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On the ameliorative potential of *Baccharis trimera* in an experimental model of pulmonary disease

Avaliação do potencial de melhoria de Baccharis trimera em um modelo experimental de doença pulmonar

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ABSTRACT
Diabetes mellitus, smoking, and dyslipidemia are more prevalent in patients with chronic obstructive pulmonary disease (COPD), the fourth leading cause of mortality worldwide. This study employed a model of lung disease in Wistar rats that incorporated these three risk factors, and investigated the effects of Baccharis trimera, a widely used medicinal plant, since no previous studies have evaluated its pulmonary effects. The diabetic and dyslipidemic rats were exposed to smoke for 4 weeks and treated with vehicle (C- group), an extract of B. trimera (HEBT), or simvastatin+insulin, for 2 weeks. The bronchoalveolar lavage was performed to evaluate inflammation. The lungs were collected for histopathological and redox state analyses. A decrease in body weight, an increase in oxidative stress, inflammation, and histopathological changes were observed in C- group. HEBT reversed these alterations and had a moderate antiinflammatory effect. Treatment with HEBT present promising effects for COPD.

Keywords: COPD. Diabetes mellitus. Dyslipidemia. Rodents. Smoking.

RESUMO
Diabetes mellitus, tabagismo e dislipidemia são mais prevalentes em pacientes com doença pulmonar obstrutiva crônica (DPOC), a quarta causa de mortalidade no mundo. Este estudo empregou um modelo de doença pulmonar em ratos Wistar que incorporou esses três fatores de risco e investigou os efeitos da *Baccharis trimera*, uma planta medicinal amplamente utilizada, uma vez que nenhum estudo avaliou seus efeitos pulmonares. Os ratos diabéticos e dislipidêmicos foram expostos à fumaça de cigarro por 4 semanas e tratados com veículo (grupo C-), extrato de *B. trimera* (HEBT), ou sinvastatina+insulina, por 2 semanas. O lavado broncoalveolar foi realizado para avaliar a inflamação. Os pulmões foram coletados para análises histopatológicas e do estado redox. Foi observada diminuição do peso corporal, aumento do estresse oxidativo, inflamação e alterações histopatológicas no grupo C-. HEBT reverteu essas alterações e apresentou efeito antiinflamatório moderado. O tratamento com HEBT apresentou efeitos promissores para a DPOC.

Palavras-chave: DPOC. Diabetes mellitus. Dislipidemia. Roedores. Tabagismo.

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INTRODUCTION

For almost 50 years, chronic obstructive pulmonary disease (COPD) has been conceptualized as a self-inflicted condition that is caused by tobacco smoke. In susceptible people, smoking leads to an abnormal inflammatory response that damages airways (leading to bronchitis and bronchiolitis) and alveola (leading to emphysema) [1]. These deleterious effects accelerate age-related physiological lung decline and cause airway flow limitation and chronic respiratory symptoms, which are difficult to reverse and may periodically manifest with exacerbations of pulmonary function. Spirometric diagnosis is mandatory; without it, the presence of COPD cannot be confirmed. Current treatments for COPD focus on increasing airway flow and improving pulmonary symptoms using bronchodilators with or without inhaled glucocorticoids[2].

Smoking is the key environmental risk factor for COPD and patients with COPD also have a higher prevalence of classic risk factors for cardiovascular disease, such as dyslipidemia, diabetes, and systemic arterial hypertension[1-3]. Comorbid conditions are highly prevalent in this disease but are not necessarily related to lung function. Thus, COPD can be considered a pulmonary component of a systemic and multimorbid syndrome[1]. Circulating inflammatory mediators can contribute to musculoskeletal loss and cachexia, in addition to initiating or worsening various comorbidities, such as ischemic heart disease, heart failure, osteoporosis, normocytic anemia, diabetes, and metabolic syndrome[2-5].

The coexistence of COPD and cardiovascular disease has an important impact on clinical outcomes. In patients with mild or moderate COPD, the most frequent causes of death are cancer and cardiovascular disease. In patients with severe COPD, the main cause of death is respiratory disease[6]. Chronic obstructive pulmonary disease is also associated with a higher risk of arrhythmias, acute myocardial infarction, and stroke[6,7]. In patients with COPD, cardiovascular disease is responsible for approximately 50% of all hospitalizations and 20% of all deaths, thus emphasizing the relationships between COPD and cardiovascular disease[8].

