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Article

4-Carvomenthenol, a monoterpene of essential oils, and its underlying effects on anti-inflammatory activity and immediate hypersensitivity reaction

Larissa Rodrigues Bernardo[1], Laércia Karla Diega Paiva Ferreira[1], Larissa Adilis Maria Paiva Ferreira[1], Cosmo Isaías Duvirgens Vieira[1], Adriano Francisco Alves[2], Pedro Thiago Ramalho Figueiredo[3], Marcia Regina Piuvezam[1*]

[1]Laboratory of Immunopharmacology, Department of Physiology and Pathology, Federal University of Paraíba, João Pessoa, Paraíba, Brazil, [2]Laboratory of Pathology, Department of Physiology and Pathology, Federal University of Paraíba, João Pessoa, Paraíba, Brazil, [3]Department of Pharmaceutical Sciences, Federal University of Paraíba, João Pessoa, Brazil.

The monoterpene 4-carvomenthenol (Carvo) is found in essential oils of plant. Here, we evaluate the Carvo oral pretreatment in acute inflammatory experimental models and *in silico* molecular docking. Mice pretreated with Carvo were challenged and submitted to the protocols: paw edema, peritonitis, scratching behavior and anaphylactic shock reaction. Besides, we used histamine H1 receptor, cyclooxygenases (COX-1 and COX-2) and phospholipase A2, as targets for molecular docking analysis. Carvo inhibited the carrageenan-induced paw edema and decreased the peritoneal influx of polymorphonuclear cells on carrageenan-challenged mice without interfering with the mononuclear cell influx. Moreover, Carvo diminished the histamine, PGE2 and compound 48/80 induced paw edematogenic effect. The monoterpene also diminished the mice scratching behavior and, surprisingly, avoided the animal death caused by compound 48/80 in 30 min. Through the docking analysis, Carvo showed favorable binding energy to the histamine H1 receptor. This study demonstrates that Carvo attenuated the allergic inflammatory process, decreasing edema, cell migration, activation of mast cells and the histamine release, probably due to interaction of Carvo with the histamine H1 receptor, ameliorating the itching and the anaphylactic shock reaction. Therefore, the results of this study indicate that Carvo has anti-inflammatory properties by reducing the histamine effects.

Keywords: 4-carvomenthenol. Monoterpene. Anti-inflammatory. Immediate hypersensitivity. Phytomedicine.

INTRODUCTION

Monoterpenes are substances structurally formed by two isoprene units and are secondary metabolites of essential oils obtained from various parts of plants. These compounds have numerous actions, which include allelochemical functions that mediate important interactions between the plant and the environment, as defense properties (Morgan, Wilson, 1999). The monoterpene 4-carvomenthenol (Carvo) or 4-terpineol is found in essential oils of aromatic plants, such as *Cinnamomum glanduliferum, Melaleuca alternifolia,* and Sc*hinus molle*. Several reports have demonstrated that the molecule presents many pharmacological properties as anti-inflammatory (Hart *et al*., 2000), anticancer (Wu *et al*., 2012), anticonvulsant (Sousa, Nóbrega, Morais, 2009), antibacterial (Zhang *et al*., 2018) and antifungal (Ninomiya *et al*., 2013).

In regards to its anti-inflammatory properties, Carvo inhibited the production of inflammatory cytokines as

***Correspondence:** M. R. Piuvezam. Departamento de Fisiologia e Patologia. Universidade Federal da Paraíba. CP 5009, Campus I, João Pessoa, PB, 58051–970, Brasil. E-mail: mrpiuvezam@ltf.ufpb.br. ORCID-ID: https://orcid.org/0000-0003-2134-1667

tumor necrosis factor α, interleukins (IL-1b, IL-8, IL-10) and prostaglandin E2 (PGE2) in lipopolysaccharide (LPS)-stimulated human macrophages, whose effect occurs due to the decreasing of the ERK1/2 and TLR4 pathway-dependent signal (Hart *et al*., 2000; Nogueira *et al*., 2014). The monoterpene also attenuated the inflammatory process in dextran sulfate-induced colitis in mice (Zhang *et al*., 2017), as well as in LPS-induced acute lung injury by PPAR-γ pathway activation and by NLRP3 pathway inhibition, respectively (Ning *et al*., 2018). Besides, Carvo regulated the edema associated with the immediate hypersensitivity response, whereas its topical application was effective in controlling the histamine-induced edema by mast cell degranulation process in mice and humans (Brand *et al*., 2002a, 2002b; Khalil *et al*., 2004).

Mast cells are associated with the immediate hypersensitivity reaction due to the presence of the FcεRI receptor that binds to the allergen-specific immunoglobulin E (IgE) in a hypersensitive person and releases histamine and other inflammatory mediators such as prostaglandins and leukotrienes. These mediators lead to vasodilation and consequently plasma leakage, resulting in fluid accumulation at the tissue site and edema formation (Amin, 2012). Histamine is the main inflammatory mediator present in the mast cell vesicles, exerting its effects through the activation of histaminergic receptors (H1-H4) coupled to G-protein. The H1 receptor is the main subtype of histamine receptors related to the major effects of allergic inflammation (Hansen Selnø *et al*., 2018).

A remarkable allergic inflammatory response is the anaphylactic shock reaction related to systemic disorders with multiple clinical manifestations, such as: cutaneous (urticaria and pruritus), cardiovascular (hypotension and tachycardia) and respiratory reactions (lack of air, cough, and dyspnea), which can lead to a potentially fatal reaction (Lee, Vadas 2011; Ring, *et al*., 2014; Lieberman, Garvey, 2016). This dangerous systemic anaphylactic reaction may be either IgE-dependent or IgE-independent, however, it is associated with the action of histamine (Finkelman, 2007). In response to IgE-mediated systemic anaphylaxis, histamine H1 and H2 receptor knockout mice were protected from the anaphylactic reaction and this response was indistinguishable from that of histamine deficient mice (Wechsler *et al*., 2013). Similarly, in an active systemic anaphylaxis model, serum histamine levels were suppressed in mast cell-deficient mice (Balbino *et al*., 2017).

