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Research Article

Anti-Inflammatory Activity of Extract Mixture of *Annona senegalensis* Pers. and *Piliostigma thonningii* (Schum.)

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Abstract

Introduction: *Annona senegalensis* Pers (Annonaceae) and *Piliostigma thonningii* (Schum.) (Leguminosae) are two medicinal plants used, often in combination, in traditional Togolese medicine for the treatment of diseases with an inflammatory component.

Objective: The aim of this study is to evaluate the anti-inflammatory effect of the hydro-ethanolic extract (EHEM) of the combination of *A. senegalensis* and *P. thonningii* (1: 1, m: m).

Methods: The ovalbumin-induced allergic airway asthma model was used. Animals made asthmatic were treated with EHEM at doses of 250 and 500 mg / kg. Inflammation markers including histamine, nitric oxide (NO), vascular leakage, leukocyte infiltration in the airways, and malondialdehyde (MDA), were measured.

Results: Compared to the SNT group, EHEM inhibits the infiltration of the airways by leukocytes ($850,00 \times 10^3 \pm 50$ cells / mL vs $1830 \times 10^3 \pm 53,85$ cells / mL for ST500 mg / kg, $P <0,05$). It inhibited vascular permeability to Evans Blue ($10,40 \pm 0,270$ µg / mL vs ST500, $p <0,05$). It inhibited histamine release ($13,95 \pm 0,937$ µg / mL vs $32,78 \pm 1,044$ µg / mL, $p <0,05$) and NO production ($0,211 \pm 0,008$ Mm vs $0,315 \pm 0,022$ µM, $p <0,05$). It finally inhibited MDA production ($14,66 \pm 0,533$ nM / mL vs $9,014 \pm 0,366$ nM / mL and $7,149 \pm 0,300$ nM / mL, $p <0,05$) in lung tissue.

Conclusion: Our results suggest that EHEM inhibits OVA-induced inflammation. These results justify the use of this combination of plants in traditional Togolese medicine.

Keywords: Inflammation, malondialdehyde, *Annona senegalensis*, *Piliostigma thonningii*.

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INTRODUCTION

Chronic inflammation is one of the causes of a number of non-infectious diseases worldwide including asthma ¹⁻². Asthma, a chronic disorder of the airways, affects individuals of all ages in countries around the world. It is a health problem that can be serious and sometimes fatal. It is characterized by permanent inflammatory reactions of the airways and especially by bronchoconstriction that can worsen over time. The prevalence of asthma is increasing everywhere, especially in urban areas with high pollution, and particularly affects children. Asthma is a significant burden, not only in terms of health costs but also in terms of lost productivity and reduced participation in family life ³. The treatment approach for inflammatory diseases is to inhibit the synthesis or action of mediators that stimulate inflammation with drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) and anticytokine therapies,

such as antitumor necrosis factor (TNF- α) ⁴. Conventional medications recommended for the treatment of asthma often do not permanently treat the disease but relieve the attacks, leading to long-term treatments with significant side effects. These chronic treatments are expensive and could weaken the economic situation of patients who, in tropical countries and particularly in Africa, are very often turned to traditional medicine based on the use of plants ⁵⁻⁶. Thus, the search for new natural molecules that are gentler, more available and above all have multiple actions, represents an undeniable hope in the resolution of these problems. Thus, in recent years, much research has been directed towards the valorization of traditional medicine, based on the use of medicinal plants, in order to verify the safety and efficacy of the plants used and to establish scientific rules for their use.

In Togo, *Annona senegalensis* Pers (Annonaceae) and *Piliostigma thonningii* (Schum.) (Leguminosae) are used in

traditional medicine for the treatment of diseases with an inflammatory component. *A. senegalensis* has been widely studied, on experimental *in vivo* and *in vitro* models of inflammatory and painful processes with total extracts, as well as phytochemical tests ⁷⁻⁸. It has been reported that leaves of *A. Senegalensis* have anticonvulsant, depressant, and central anxiolytic properties attributable to flavonoids ⁹. An anticonvulsant effect of kaurenoic acid isolated from root bark *A. Senegalensis* ¹⁰. A recent study had shown toxicity of the N-hexane and chloroform fractions of leaf extract of *A. Senegalensis* on the immature stage of mosquito species such as *Anopheles gambiae* and *Culex quinquefasciatus* ¹¹.