Based on the high prevalence of diseases and risk factors that are related to the cardiovascular and respiratory systems, scientific studies need to evaluate alternative means of treatment and develop more effective drugs that are more cost-effective and viable for treating cardiac and pulmonary symptoms. One promising treatment alternative is medicinal plants. Among the promising plant species is *Baccharis trimera* (Asteraceae), popularly known as “carqueja.” It is a perennial shrub that originated in South America and is grown mainly in Brazil, Argentina, Paraguay, and Uruguay[9,10]. The plant infusion is popularly used for the treatment of liver and digestive problems, malaria, diabetes, anemia, diarrhea, inflammation, worms, hypercholesterolemia, and

rheumatism[10-13]. Pharmacological studies have reported its antioxidant, antiinflammatory, gastroprotective, and hepatoprotective effects[10,14-16]. Its effects on weight loss and hypolipidemic and hypoglycemic activity are also well described, in addition to antihemorrhagic, anti-Alzheimer's[10], and cardioprotective actions[17,18]. Despite various comprehensive studies of the therapeutic effects of *B. trimera*, its pneumoprotective activity has not yet been scientifically investigated.

The present study employed a rat model of pulmonary disease by incorporating various risk factors for dyslipidemia and diabetes, combined with one modifiable risk factor, smoking. *B. trimera* has been previously shown to have cardio- and hepatoprotective actions in this experimental model. We extended this model to evaluate its pneumoprotective effects.

METHODS

BOTANICAL MATERIAL AND EXTRACT PREPARATION

Aerial parts of *Baccharis trimera* (Less.) DC were collected in February 2018 from the Medicinal Plants Garden of Paranaense University, which is located 430 m above sea level (coordinates: 23°46'11.3"S, 53°16'41.2"W) in Umuarama, Paraná, Brazil. A voucher specimen was deposited in the herbarium of UNIPAR (no. 07). The extract was prepared by infusion according to the ethnomedicinal form of use and then purified with ethanol according to previously described methods and the phytochemical profile was previously published[17]. The plant name was checked at http://www.theplantlist.org and found to be approved.

ANIMALS

Male Wistar rats, weighing 200-250 g, were obtained from the State University of Maringá. They were housed in the vivarium of the Preclinical Research Laboratory for Natural Products of the University of Paraná with free access to a liquid and solid diet under controlled environmental conditions (temperature: 20°C ± 2°C; relative humidity: 50% ± 5%; 12 h/12 h light/dark cycle) with environmental enrichment . The experimental protocol was approved by the Ethics Committee on the Use of Animals of the Paranaense University (protocol no. 1000/2018) and all international guidelines and recommendations that ensure animal welfare and reduce the number of animals that are used in experimental research. All national and international guidelines were followed. The reporting of animal investigations was performed and interpreted according to Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines[19].

EXPERIMENTAL DESIGN AND TREATMENTS

To induce diabetes, after a 12-h fast, the rats received streptozotocin (60 mg/kg), diluted in citrate buffer (10 mM, pH 4.5, i.p.). Three days later, glycemia was measured with a glucose meter in a small volume of peripheral blood that was collected from the tail. Rats with glycemia ≥ 250 mg/dL were considered diabetic. For the induction of dyslipidemia, the diabetic animals received a standard diet that was enriched with 0.5% cholesterol *ad libitum* for 4 weeks[17]. Concomitantly, the animals were exposed to nine commercial tobacco cigarettes (0.8 mg nicotine, 10 mg tar, and 10 mg carbon monoxide) for 1 h/day, 5 days/week[20]. The experiment lasted 4 weeks. During the last 2 weeks, the animals were randomized to different groups (n = 8/group) and orally treated with vehicle (filtered water by gavage; negative control [C-] group), three doses of an ethanol-soluble fraction of *B. trimera* (30, 100, and 300 mg/kg by gavage), or simvastatin (2.5 mg/kg, s.c.) plus insulin (6 IU, s.c.; SIM+INS group). A group of normoglycemic rats that was not exposed to dyslipidemia or tabagism and was treated with vehicle (basal group) was also included. The drugs were prepared immediately before use and administered once daily.