In addition, other symptoms of anaphylaxis are related to histamine. For instance, the vasodilation induced by this mediator causes a decrease in blood pressure and an increase in the blood vessel wall permeability, which causes plasma and protein leakage into peripheral tissues, resulting in an anaphylactic reaction. This reaction leads to urticarial, angioedema, as well as laryngeal and intestinal edema, whereas the systemic smooth muscle contraction results in wheezing, abdominal cramps, diarrhea (Lee, Vadas 2011; Ring, *et al*., 2014), and may cause death. Therefore, a clinical diagnosis must be efficiently made so that immediate pharmacological intervention is performed to minimize morbidity and mortality. These data above showed the remarkable action of mast cells and histamine in anaphylactic reactions.

Considering all this information, the present study aimed to investigate the mechanism underlying the previously described anti-histamine effect of oral treatment with Carvo on experimental acute and allergic inflammatory models as an anaphylactic shock reaction. In addition, we investigated the binding energy of the monoterpene on the histamine H1 receptor involved in the allergic process by molecular modeling strategy.

MATERIAL AND METHODS

Animals

Female Swiss mice (6 to 8 weeks old) weighing between 25-30 g were used in the experiments. The animals, housed in polypropylene cages at a temperature of 25 ± 2° C, were kept on light/dark cycles of 12 h with free access to water and food during the experimental period. The mice were obtained from the Vivarium Professor Thomas George of the Institute of Research for Drugs and Medicines/UFPB, João Pessoa, PB, Brazil. All experimental procedures were conducted according to the guidelines of the National Animal Experimentation Control Council (CONCEA) and the Brazilian Law no.

11.794/2008, which establishes rules for the use and care of animals. The Ethical Committee of the Health Science Center at the Federal University of Paraiba, Brazil, approved the experimental models (Protocol No. 9820210318). Each experimental group consisted of six to eight mice, depending on the experimental protocol.

4-Carvomenthenol (Carvo) treatment and paw edema induced by phlogistic agents

The animals were orally (po) treated with 4-carvomenthenol (Sigma-Aldrich, 95% purity) (Figure 1) at doses of 12.5, 25, 50, 75 or 100 mg/kg (Carvo group), indomethacin at 10 mg/kg (Indo group) or saline (saline group), one-hour prior to the phlogistic agent challenges. The doses of Carvo used in this study were based on a previous study (Oliveira *et al*., 2012). The animals received in the left paw, by intraplantar (ipl) administration, 20 µL of carrageenan (1% w/v, CG group), histamine (100 µg/paw, HIST group), prostaglandin E2 (5 µg/paw, PGE2 group) or compound 48/80 (100 ng/paw, C 48/80 group) and, in the right paw, received the same volume of saline. A digital micrometer measured the paw volume. The difference between right and left paws was defined as the edema index at different times, according to the phlogistic agent: carrageenan paw edema measured at 2, 4 and 6 h and histamine, prostaglandin E2 or compound 48/80 paw at 30 and 60 min; at 15, 30 and 60 min; or at 30 and 60 min, respectively.

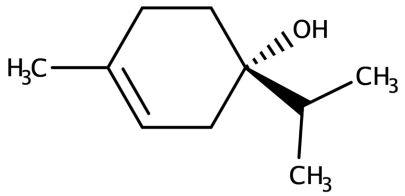

FIGURE 1 - Chemical structure of 4-carvomenthenol.

Carrageenan-induced peritonitis

The animals were orally treated with Carvo (25 mg/kg), Indo (10 mg/kg), or saline and, one hour later, the animals received carrageenan at 1% (intraperitoneal). Four hours later, the animals were euthanized and the peritoneal lavage was performed using 3 mL of cold PBS. The cell suspensions were centrifuged (1500 rpm, 10 min, 4 °C); the supernatants were kept at − 20 °C for further protein quantification and the pellet with cells were counted in a Neubauer chamber to characterize the cell populations; hematoxylin and eosin dye was used. The protein content was measured by pyrogallol red–molybdate, according to the kit manufacturer's protocol (Sensiprot).

Scratching behavior

To evaluate the anti-itch potential of Carvo, mice were pretreated subcutaneously (*sc*), and, one hour later, these animals received an intradermal injection of compound 48/80 (10 µg in 20 µL saline) into the rostral part of the back. Immediately after the injection, the scratching behavior was observed for a period of 60 min by filming the animals of all experimental groups. The number of scratching was standardized as the number of times the animal scratches the injection site with the hind paw.

Tissue collection and histological analysis

The rostral tissue of the back of the animals from the different groups (saline, C 48/80 and Carvo 25) was fixed in 10% formalin, for 24 hours. Subsequently, the tissue was dehydrated, diaphanized and then paraffinized. Tissue sections of 5 µm were generated by microtomy and each tissue section was fixed on slides and stained with Hematoxylin & Eosin for inflammatory parameter analysis or with Toluidine Blue dye for skin mast cell analysis. The images were obtained by optical microscopy with a total magnification of 40X (56,000 μm^2/field) by a microchamber Multicam 5 (5MP), and analyzed with the Motic Images Plus 2.0 software.