Previous studies showed that *P. thonningii*, root bark fractions exhibited significant anti-inflammatory and analgesic activity against phenylquinone-induced contortions ¹². According to Rist ¹³, *P. thonningii* root bark showed anti helminthic activity in veterinary medicine. According to Kerharo ¹⁴, aqueous stem bark extract of *P. thonningii* showed antibacterial activity against the bacterial strain *Sarcina lutea*. While defibrated roots are used as a purgative, deworming agent and to alleviate dysentery, malaria, tuberculosis ¹⁵.

In this study, we were interested in studying the anti-inflammatory activity of the combination of these two plants.

MATERIALS AND METHODS

Plant material and extraction

Plant material is constituted of root bark *A. senegalensis* and stem bark *P. thonningii*. Both *A. senegalensis* and stem bark for *P. thonningii* were collected from Danyivi, southwest Togo in September 2017 in in. Botanical identification has been performed at the herbarium of the Botany Department of the Faculty of Sciences, University of Lomé where a voucher specimen of each plant has been deposited under the numbers TOGO15567 and TOGO15568 for *A. senegalensi* and *P. thonningii* respectively.

Fresh samples were washed with tap water and dried under air conditioning in the sunshine in the Laboratory of Physiology and Pharmacology of Natural Substances of the Université de Lomé. The dried samples were crushed using an electric mill. We made a combination of the two powders obtained (1/1; m/m) and macerated in an ethanol and water solvent (80/20; v/v) in the proportions of 10 g per 100 mL of solvent. The mixture is stirred occasionally at room temperature for 72 hours. The extract is filtered and dry evaporated at 40°C using a rotary evaporator (BÜCHI, Switzerland). The resulting hydroethanol extract of the mixture (EHEM) was stored in a refrigerator.

Experimental animals

Wistar strain rats of both sexes weighing between 140 and 160 g were used. Animals were reared in the animal house of the Laboratory of Physiology and Pharmacology of Natural Substances of the University of Lomé, at room temperature with a light/dark cycle of 12 hours with free access to food and water.

Sensitization of rats to ovalbumin

Rats were divided into four groups (n = 5): unsensitized or normal control group (NS), untreated sensitized group or control (SNT), and two groups treated with EHEM 250 and 500 mg/kg respectively (ST250 and ST500). Sensitization was performed by intraperitoneal injection of 10 mg/kg OVA (grade V; Sigma, St.Louis, MO, USA) mixed with 40 mg/kg aluminum hydroxide as adjuvant in normal saline (0.9%) on days 0, 3, 7 and 21. Unsensitized animals were injected with

aluminum hydroxide (40 mg / kg) only. On days 24 to 27, SNT and sensitized groups treated with EHEM, under mild ether anaesthesia were given intranasal instillations of 50 µl of OVA (20%) in normal saline. The NS group received normal saline as well.

Rats in the EHEM-treated groups received orally 10 ml/kg of 250 and 500 mg/kg 30 minutes prior to OVA challenge, while the others were administered with normal saline. Rats were sacrificed on day 28. Bronchoalveolar lavage was performed by cannulating the trachea with a PE-240 polyethylene catheter (DI: 1.67 mm, OD: 2.41 mm) and infusing the lung with 5 ml of 0.9% sterile saline. Bronchoalveolar fluid (BAL) was obtained by two tracheal cannulation aspirations and the BAL recovery rate was approximately 78-84% ¹⁶.

Leukocyte cell count in BAL

The activity of EHEM on the infiltration of cells in the airways was evaluated, following the induction of allergy by OVA according to the method described by ¹⁶⁻¹⁷. Leukocyte cell count in the BAL was carried out using the Malassez cell mounted on an photomicroscope (OPTICA, Italy) connected to a microcomputer equipped with software (Optika Micro Image Analysis Software). This technique allows to count directly the leukocyte cells on the computer screen. results were expressed as the number of cells per mL of BAL volume recovered.