BRONCHOALVEOLAR LAVAGE FOR INFLAMMATORY CELL EVALUATION

At the end of the 4-week experimental period, the rats were subjected to bronchoalveolar lavage to evaluate inflammatory cell infiltrates in the lungs. The animals were anesthetized with ketamine and xylazine (80 and 20 mg/kg, i.p., respectively). The trachea was then exposed and cannulated with a #22 catheter, and 2.5 ml of saline solution was injected. After massaging the chest, the fluid was aspirated through the catheter. The cell content was stained with Turk fluid and analyzed under an optical microscope to count the number of inflammatory cells in the aspirate.

EUTHANASIA AND TISSUE COLLECTION

After bronchoalveolar lavage, the rats were euthanized by deep anesthesia with isoflurane in a saturation chamber (1-3%). The lungs were collected and processed for antioxidant system and histopathological analyses.

PULMONARY OXIDATIVE STRESS ANALYSIS

To evaluate the antioxidant potential of *B. trimera*, lung homogenates were analyzed to assess levels of catalase, superoxide dismutase (SOD), lipoperoxidation, and reduced glutathione

(GSH), all of which are indicators of cellular redox status. Lung samples were homogenized and centrifuged at 13,000 rotations per minute for 20 min. Homogenization was performed on ice, and the supernatant was refrigerated at 4ºC. Catalase and SOD were analyzed in the supernatant according to Aebi[21] and Gao *et al*.[22], respectively. The rate of lipoperoxidation was measured using the FOX2 method[23]. Reduced glutathione levels were measured according to Sedlak & Lindsay[24].

PULMONARY HISTOPATHOLOGICAL ANALYSIS

For the histopathological analyses, a sample of the lung was quickly harvested and fixed in 10% buffered formalin solution, fixed, sectioned (6 μm), and stained with hematoxylin/eosin. The slides were analyzed by optical microscopy (Leica DM 2500) to evaluate cellular alterations, such as congestion, inflammation, polymorphonuclear cell infiltrates, bronchitis, alveolar wall thickening, the presence of bronchiolar content, bronchoconstriction, hyperplasia, and anthracnose. The histopathological analyses were performed blindly by a veterinarian pathologist. Lesions were classified as the following: 0 (absence of lesions), 0.5 (minor lesions), 1 (moderate lesions), 2 (marked lesions), and 3 (massive lesions).

STATISTICAL ANALYSIS

The data were first analyzed for homogeneity of variance and a normal distribution. The data were then analyzed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls *post hoc* test. Values of $p < 0.05$ were considered statistically significant. The statistical analysis was performed using GraphPad Prism 5.0 software.

RESULTS

THE EXPERIMENTAL MODEL ALTERED BODY WEIGHT

The combination of diabetes, dyslipidemia, and smoking reduced final body weights (Figura 1) in the C- group (248.80 ± 5.24 g) compared with the basal group (320.00 ± 6.68 g). Treatment with 30 mg/kg *Baccharis trimera* prevented weight loss (301.70 ± 4.00 g). However, the other doses of *B. trimera* did not influence body weight loss. Treatment with SIM+INS induced a moderate effect on body weight (281.80 ± 13.17 g).