Anaphylactic Shock Reaction Induced by Compound 48/80

The anaphylactic shock reaction was induced with a lethal dose of compound 48/80. Hence, animals were

pretreated with Carvo (25 mg/kg), and, one hour later, they were intraperitoneally challenged with compound 48/80 (10 mg/kg). The saline group received only saline. The animals were observed for one hour and the mortality was evaluated at 10, 30, and 60 min.

Molecular docking assays

The chemical structure of Carvo was drawn using the MarvinSketch 18.5 software, had its geometry optimized by the Hyperchem v. 8.0.3 program, using the molecular mechanic method (MM +), and the semi-empirical method AM1 (Austin Model 1). The receptor and enzymes were obtained from the Protein Data Bank Data Bank (https://www.rcsb.org/) together with their co-crystallized inhibitors, presenting the following codes: 3RZE (3.1 Å), 1CQE (3.1 Å), 3PGH (2.5 Å), 5IXC (2.65 Å), corresponding to the histamine H1 receptor and the cyclooxygenases (COX-1/COX-2) and phospholipase A2 (PLA2) enzymes, respectively. Molecular docking was performed with the aid of the Molegro Virtual Docker (MVD) software (v 6.0.1, Molegro ApS, Aarhus, Denmark), using the standard parameters of the software, removing the water molecules and generating a template for each protein. The molecular docking was validated through redocking using RMSD (Root Mean Standard Deviation) values. To be considered a valid docking, the RMSD values must remain in the range 0-2 Å.

Statistical analysis

Data were expressed as a mean ± standard error of the mean and analyzed with the GraphPad Prism 7.0 software using the one-way analysis of variance (ANOVA), followed by the Tukey's test. The results were considered statistically significant when $p < 0.05$.

RESULTS

Effect of Carvo on the carrageenan-induced paw edema and peritonitis models

The pretreatment with the Carvo (25 - 75 mg/kg) one hour before the carrageenan-challenge induced a significant inhibition ($p < 0.0001$) of the paw edema when compared to non-treated animals (CG group), and sustained the effect for a period of six hours (Figure 2A). Due to the absence of significant differences in the paw edema inhibition among the doses, the lowest dose (25 mg/kg) was chosen to proceed all further analyses. Therefore, in the peritonitis model, Carvo reduced in about 63% ($p < 0.0001$) the inflammatory cell migration to the peritoneal cavity (Figure 2B). The reduction polymorphonuclear corresponds to about 55.2% when compared to the CG group (Figure 2C). The Carvo treatment did not interfere either in the mononuclear cell migration (Figure 2D) or in the protein content (Figure 2E).