Measurement of vascular permeability

Plasma exudation is demonstrated by intravenous injection of Evans blue, which binds to plasma proteins. The effect of the ethanolic extract of the root of EHEM on OVA-induced vascular permeability in rats was evaluated according to the method described by Lilly et al and Agbonon et al ¹⁶⁻¹⁷. Four groups of rats were constituted as above. These rats were also sensitized and instilled with ovalbumin as described above. Twenty-four hours after the last intranasal instillation, animals were anaesthetized with 20% urethane (Sigma Chemical, St. Louis, MO, USA) at a dose of 1 g/kg. Evans blue (30 mg/kg dissolved in 0.9% NaCl solution) was administered to the rats via the tail vein. Five minutes later, the rats were sacrificed by urethane overdose. Bronchoalveolar fluid was collected as described above. The concentration of Evans blue was determined in the BAL by spectrophotometry using Genesys (Genesys, France) at a wavelength of 620 nm against the blank composed of a 0.9% NaCl solution. The concentration of Evans blue in the BAL was determined from the linear regression equation of the calibration curve generated from Evans blue dissolved in 0.9% NaCl (0 - 10 µg/mL).

The concentration of Evans blue expresses vascular permeability. The percentage inhibition of vascular permeability is calculated according to the following formula:

$$\% \text{ inhibition} = [((\text{SNTC}) - (\text{STC})) / (\text{SNTC})] \times 100.$$

- SNTC: Concentration of Evans Blue in the BAL of untreated sensitized rats.

- STC: concentration of Evans blue in the BAL of sensitized and treated rats

Determination of histamine in the BAL

Rats were sensitized as before, but instillation with ovalbumin was performed from day 24 to day 28. The BAL was taken 30 minutes after the last instillation. Histamine determination assay was performed using the colorimetric method described by Yang et al. ¹⁸. In short, to 0.5 mL of the

BAL or standard histamine dihydrochloride were simultaneously added a 0.1 mL of 1% sulfanilic acid and 0.1 mL of a 5% sodium nitrite. The mixture was incubated for 10 minutes and afterwards 1.3 mL of the 5% sodium carbonate solution was added. Two (02) minutes later, 1 mL of 75° ethanol was added to the mixture. The absorbance was read at 530 nm in the first 20 minutes using the UV-visible spectrophotometer. The concentration of histamine was determined from the calibration curve generated from regression equation established with histamine dihydrochloride (Sigma Chemical, St. Louis, MO, USA) dissolved in 0.9% NaCl (0 - 50 µg/mL).

Determination of lipid peroxidation

The concentration of malondialdehyde (MDA) was determined as an indicator of lipid peroxidation. Whole lung samples were dissected 24 hours after the last OVA instillation and washed immediately with ice saline to remove as much blood as possible. They were weighed and 2 g of tissue was homogenized in 5 ml of a cold KCl solution (1.5%). The homogenate was centrifuged at 3000 rpm for 10 min. Subsequently, the MDA content in the supernatants was measured according to the method described by Agbonon and Gbeassor ¹⁹⁻²⁰. Briefly, 200 µl of supernatant was exposed to 0.6 ml of phosphoric acid (1%) and 1 ml of thiobarbituric acid (1%) and the mixture was heated at 100°C for 50 min. At the end of the incubation period, the mixture was cooled in ice for 10 min and 2 ml of 1-butanol was added and the mixture was centrifuged as described above. After centrifugation, the supernatant was removed and the absorbance was read at 535 nm using a UV-visible recording spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, France). 1,1,3,3-tetramethoxypropane (MDA) was used as standard to obtain the standard curve (0 - 60 nM; $y = 0.03860x + 0.04205$; $r^2 = 0.998$).

Measurement of NO

Pulmonary NO production was determined spectrophotometrically by assaying BAL liquid for nitrite using Griess reagent (1% sulfanilic acid, 0.1% N-1 naphthylenediamine dihydrochloride, 2.5% phosphoric acid). In each test tube were introduced in the order 100 µL of BAL and 100 µL of Griess reagent. After homogenization, the absorbance of each tube was read at 570 nm after 10 minutes incubation against the blank. The concentration of the NO solution was measured spectrophotometrically against the blank. The nitrite concentration was determined using sodium nitrite as standard ²¹.

Statistical analyses

Results are expressed as mean ± standard error of the mean (mean ± SEM). Comparisons between the means of different groups were performed using one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test. Results were considered significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 5.00 (GraphPad Software Inc., CA, USA).