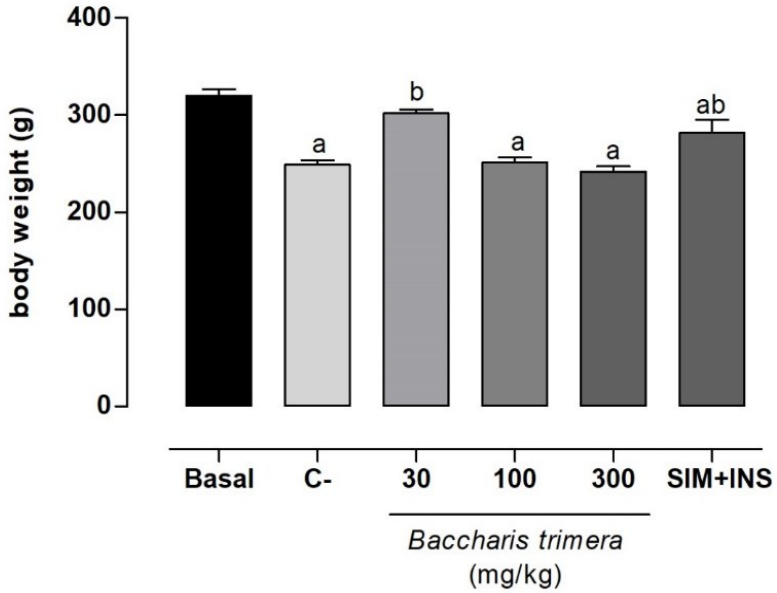

Figure 1. Body weight of normoglycemic, non-dyslipidemic, and non-smoker Wistar rats (basal group) and diabetic, dyslipidemic, and smoker Wistar rats that were treated with vehicle (negative control [C-]), *Baccharis trimera* (30, 100, and 300 mg/kg) or simvastatin + insulin (SIM+INS). The data are expressed as mean ± SEM. $^{a}p < 0.05$, *vs*. basal; $^{b}p < 0.05$, *vs.* C- (one-way ANOVA followed by Newman-Keuls post hoc test).

Baccharis trimera NORMALIZED PULMONARY REDOX STATE IN RATS

The three risk factors induced pulmonary oxidative stress in rats. A decrease in GSH levels (17.35 ± 2.15 µg GSH/g of tissue; Figura 2A) and an increase in lipoperoxidation levels (78.49 ± 8.50 mmol hydroperoxides/min/g of tissue; Fig. 2B) were observed in the C- group compared with the basal group (51.66 ± 4.10 µg GSH/g of tissue and 36.79 ± 1.63 mmol hydroperoxides/min/g of tissue, respectively). Treatment with all doses of *B. trimera* normalized lipoperoxidation levels, but only 30 mg/kg *B. trimera* normalized GSH levels. Treatment with *B. trimera* (30 mg/kg) reversed the increases in SOD activity (Figura 2C) and catalase (Figura 2D) that were observed in the C- group compared with the basal group (SOD activity: 1182.00 ± 26.43 U SOD/g of tissue; catalase: 136.90 ± 10.94 mmol/min/g of tissue). Treatment with SIM+INS normalized GSH levels and partially reversed SOD activity but did not reverse lipoperoxidation or catalase activity.

