111/j.1742-7843.2010.00609.x

Guimarães AG, Xavier MA, de Santana MT, Camargo EA, Santos CA, Brito FA, Barreto EO, Cavalcanti SCH, Antoniolli ÂR, Oliveira RCM, Quintans-Júnior LJ (2012) Carvacrol attenuates mechanical hypernociception and inflammatory response. Naunyn Schmiedeberg's Arch Pharmacol 385:253–263. https://doi.org/10.1007/s00210-011-0715-x

Guimarães AG, Serafini MR, Quintans-Júnior LJ (2014) Terpenes and derivatives as a new perspective for pain treatment: a patent review. Expert Opin Ther Pat 24:243–265. https://doi.org/10.1517/13543776.2014.870154

Hashemipour H, Kermanshahi H, Golian A, Khaksar V (2014) Effects of carboxy methyl cellulose and thymol + carvacrol on performance, digesta viscosity and some blood metabolites of broilers. J Anim Physiol Anim Nutr (Berl) 98:672–679. https://doi.org/10.1111/jpn.12121

Hogg JC, Senior RM (2002) Chronic obstructive pulmonary disease - part 2: pathology and biochemistry of emphysema. Thorax 57:830–834

Hotta M, Nakata R, Katsukawa M, Hori K, Takahashi S, Inoue H (2010) Carvacrol, a component of thyme oil, activates PPARα and γ and suppresses COX-2 expression. J Lipid Res 51:132–139. https://doi.org/10.1194/jlr.M900255-JLR200

Jayakumar S, Madankumar A, Asokkumar S, Raghunandhakumar S, Gokula dhas K, Kamaraj S, Josephine Divya MG, Devaki T (2012) Potential preventive effect of carvacrol against diethylnitrosamine-induced hepatocellular carcinoma in rats. Mol Cell Biochem 360:51–60. https://doi.org/10.1007/s11010-011-1043-7

Kirimer N, Başer KHC, Tiimen G (1995) Carvacrol-rich plants in Turkey. Chem Nat Compd 31:37–41

Koparal AT, Zeytinoglu M (2003) Effects of carvacrol on a human non-small cell lung cancer (NSCLC) Cell Line, A549. Cytotechnology 43:149–154. https://doi.org/10.1023/B:CYTO.0000039917.60348.45

Landa P, Kokoska L, Pribylova M, Vanek T, Marsik P (2009) In vitro anti-inflammatory activity of carvacrol: inhibitory effect on COX-2 catalyzed prostaglandin E2 biosynthesisb. Arch Pharm Res 32:75–78. https://doi.org/10.1007/s12272-009-1120-6

Li W, Qiu X, Wang J, Li H, Sun Y, Zhang F, Jin H, Fu J, Xia Z (2013) The therapeutic efficacy of glutamine for rats with smoking inhalation injury. Int Immunopharmacol 16:248–253. https://doi.org/10.1016/j.intimp.2013.02.022

Lima M d S, Quintans-Júnior LJ, de Santana WA, Martins Kaneto C, Pereira Soares MB, Villarreal CF (2013) Anti-inflammatory effects of carvacrol: evidence for a key role of interleukin-10. Eur J Pharmacol 699:112–117. https://doi.org/10.1016/J.EJPHAR.2012.11.040

Lima CM, Lima AK, Melo MGD et al (2014) Valores de referência hematológicos e bioquímicos de ratos (Rattus novergicus linhagem Wistar) provenientes do biotério da Universidade Tiradentes, vol 10, pp 1–9

Mahtaj LG, Feizpour A, Kianmehr M, Soukhtanloo M, Boskabady MH (2015) The effect of carvacrol on systemic inflammation in guinea pigs model of COPD induced by cigarette smoke exposure. Pharmacol Rep 67:140–145. https://doi.org/10.1016/j.pharep.2014.08.017

Mastelić J, Jerković I, Blažević I et al (2008) Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. J Agric Food Chem 56:3989–3996. https://doi.org/10.1021/jf073272v

Melo MS, Sena LCS, Barreto FJN, Bonjardim LR, Almeida JRGS, Lima JT, de Sousa DP, Quintans-Júnior LJ (2010) Antinociceptive effect

of citronellal in mice. Pharm Biol 48:411–416. https://doi.org/10.3109/13880200903150419

Miguel MG, Dandlen SA, Figueiredo AC, Pedro LG, Barroso JG, Marques MH (2010) Comparative evaluation of the antioxidant activities of thymol and carvacrol and the corresponding β-cyclodextrin complexes. Acta Hortic:363–368. https://doi.org/10.17660/ActaHortic.2010.853.44