RESULTS

Effect of EHEM on the infiltration of leukocytes in the respiratory tract

Exposure of rats to OVA caused a significant increase in the number of leukocytes in the BAL of the sensitized control group (NST) compared to the unsensitized control group (NS) ($3190 \times 10^3 \pm 100.50$ cells / mL vs. $850.00 \times 10^3 \pm 50$ cells / mL, $P < 0.001$). In contrast, treatment of rats in the (ST250 and ST500) groups with EHEM prior to OVA exposure resulted in a significant decrease ($P < 0.001$) in the number

of leukocyte cells compared to the sensitized control (SNT) group ($850.00 \times 10^3 \pm 50$ cells / mL for the control group vs. $2170 \times 10^3 \pm 134.70$ cells / mL and $1830 \times 10^3 \pm 53.85$ cells / mL for ST250 and ST500 mg/kg extract respectively, $P < 0.001$) (Figure 1).

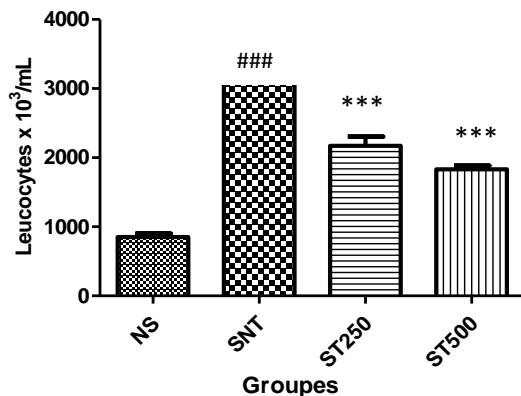


Figure 1: Total number of leukocytes in the BAL

Values are expressed as mean ± ESM (n = 5); NS = unsensitized group; SNT = group sensitized and instilled with OVA; ST250, ST500: groups sensitized and treated with EHEM at 250 and 500 mg/kg; # # $P < 0.001$ compared to NS; *** $P < 0.001$: significant differences from SNT (one-factor ANOVA followed by Tukey's multiple comparison test).

Effect of EHEM on vascular permeability

Intranasal instillation with OVA in OVA-sensitized control rats (SNT) increased vascular permeability compared to non-sensitized rats (NS). Values for the concentration of Evans Blue in BAL are 7.077 ± 0.238 µg/mL in the unsensitized control rats and 10.40 ± 0.270 µg/mL in the sensitized control rats. Treatment of ST250 and ST500 groups prior to each instillation resulted in significant ($P < 0.001$) inhibition of vascular permeability in the airway compared to the untreated sensitized group. The concentrations of Evans Blue in the BAL are 3.506 ± 0.175 µg/mL and 1.683 ± 0.272 µg/mL for ST250 and ST500 respectively (Figure 2).

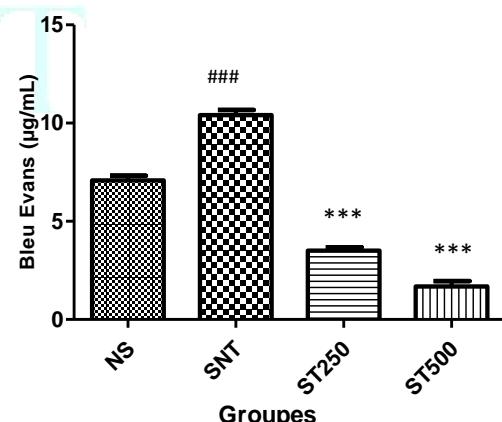


Figure 2: Effect of EHEM on Vascular Permeability

Values are expressed as mean ± ESM (n = 5); NS: non-sensitized group; SNT: sensitized group treated with 0.9% NaCl; ST250, ST500: groups sensitized and treated with EHEM at 250 and 500 mg/kg; # # $P < 0.001$: significant difference from NS; *** $P < 0.001$: significant difference from SNT (single-factor ANOVA followed by Tukey's multiple comparison test).

Effect of EHEM on histamine concentration in the BAL

The histamine assay showed that exposure of OVA-sensitized rats to OVA caused a significant ($P < 0.001$) increase in the concentration of histamine in the BAL

compared to the non-sensitized control group ($32.78 \pm 1.044 \mu\text{g/mL}$ vs. $19.11 \pm 1.220 \mu\text{g/mL}$). On the other hand, we find a significant reduction ($P < 0.001$) in histamine concentration only in the ST500 group's BAL compared to the SNT group ($13.95 \pm 0.937 \mu\text{g/mL}$ vs. $32.78 \pm 1.044 \mu\text{g/mL}$) (Figure 3).