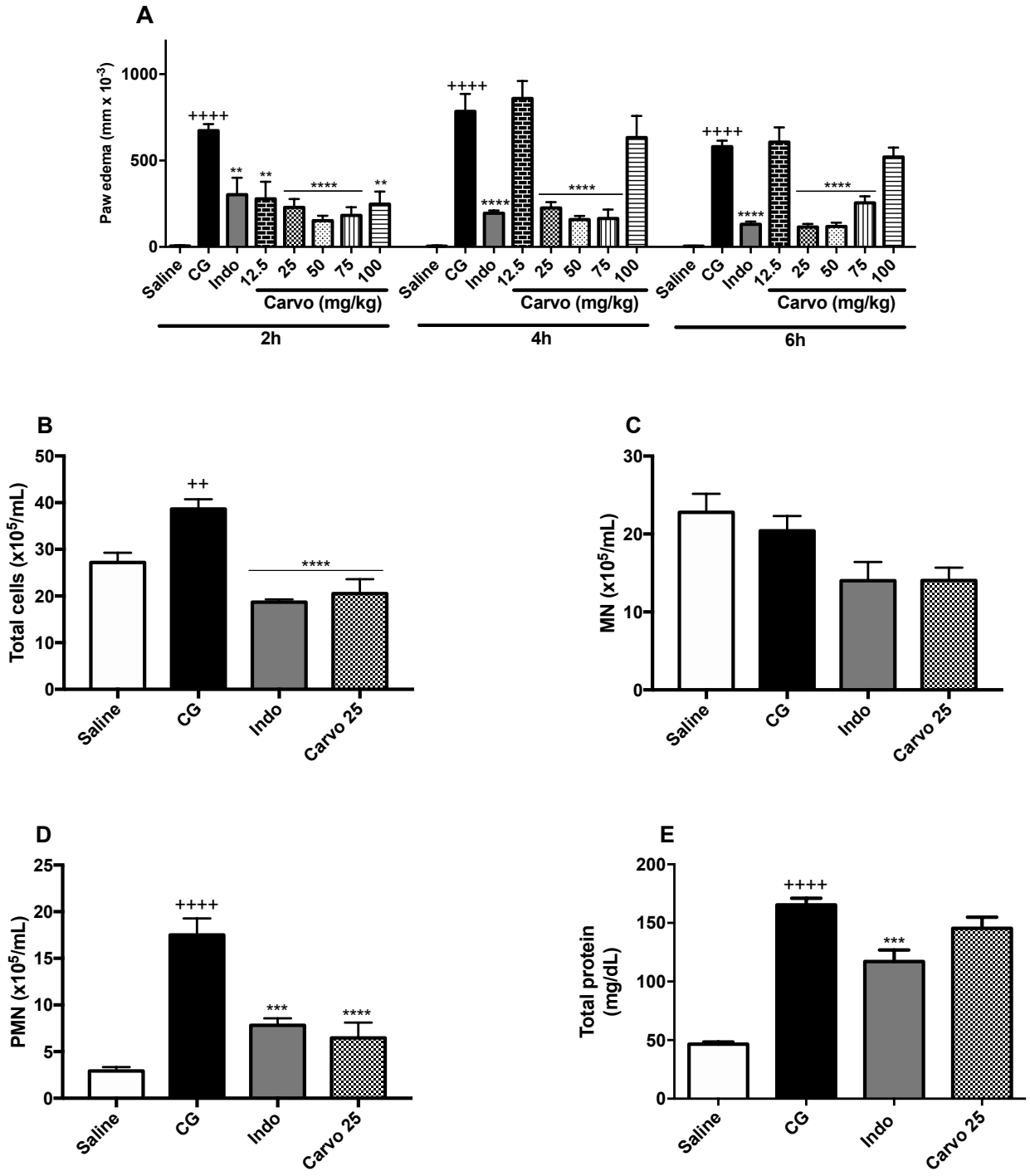

FIGURE 2 - Effect of 4-carvomenthenol (Carvo) on the carrageenan-induced hind paw edema and peritonitis models. Mice (n=6) received intraplantar injection (20 µl) of (A) carrageenan (CG, 1% w/v) in the left hind paw and saline in the right hind paw one hour after 4-carvomenthenol (Carvo, 12.5 - 100 mg/kg), Indomethacin (Indo, 10 mg/kg) or Saline treatments. The Saline group received only saline in both paws. The edema was measured after 2, 4 and 6 h. For the peritonitis model, mice received in the peritoneal cavity, 300 µL of carrageenan 1% (0.1/10 g) 1 h after the Carvo (25 mg/kg, *po*), Indomethacin (Indo, 10 mg/kg, *po*) or Saline treatments. After 4 h of the carrageenan injection, the peritoneal lavage was obtained to determine (B) the total inflammatory cells; (C) mononuclear cells (MN); (D) polymorphonuclear (PMN); (E) protein content in the peritoneal lavage. Data are presented as a mean ± standard error of the mean, analyzed by One-way ANOVA followed by the Tukey post-test. ++ $p < 0.01$ or ++++ $p < 0.0001$ for the comparison of the CG group vs Saline group. **** $p < 0.0001$, *** $p < 0.001$ or ** $p < 0.01$ for the comparison of the treated groups vs CG group. The data are representative of two experiments.

Effect of Carvo on the paw edema induced by histamine and prostaglandin E2

We study the Carvo effect on the paw edema formation triggered by histamine and PGE2. As shown in Figure 3A (Histamine) and Figure 3B (PGE2), Carvo reduced ($p < 0.0001$) the paw edema induced by both inflammatory mediators during the period of analysis (15 – 60 min) when compared to the HIST and PGE2 groups, respectively. In addition, both standard drugs, indomethacin, and promethazine, inhibited the paw edema formation as expected.

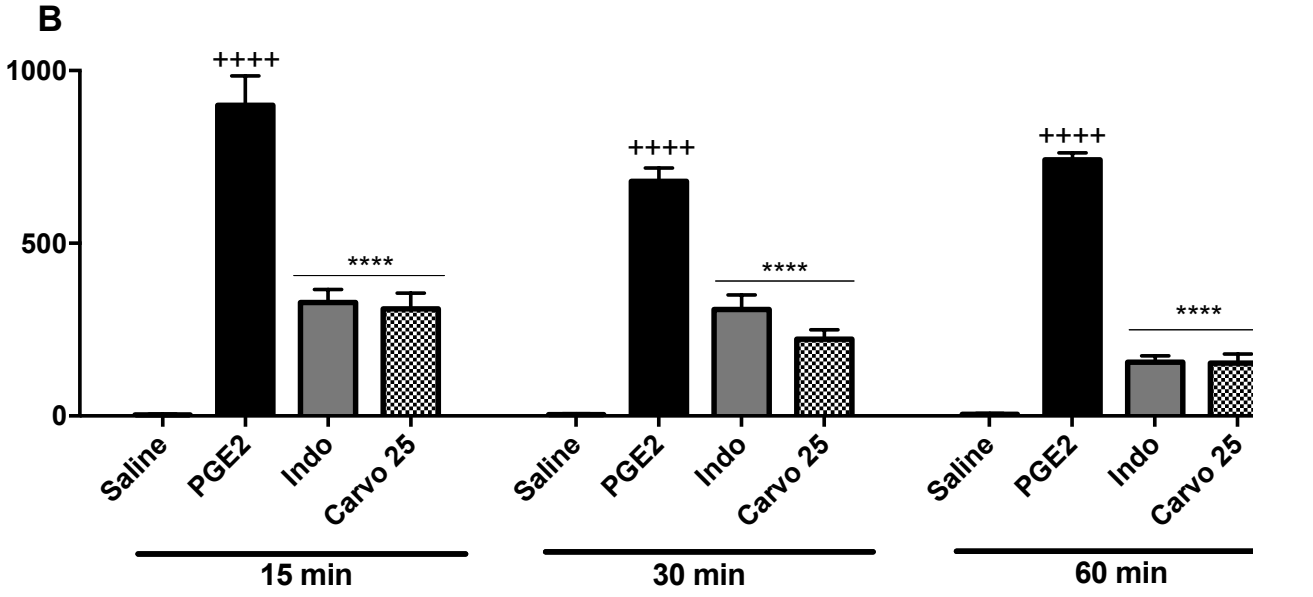

FIGURE 3 - Effect of 4-carvomenthenol (Carvo) on the hind paw edema formation challenged with phlogistic agents. Mice (n=7) received intraplantar injections (20 µl) of: (A) histamine (HIST, 100 ug/paw) or (B) prostaglandin E2 (PGE2, 5 µg/paw) in the left hind paw and saline in the right hind paw 1 h after the 4-carvomenthenol (Carvo 25 mg/kg), Indomethacin (Indo, 10 mg/kg), Promethazine (Prom, 10 mg/kg) or saline treatments. The Saline group received only saline in both hind paws. The edema was measured after the phlogistic agent injection at the selected time intervals. Data are presented as a mean ± standard error of the mean, analyzed by One-way ANOVA followed by the Tukey post-test. ++++ $p < 0.0001$ for the comparison of the HIST or PGE2 group vs Saline group. **** $p < 0.001$ for the comparison of the Carvo group vs HIST or PGE2 groups. The data are representative of two experiments.