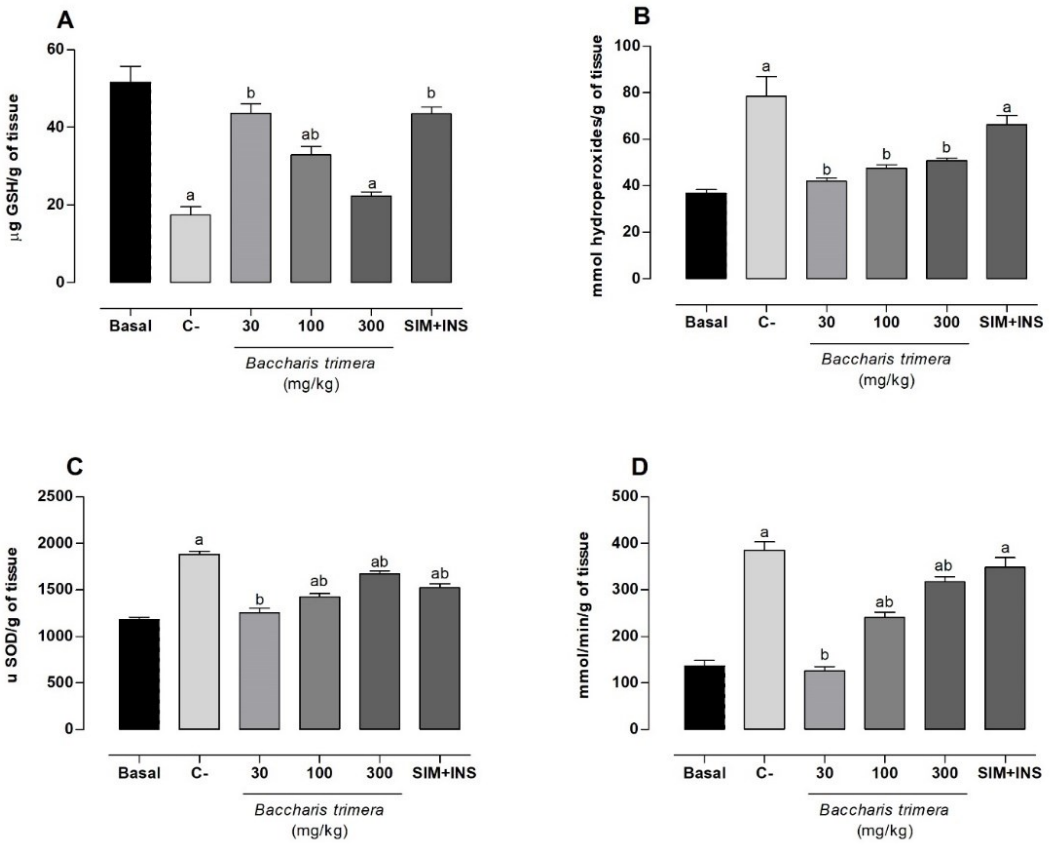

Figure 2. Antioxidant effects of *Baccharis trimera*. Pulmonary levels of **(A)** reduced glutathione, **(B)** lipoperoxidation, **(C)** superoxide dismutase, and **(D)** catalase in normoglycemic, non-dyslipidemic, and non-smoker Wistar rats (basal group) and diabetic, dyslipidemic, and smoker Wistar rats that were treated with vehicle (negative control [C-]), *Baccharis trimera* (30, 100, and 300 mg/kg), or with simvastatin + insulin (SIM+INS). The data are expressed as mean ± SEM. $^{a}p< 0.05$, *vs.* basal; $^{b}p< 0.05$, *vs.* C- (one-way ANOVA followed by Newman-Keuls *post hoc* test).

Baccharis trimera PROMOTES MODERATE PULMONARY ANTIINFLAMMATORY EFFECTS IN RATS

The presence of leukocytes in the lungs is shown in Figura 3. The combination of diabetes, tabagism, and dyslipidemia induced a pulmonary inflammatory response (85.00 ± 5.00 leucocytes/10 fields) compared with the basal group (13.33 ± 6.66 leucocytes/10 fields). Treatment with *B. trimera* (30 mg/kg) promoted a moderate pulmonary antiinflammatory effect (40 ± 8.16 leucocytes/10 fields). The other treatments did not exert antiinflammatory effects.

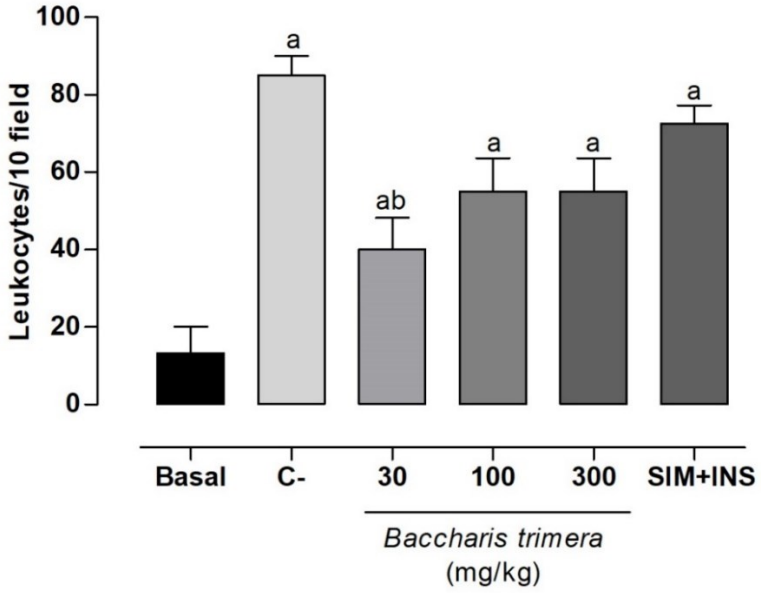

Figure 3. Pulmonary anti-inflammatory effects of *Baccharis trimera*. Number of leucocytes found in 10 fields/slides of lung in normoglycemic, non-dyslipidemic, and non-smoker Wistar rats (basal group) and diabetic, dyslipidemic, and smoker Wistar rats that were treated with vehicle (negative control [C-]), *Baccharis trimera* (30, 100, and 300 mg/kg), or with simvastatin + insulin (SIM+INS). The data are expressed as mean ± SEM. [a]p< 0.05, *vs*. basal; [b]p< 0.05, *vs.* C- (one-way ANOVA followed by Newman-Keuls post hoc test).