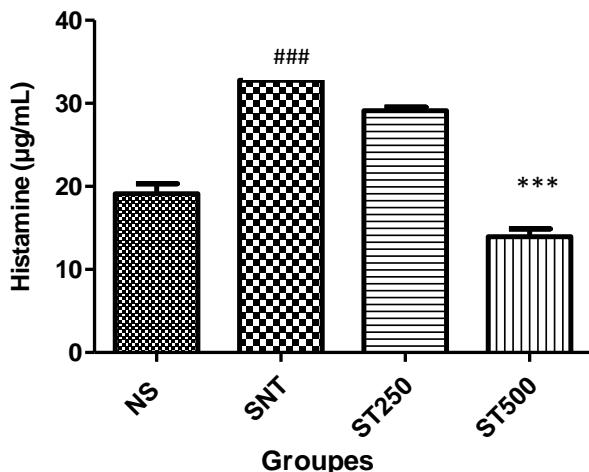


Figure 3: Effect of EHEM on Histamine Concentration

Values are expressed as mean \pm ESM ($n = 5$); NS: non-sensitized group; SNT: sensitized group treated with 0.9% NaCl; ST250, ST500: sensitized groups treated with EHEM at 250 and 500 mg/kg; # # $P < 0.001$: significant difference from NS; *** $P < 0.001$: significant difference from SNT (single-factor ANOVA followed by Tukey's multiple comparison test).

Effect of EHEM on Lipid Peroxidation in Lung Tissue

Estimation of lipid peroxidation by estimating MDA levels showed a significant increase ($P < 0.001$) in the concentration of MDA in the lung tissue of untreated sensitized rats (SNT) compared to unsensitized rats (NS) i.e. ($14.66 \pm 0.533 \text{ nM}$ vs. $9.273 \pm 0.337 \text{ nM}$). Treatment of ST250 and ST500 rats with EHEM prior to each instillation significantly reduced MDA formation in these lots compared to the SNT group ($9.014 \pm 0.366 \text{ nM}$ and $7.149 \pm 0.300 \text{ nM}$ vs. $14.66 \pm 0.533 \text{ nM}$) (Figure 4).

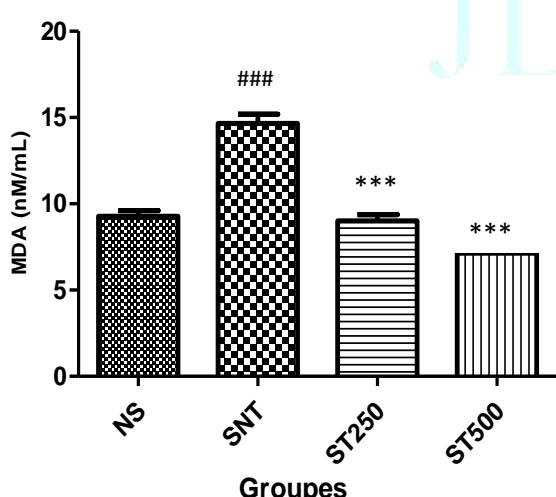


Figure 4: Effect of EHEM on Lipid Peroxidation in Lung Tissues

Values are expressed as mean \pm ESM, $n = 5$; NS: non-sensitized group; SNT: sensitized group treated with 0.9% NaCl; ST250, ST500: groups sensitized and treated with EHEM at 250 and 500 mg/kg; # # $P < 0.001$: significant difference from NS; *** $P < 0.001$: significant difference from SNT (one-factor ANOVA followed by Tukey's multiple comparison test).

Effect of EHEM on the production of nitric oxide (NO)

The NO concentration in BAL is $0.181 \pm 0.014 \mu\text{M}$ in NS rats. This value increased significantly ($p < 0.001$) in OVA-sensitized and OVA-induced (SNT) rats ($0.315 \pm 0.022 \mu\text{M}$). In rats sensitized and treated with EHEM, there was a significant decrease ($p < 0.01$) in NO concentration only in the ST500 lot compared to untreated sensitized rats (SNT), i.e. ($0.211 \pm 0.008 \mu\text{M}$ vs $0.315 \pm 0.022 \mu\text{M}$) (Figure 5).