Effect of Carvo on the paw edema induced by compound 48/80

We also analyzed the Carvo effect on the paw edema formation using a mast cell degranulating compound 48/80, and observed that the treatment reduced the edema formation in about 58% ($p < 0.01$), at 30 min, and sustained its effect until 60 min when compared to the C48/80 group (Figure 4). The standard drug, promethazine, inhibited ($p < 0.001$) the edema formation during the analyzed period as expected.

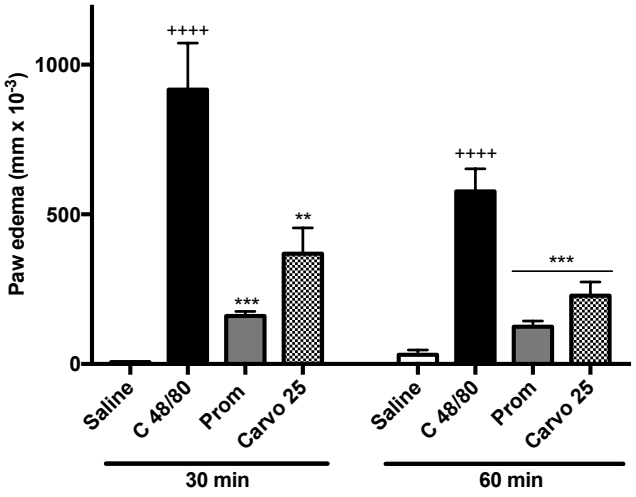

FIGURE 4 - Effect of 4-carvomenthenol (Carvo) on the hind paw edema formation by compound 48/80. Mice (n=6) received intraplantar injections (20 µl) of compound 48/80 (C 48/80, 100 ng/paw) in the left hind paw and saline in the right hind paw 1 h after the Carvo (25 mg/kg), promethazine (Prom, 10 mg/kg) or saline treatments. The Saline group received only saline in both paws. The edema was measured after the phlogistic agent injection at 30 and 60 min. Data are presented as a mean ± standard error of the mean, analyzed by the ANOVA one-way analysis of variance, followed by the Tukey post-test. ++++ $p < 0.001$ for the comparison of the C 48/80 group vs Saline group; *** $p < 0.001$ or ** $p < 0.01$ for comparison among the treated groups and the C 48/80 group. The data are representative of two experiments.

Effect of Carvo on the scratching behavior induced by compound 48/80

Itching is one of many effects of histamine, so that we used compound 48/80 to induce skin mast cell degranulation and measured the number of times the animal scratches the inoculated skin site. In Figure 5A, we observe that the Carvo treated animals showed a significant decrease ($p < 0.01$) in the number of scratches (about 43%) when compared to the C48/80 group. The histological analysis of the skin sites of the animals demonstrated that the Carvo treatment decreased significantly ($p < 0.01$) the number of inflammatory cells, as well as the number of mast cells as indicated by the black arrows (Figure 5B, C and D). Surprisingly, the morphological aspects of these skin tissues were similar to those of the saline group.