Baccharis trimera REVERSED HISTOPATHOLOGICAL PULMONARY ALTERATIONS IN RATS

No alterations were found in the lungs in rats in the basal group (score of 0; Figura 4A). Diabetes, tabagism, and dyslipidemia induced congestion in peribronchial vessels, bronchiole inflammation, polymorphonuclear cell infiltrates in peribronchial connective tissue, morphonuclear bronchitis, alveolar wall thickening, the presence of bronchiolar content, bronchoconstriction, extensive focal bronchopneumonia, follicular lymphocytic hyperplasia, and anthracnose (score of 3; Figura 4B). Treatment with 30 mg/kg *B. trimera* attenuated these alterations (score of 0.5; Figura 4C). The 100 mg/kg (score of 1; Figura 4D) and 300 mg/kg (score of 1; Figura 4E) doses of *B. trimera* were less effective in reversing these changes. Treatment with SIM+INS exerted a moderate pneumoprotective effect (score of 2; Figura 4F).

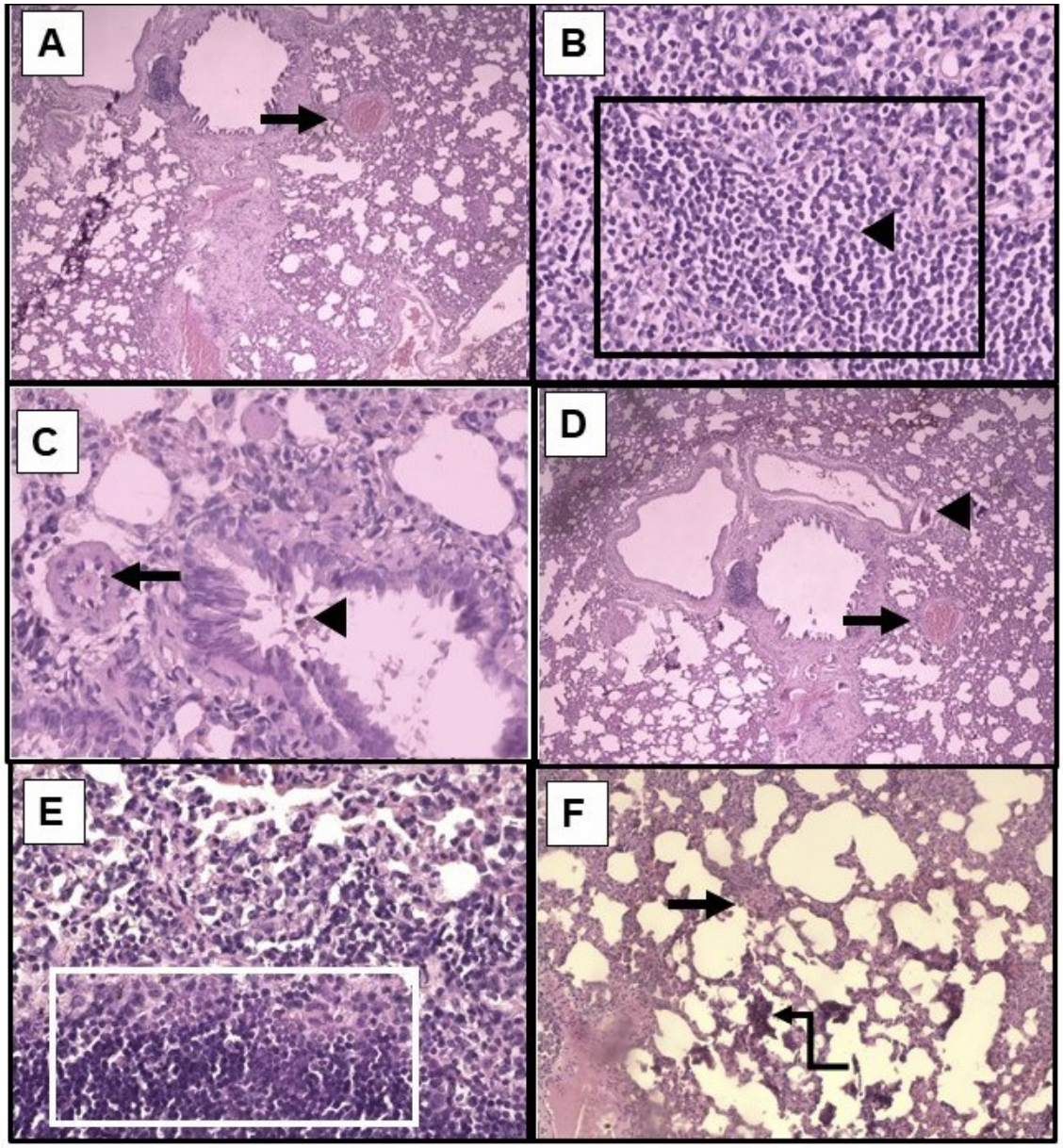

Figure 4. Histopathological pulmonary analysis. **(A)** normoglycemic, non-dyslipidemic, and non-smoker Wistar rats (basal group) and diabetic, dyslipidemic, and smoker Wistar rats that were treated with **(B)** vehicle (negative control [C-]), **(C)** 30 mg/kg *Baccharis trimera*, **(D)** 100 mg/kg *Baccharis trimera*, **(E)** 300 mg/kg *Baccharis trimera* and **(F)** simvastatin + insulin. Black arrows indicate vessel congestion. Arrowheads show polymorphonuclear cells. Angled arrow indicates anthracnose. Black square show extensive bronchopneumonia. White square show Congestion and lymphocyte follicular hyperplasia. 20× magnification, hematoxylin/eosin staining.
Source: Souza *et al*.[17]

DISCUSSION

Animal models that incorporate multiple risk factors simultaneously are relatively uncommon. Such models can better mimic reality in the population today compared with assessments of individual risk factors alone. Such combinations of risk factors can result in greater pathophysiological aggressiveness through