Nguyen TT, Cox CS, Herndon DN et al (1995) Effects of manganese superoxide dismutase on lung fluid balance after smoke inhalation. J Appl Physiol 78:2161–2168. https://doi.org/10.1152/jappl.1995.78.6.2161

Peters WJ (1981) Inhalation injury caused by the products of combustion. Can Med Assoc J 125:249–252

Qiu X, Ji S, Wang J, Li H, Xia T, Pan B, Xiao S, Xia Z (2012) The therapeutic efficacy of Ulinastatin for rats with smoking inhalation injury. Int Immunopharmacol 14:289–295. https://doi.org/10.1016/j.intimp.2012.08.002

Quintans-Júnior L, Guimarães A, Araújo B et al (2002) Carvacrol, (−)-borneol and citral reduce convulsant activity in rodents. African J Biotechnol 9:6566–6572

Quintans-Júnior LJ, Souza TT, Leite BS, Lessa NMN, Bonjardim LR, Santos MRV, Alves PB, Blank AF, Antoniolli AR (2008) Phythochemical screening and anticonvulsant activity of Cymbopogon winterianus Jowitt (Poaceae) leaf essential oil in rodents. Phytomedicine 15:619–624. https://doi.org/10.1016/j.phymed.2007.09.018

Rajpura A (2002) The epidemiology of burns and smoke inhalation in secondary care: a population-based study covering Lancashire and South Cumbria. Burns 28:121–130

Ramírez Rivero CE, Judith Rivera María Consuelo Cabezas Leonelo Bautista Lorenzo JG, Armando Uribe Carvajal J (n.d.) Asociacion Colombiana De Facultades De Medicina-Ascofame

Ramos C, Pedraza-Chaverri J, Becerril C, Cisneros J, González-Ávila G, Rivera-Rosales R, Sommer B, Medina-Campos ON, Montaño M (2013) Oxidative stress and lung injury induced by short-term exposure to wood smoke in guinea pigs. Toxicol Mech Methods 23:711–722. https://doi.org/10.3109/15376516.2013.843113

Santana DG, Santos CA, Santos ADC et al (2012) Beneficial effects of the ethanol extract of Caesalpinia pyramidalis on the inflammatory response and abdominal hyperalgesia in rats with acute pancreatitis. J Ethnopharmacol 142:445–455. https://doi.org/10.1016/j.jep.2012.05.015

Seo E-J, Min S-G, Choi M-J (2010a) Release characteristics of freeze-dried eugenol encapsulated with β-cyclodextrin by molecular inclusion method. J Microencapsul 27:496–505. https://doi.org/10.3109/02652041003681398

Seo M, Gorelikov I, Williams R, Matsuura N (2010b) Microfluidic assembly of monodisperse, nanoparticle-incorporated perfluorocarbon microbubbles for medical imaging and therapy. Langmuir 26:13855–13860. https://doi.org/10.1021/la102272d

Shegokar R, Singh KK, Müller RH (2011) Production & stability of stavudine solid lipid nanoparticles—From lab to industrial scale. Int J Pharm 416:461–470. https://doi.org/10.1016/j.ijpharm.2010.08.014

Souto EB, Severino P, Santana MHA, Pinho SC (2011) Nanopartículas de lipídios sólidos: métodos clássicos de produção laboratorial. Quim Nova 34:1762–1769. https://doi.org/10.1590/S0100-40422011001000009

Souza R, Jardim C, Salge JM, Carvalho CRR (2004) Lesão por inalação de fumaça. J Bras Pneumol 30:557–565. https://doi.org/10.1590/S1806-37132004000600011

Trevaskis NL, Charman WN, Porter CJH (2008) Lipid-based delivery systems and intestinal lymphatic drug transport: a mechanistic update. Adv Drug Deliv Rev 60:702–716. https://doi.org/10.1016/j.addr.2007.09.007

Wagner H, Wierer M, Bauer R (1986) In vitro inhibition of prostaglandin biosynthesis by essential oils and phenolic compounds. Planta Med 52:184–187

Wang Y, Wang W, Zhang Y, Zhao X, Yang D (2010) Clinical significance of a myeloperoxidase gene polymorphism and inducible nitric oxide synthase expression in cirrhotic patients with hepatopulmonary syndrome. J Huazhong Univ Sci Technol Med Sci 30:437–442. https://doi.org/10.1007/s11596-010-0445-1

Zhu F, Qiu X, Wang J, Jin Y, Sun Y, Lv T, Xia ZF (2012) A rat model of smoke inhalation injury. Inhal Toxicol 24:356–364. https://doi.org/10.3109/08958378.2012.673179