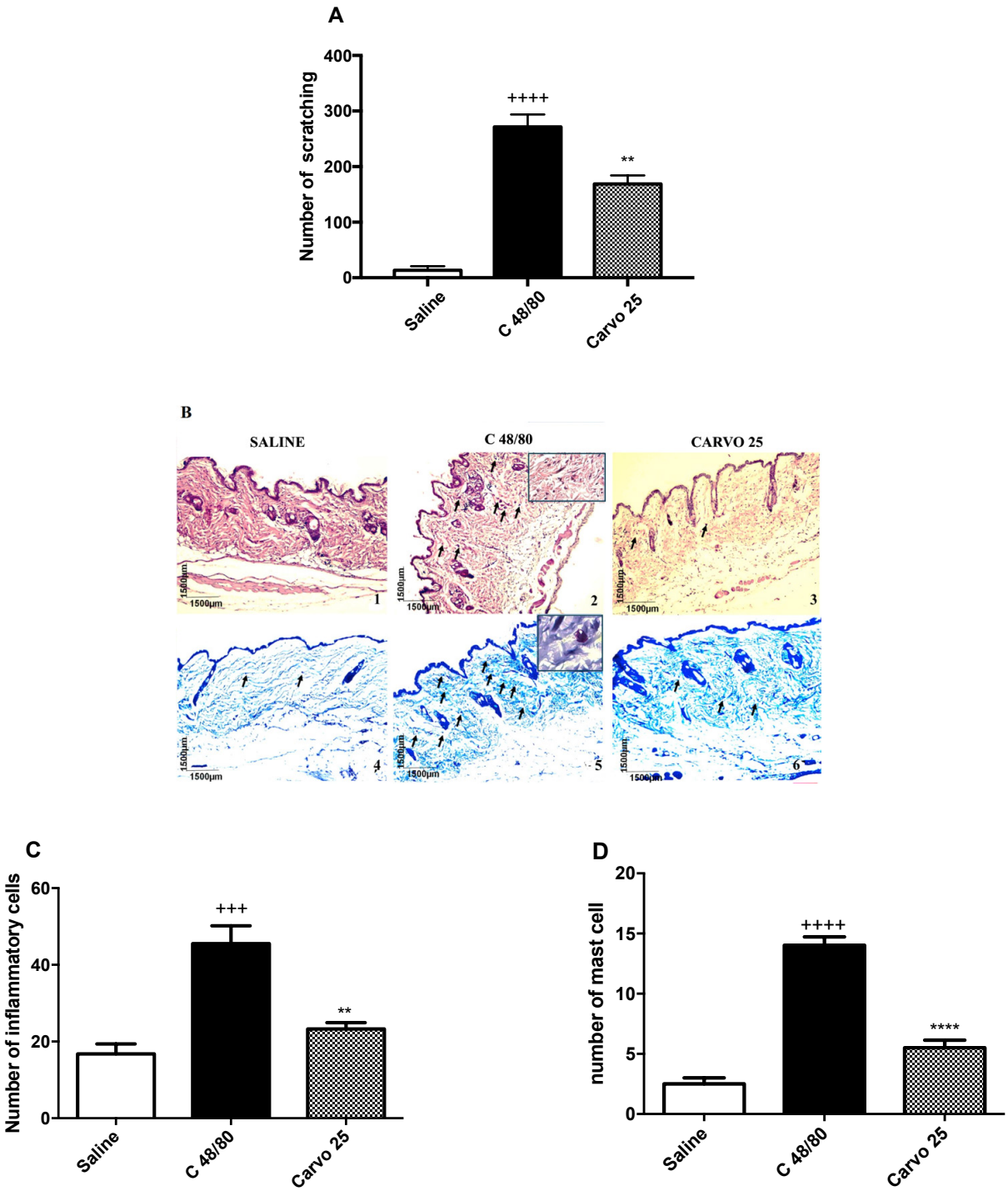

FIGURE 5 - Effect of 4-carvomenthenol (Carvo) on the scratching behavior. Mice (n=6) were pretreated subcutaneously with Carvo (25 mg/kg) or saline, and 1 h later the animals received an intradermal injection of compound 48/80 (C 48/80, 10 µg in 50 µl) in the rostral region of the back. The Saline groups received only saline. The number of scratches was shown as the number of times the animal scratches the inflamed local using the hind paw along 60 min. A. The number of scratches. B. Skin tissues analysis of Saline, compound 48/80 and Carvo (25 mg/kg) groups. The panels 1, 2 and 3 show inflammatory cells, indicated with black arrows, of skin tissues stained with hematoxylin and eosin, and panels 4, 5 and 6 exhibit mast cells, pointed by black arrows, of skin tissues stained with toluidine blue. C. and D. Number of inflammatory cells and mast cells, respectively. Data are presented as a mean ± standard error of the mean, analyzed by the ANOVA one-way analysis of variance, followed by the Tukey post-test. +++ $p < 0.001$ or ++++ $p < 0.0001$ for the comparison of the Saline group vs C 48/80 group; ** $p < 0.01$ or **** $p < 0.0001$ for the Carvo group vs C 48/80 group. The data are representative of two experiments.

Effect of Carvo on the anaphylactic shock reaction induced by compound 48/80.

The anaphylactic shock reaction is mediated by the immediate systemic release of histamine. Then, we induced the anaphylactic shock in animals with a lethal dose of compound 48/80 and observed that the animals challenged with the substance died after a time interval between 10 and 30 min. On the other hand, 50% of the Carvo treated animals survived the anaphylactic shock reaction after 30 min of observation (Table I).

TABLE I - Effect of 4-carvomenthenol (Carvo) on compound 48/80 - induced systemic anaphylactic reaction

Groups†	Compound 48/80 (10mg/kg) ‡	Number of survival of animals at different times (min) §				Survival (%, at 30 min) ¶
		0'	10'	30'	60'	
Saline	-	**8**	8	8	8	100
Compound 48/80	+	8	8	0	0	0
Carvo 25	+	8	8	4	0	50

† The groups of mice (n=8) were orally administrated with saline or Carvo (25 mg/kg) 1h before the compound 48/80 or saline injection
‡ The compound 48/80 solution was intraperitoneally injected to the groups of mice
§ The number of animal survivors was quantified at 0, 10, 30, and 60 min after compound 48/80 or saline challenges
¶ Survival (%) was presented at 30 min as the 'Number of survival mice x 100

Molecular modeling analysis of Carvo

Molecular docking technology was used to assess the possible location of Carvo action. The interaction of the monoterpene with the proteins was performed by selecting three enzymes and the histamine H1 receptor, all associated with the inflammatory process. The RMSD values for the four proteins tested were 3RZE (0.51 Å), 1CQE (0.21 Å), 3PGH (0.15 Å), 5IXC (0.98 Å), confirming the validation of the protocol. Carvo showed better binding energy with the histamine H1 receptor (-73.7 kcal/mol), followed by the cyclooxygenases COX-1 (-60.0 kcal/mol), COX-2 (-61.6 kcal/mol) and PLA2 (-41.3 kcal/mol). Also, Carvo performed a hydrophobic and hydrogen bonding interaction on the H1 histamine receptor, highlighting the hydrogen bonding interaction with the amino acid Tyr108 residue, and the van der Waals interaction with the Trp428 and Ile115 residues, supporting an adequate anchoring of the molecule to the active site of the receptor (Figure 6A). The interactions with the other targets are seen in Figure 6 (B - D).