synergistic effects among risk factors and the acceleration of disease. The multifactorial experimental design that was employed in the present study included three specific cardiovascular risk factors for COPD: diabetes, dyslipidemia, and smoking. The present experimental model decreased body weight, induced pulmonary oxidative stress, and triggered an inflammatory response in lung parenchyma. According to Gold[5], COPD is characterized by persistent respiratory symptoms and flow limitation, which occur through alveolar or airway abnormalities. It is usually caused by exposure to harmful smoke particles or gases. For the diagnosis of COPD, spirometry is mandatory, which reveals the limited reversibility of airway flow. The histopathological basis of COPD is an abnormal inflammatory response, which damages the airways and triggers bronchitis, bronchiolitis, elevations of polymorphonuclear cells, and interstitial and peribronchial fibrosis, leading to thickening of the alveolar wall[5]. All of these changes were observed in the rats in the present study. However, we cannot specifically diagnose COPD in these rats because spirometry cannot be performed. Nonetheless, our model reliably mimicked the human condition by causing histopathological alterations that are commonly observed in human COPD.

Structural and inflammatory changes that occur in the airways during COPD persist even after smoking cessation. Increases in oxidative stress and excess proteinases in the lungs are likely responsible for modifying lung inflammation, thereby perpetuating the cycle[25]. In the present study, we observed oxidative stress in the lungs, which is likely responsible for the pulmonary inflammatory condition and was confirmed by the histopathological evaluation. Treatment with *B. trimera* effectively reversed oxidative stress and thus inflammation at the dose of 30 mg/kg, indicating the reversal of histopathological changes that were caused by the three risk factors.

Kozma *et al*.[20] reported that 8-36 weeks (mean: 6 months) of cigarette smoke exposure is necessary to induce emphysema in Wistar rats. The present study exposed the rats to cigarette smoke for 4 weeks, and the pulmonary cellular evaluation showed significant inflammation. A potentiating effect between the multiple risk factors can be inferred, which differs from simply the sum of isolated effects of individual risk factors alone. We also analyzed weight loss during the systemic process of pulmonary inflammation. Inflammatory mediators in blood circulation in patients with COPD can contribute to the loss of skeletal muscle, thus justifying weight loss that was observed in the animals. However, a possible effect on weight loss that is popularly attributed to *B. trimera* was not substantiated in the present study because the 30 mg/kg dose preserved the animals' body weight.

Souza *et al*.[17] employed the same multifactorial model as the one that was used in the present study to investigate

atherogenesis, which is a worsening of hemodynamic variables that causes endothelial dysfunction, thickening of the abdominal aorta, and the presence of mesenteric vascular hyperactivity. Furthermore, these authors described important alterations of cardiac and renal redox states, reflected by an increase in lipoperoxidation levels and decreases in GSH levels and SOD activity. In the present study, we observed these alterations, and treatment with *B. trimera* (30 mg/kg) reversed these changes, whereas the other doses of *B. trimera* did not. Notably, similar to Souza *et al*.[17], the most effective dose of *B. trimera* was the lowest dose (30 mg/kg), which had better antiinflammatory effects than even the conventional treatment. The lowest dose of *B. trimera* also exerted the most effective antioxidant effects. Although all doses decreased lipoperoxidation, only the lowest dose normalized GSH levels and reversed the increase in SOD and catalase activity. Treatment with insulin plus simvastatin normalized GSH levels but only partially reversed SOD activity, again demonstrating the superiority of this dose of *B. trimera* over the other doses and the conventional treatment.

The antioxidant and antiinflammatory effects of *B. trimera* may explain the improvements in pulmonary inflammation, reflected by reversal of the histopathological condition, the reversal of oxidative stress, a decrease in lipid peroxidation, and the normalization of GSH and SOD. The antioxidant effect of *B. trimera* occurs through its reactive oxygen and nitrogen species scavenging ability and increases in antioxidant system activity[10,14-18].

According to Arnett *et al*.[26], impoverished populations have limited access to healthy lifestyles and exhibit a high prevalence of smoking, which predisposes to the development of COPD. Patients with COPD also have an increased prevalence of classic risk factors for cardiovascular disease, such as dyslipidemia, diabetes, and systemic arterial hypertension[27]. The coexistence of COPD and cardiovascular disease has an important impact on clinical outcomes, since in patients with mild and moderate COPD, the most frequent causes of death are cancer and cardiovascular disease, while in those with severe COPD the main cause of death is respiratory disease[6]. In fact, in COPD patients, cardiovascular diseases are responsible for approximately 50% of all hospitalizations and 20% of all deaths, emphasizing the interrelationship between COPD and cardiovascular diseases[8].

Due to the high prevalence of diseases and risk factors related to the cardiovascular and respiratory system, scientific studies that evaluate alternative means of treatment should be considered to develop more effective, cost-effective, and viable drugs for the treatment of heart and lung diseases. The pharmacological effects of *B. trimera*, the rusticity and ease of obtaining *B. trimera* biomass associated with its medicinal potential, in addition to the fact that it is one of the 71 plants listed in the National List of Medicinal Plants of

interest to the Unified Health System of Brazil, make carqueja a possible pneumoprotective pharmacological agent, with special attention to diseases triggered by the association of dyslipidemia, diabetes and smoking, since there are few studies correlating them. Considering the important pneumoprotective activity of *B. trimera* and the medicinal plant's low production costs and easy accessibility, the present results support the therapeutic use of *B. trimera* as an adjunctive treatment and/or prevention for COPD, especially conditions that are associated with multiple cardiovascular risk factors.

CONCLUSION

The combination of diabetes mellitus, dyslipidemia, and smoking induced oxidative stress, inflammation, and pulmonary cell changes in rats. Treatment with *Baccharis trimera* (30 mg/kg) partially reversed inflammation and completely reversed cellular and oxidative alterations, thus demonstrating its pneumoprotective actions. Further studies are needed to elucidate the mechanisms by which *B. trimera* exerts these beneficial effects.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this research.

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