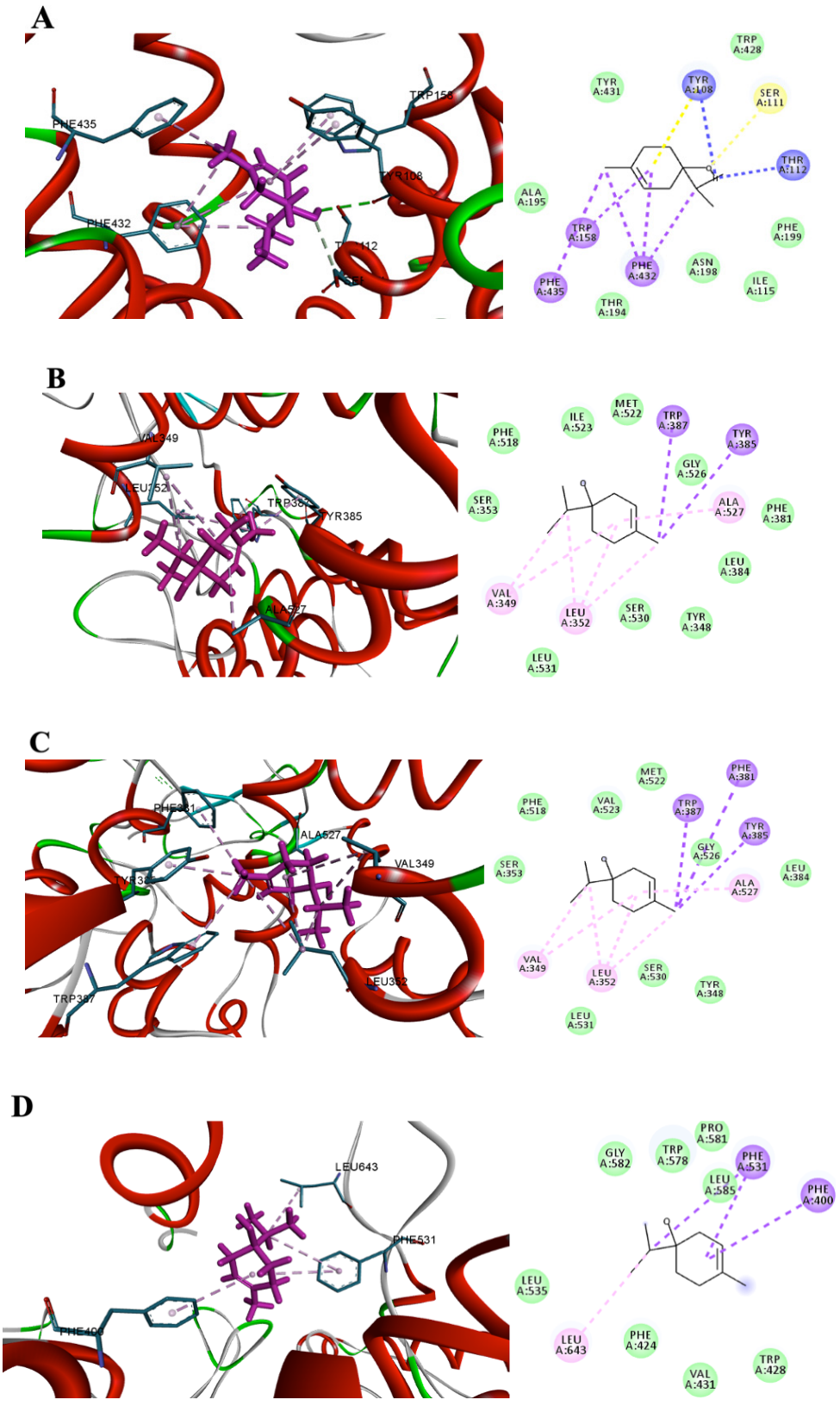

FIGURE 6 - Molecular docking study. Data showing molecular docking studies of binding sites and ligand maps of the 4-carvomenthenol with (A) histamine H1 receptor; (B) cyclooxygenase-1; (C) cyclooxygenase-2; (D) phospholipase A2. Interactions: green - van der Waals, blue - conventional hydrogen bond, purple - pi-alkyl, yellow - carbon hydrogen bond and pink – alkyl.

DISCUSSION

In the last decades, the discovery of new molecules from medicinal plants, such as terpenes, has increased the arsenal of pharmacological drugs to treat degenerative diseases (Newman, Cragg 2016). For instance, the artemisinin, a lactone sesquiterpene, is commonly used as anti-malaria; taxol, a diterpene, is used as antineoplastic (Croteau *et al*., 2006; Pollier, Moses, Goossens, 2011); and α- and γ- terpineol (Carvo isomers) in combination with linalool, both monoterpenes, are used as anti-edema (Moretti, Peana, Satta,, 1997). Moreover, Carvo (or 4-terpineol), an alcohol monoterpene, modulated the inflammatory cytokine production in human LPS-stimulated macrophages (Hart *et al*., 2000; Nogueira *et al*., 2014), the inflammatory cell migration to the gut in dextran sulfate-induced colitis (Zhang *et al*., 2017), and the lung in LPS-induced acute lung injury (Ning *et al*., 2018). Carvo also regulated the edema formation associated with the immediate hypersensitivity reaction (Brand *et al*., 2002a; Khalil *et al*., 2004).

In this context, the present study aimed to analyze Carvo oral treatment in allergic processes. We demonstrated that the monoterpene inhibited the hind paw edema in mice triggered by histamine, PGE2 as well as the mast cell degranulating compound 48/80. We also showed that the monoterpene had a better binding affinity with the histamine 1 receptor, and reduced the immediate hypersensitivity reactions as the scratching behavior and anaphylactic shock reaction, both dependent on the mast cell degranulation process. Hence, Carvo treatment may prevent the histamine release, an important mechanism involved in allergic disorders.

Previously, we screened the anti-edematogenic property of Carvo through the carrageenan-induced hind paw edema experimental model. Carrageenan, as a phlogistic agent, triggers two well-defined inflammatory phases. The first phase is the release of vasoactive amines, such as histamine and serotonin, followed by bradykinin and prostaglandins and edema formation. The second phase is the maintenance of the edema with fluid extravasation (exudate), cytokine releases and polymorphonuclear cell migration to the inflamed site. Both phases can be analyzed by experimental models as hind paw edema and peritonitis with polymorphonuclear cell migration and exudate to the inflamed site (Posadas *et al*., 2004; Necas, Bartosikova, 2013).

We observed that Carvo reduced both phases of the carrageenan-induced hind paw edema and peritonitis, without interfering in the protein extravasation to the inflamed site. No significant differences of these effects were observed among the Carvo doses (25, 50 and 75 mg/kg), therefore, we chose the lowest dose (25 mg/kg) to continue the investigation of Carvo activity. These data also showed the potential of the monoterpene. Indeed, in another study it was demonstrated the anti-edematogenic effect of Carvo, in a dose-dependent manner, but using a different route of administration (topical route) (Pongprayoon *et al*., 1996).

By using *in silico* molecular docking analysis, we demonstrate that Carvo presented high binding energy with the histamine H1 receptor (-73, 7 kcal/mol), with lower binding energies to other inflammatory enzymes as COX-1 and COX-2 and PLA2. Histamine is a biogenic amine synthesized and stocked in intracellular vesicles by several types of cells including mast cells, basophils, and eosinophils involved directly in allergic processes (Branco *et al*., 2018). Yet, histamine is related to PLA2 activation, which is associated with PGE2 formation by COX activation (Juan, Sametz, 1980; Jutel, Akdis, Akdis, 2009). Both histamine and PGE2 cause, among several effects, the vasodilation and increase of the vascular permeability and fluid exudation to the inflamed site (Kalinski, 2012). Our study clearly showed that Carvo, at the lowest dose tested, inhibited the edema formation induced by both vasoactive mediators, histamine, and PGE2.

Therefore, the anti-edematogenic effect of Carvo may be associated with the interaction of the histamine H1 receptor, interfering with the signaling pathway breakpoints induced by both mediators: histamine and PGE2, and/or due to the inhibition of histamine release by tissue cells. Thus, to demonstrate the second hypothesis, we used the mast cell degranulating compound 48/80 to induce hind paw edema and, as expected, Carvo inhibited the edema formation. This finding was similar to those reported with other monoterpenes as myrtenol, limonene epoxide and carvacrol acetate (Damasceno *et al*., 2014; Almeida *et al*., 2017; Silva, Salvadori, Sousa, 2017), which

successfully inhibited and modulated the edema induced by compound 48/80. Indeed, the monoterpene α-terpineol was capable of modulating the mast cell degranulation process by inhibiting the histamine release of RPMCs (rat peritoneal mast cells) (Moon *et al*., 2013). Furthermore, Brand and col. (2002b) and Khalil and col. (2004) reported that the topical use of the essential oil or the 4-terpineol (Carvo) suppressed the mouse ear edema induced by histamine and regulated the histamine-induced wheal and flare in humans, respectively (Brand *et al*., 2002b; Khalil *et al*., 2004).

To corroborate with the above-mentioned data, we used two well-described *in vivo* anaphylactic experimental models, where the injection of compound 48/80 induces skin scratching behavior and systemic anaphylactic shock reaction. Both sick behaviors are related to allergic reactions, histamine release and, sometimes, these reactions make the pharmacological control difficult. Compound 48/80 is a basic polyamine, which interacts with a G protein in the mast cell cytoplasm and causes an influx of Ca^{2+}, culminating in the degranulation process and release of histamine and serotonin that induce the vascular dilatation, fluid extravasation, edema formation, and local itching (Palomaki, Ville, Laitinen, 2006), which are common characteristics in the allergic process. Thus, the use of compound 48/80 is an important experimental tool in allergy studies to assess mast cell functions and histamine release.

We intradermally injected compound 48/80 into the rostral region of the animals to induce a local scratching behavior. It was observed that Carvo treatment decreased the itching behavior, clearly indicating that the molecule is inhibiting, at least, the histamine release. The histological analysis of the skin of these animals corroborated with this finding showing that Carvo prevented the tissue inflammatory reactions, such as vasodilation, inflammatory cell migration, and mast cell activation. On the other hand, Santos and Rao (1997, 2002) showed that high doses of 1,8-cineol, a similar monoterpene, induced local edema and itching after subcutaneous or intradermal applications (Santos, Rao, 1997; Santos, Rao, 2002). We believe that these observations can be associated with the structures of the molecules since 1,8 cineol and Carvo belong to different monoterpene classification, which may confer differences in the pharmacological properties, as well as the doses used in these studies.

Another life-threatening allergic disorder mediated by mast cell degranulation and histamine release is the anaphylactic shock, which is potentially fatal. The experimental model that mimics this disorder can be developed by compound 48/80 (Lieberman, Garvey, 2016). Therefore, Carvo treatment in the compound 48/80-challenged animals, in a dosage of the compound that causes animal death, was capable of increasing the animal survival at 30 min of observation when compared to the animals without treatment. According to these data, Sá and col. (2013) described several classes of monoterpenes such as l-menthol, menthone and limonene that modulate actively the immediate hypersensitivity reaction in a passive cutaneous anaphylaxis model by suppressing mast cell activation and degranulation (Sá, Andrade, de Sousa, 2013).

In conclusion, we demonstrate that the anti-inflammatory effect of Carvo takes place by reducing the migration of inflammatory cells and inflammatory edema, in addition to preventing mast cell degranulation and histamine release and possibly interacting with the histamine receptor (H1). These preliminary findings provide a solid scientific basis in the context of Carvo to become a phytomedicine in the treatment of histamine-induced diseases such as allergic disorders.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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