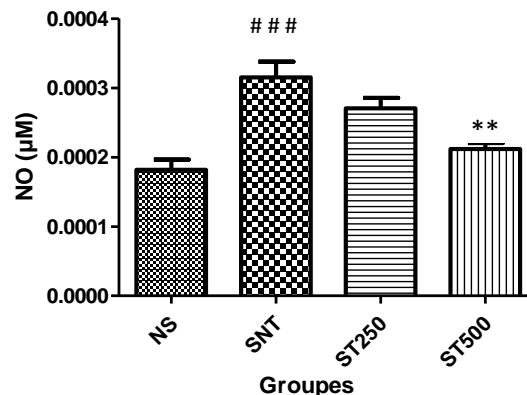


Figure 5: Effect of EHEM on NO

SNT: group sensitized, OVA-induced and treated with 0.9% NaCl; ST250, ST500: groups sensitized, OVA-induced and treated with EHEM at 250 and 500 mg/kg; # # # $P < 0.001$: significant difference from NS; ** $P < 0.01$: significant difference from SNT (one-factor ANOVA followed by Tukey's multiple comparison test).

DISCUSSION

The objective of this study was to assess the effect of EHEM from the combination of *A. senegalensis* and *P. thonningii*, on some parameters of chronic inflammation, with a view to scientific validation of its traditional use. EHEM inhibited dose dependent leukocyte infiltration into the respiratory tract, inhibited the permeability of Evans Blue to vascularized vessels, significantly reduced histamine production in rats treated with this extract. EHEM also showed a significant dose-dependent reduction in MDA and NO levels in rats treated with this extract.

We used an animal model of allergic asthma in which OVA is used as an allergen. Ovalbumin-induced asthma results from chronic airway inflammation associated with infiltration of leukocytes such as macrophages, lymphocytes, mastocytes, neutrophils and eosinophils into the lumen of the bronchial tract ²²⁻²³. Depletion of leukocytes in the airways in ovalbumin-sensitized rats treated with EHEM inhibits the asthmatic response ²⁴. Results from, this present study suggest that EHEM inhibited leukocytes passage across rats' airways wall. These leukocytes, once they arrive at the inflammation area, release pro-inflammatory substances that lead to vasodilation, increased vascular permeability and vascular leakage ²⁵⁻²⁶.

Our results revealed an increased total leukocyte cells count in OVA-sensitized rats, characterizing asthmatic inflammation. Moreover, the number of total leukocyte cells count was much higher in the OVA-sensitized rats as compared to non-sensitized group due to the increase in other cell types such as lymphocytes and basophils. These cells amplify the immune response and thus participate in the development of inflammation with destruction of the bronchial epithelium leading to remodeling of lung tissue ²⁷.

However, administration of EHEM in OVA-sensitized rats significantly reduced pulmonary infiltration by these inflammatory cells. This suggests that treatment with EHEM

might reduce the inflammatory state by bringing white blood cell count back to normal and by decreasing the recruitment of white blood cells in the lungs.

Our results also showed that EHEM inhibits the permeability of Evans blue to vascular tissue. Indeed, during the inflammatory process, vascular permeability is exacerbated. This permeability leads to plasma exudation, which contributes to enriching the extravascular environment with factors designed to maintain and amplify the inflammatory response²⁸. This study shows that EHEM inhibited vascular permeability, indicating that it can modulate the amplitude of the inflammatory response.

Histamine is a pro-inflammatory substance released by leukocyte cells. It is an amine released during mast cell degranulation. The significant decrease ($P < 0.001$) of its concentration in the bronchoalveolar fluid of instilled and treated rats shows that the extract inhibited the release of this mediator or prevented the passage of mast cells into the respiratory tract. Since histamine is a vasodilator, it therefore increases vascular permeability. Results of histamine measurement in BAL suggest the physiological mechanism involved in anti-inflammatory effect of EHEM on OVA-induced chronic in rats. This would likely be due to secondary metabolites, mainly tannins, on cAMP and intracellular calcium-mediated histamine release²⁹. Or *A. senegalensis* and *P. thonningii* contain tannins³⁰⁻³¹.

In individuals with inflammatory diseases, a permanent state of oxidative stress has been demonstrated and is characterized by an imbalance between the biochemical processes involved in the production of reactive oxygen species (ROS) and those responsible for their control and elimination³²⁻³³. During the inflammatory process, neutrophils and macrophages recruited at the inflammatory site release oxygenated and nitrated free radicals indicating the presence of oxidative stress³⁴.

This imbalance between the defense and free radical production systems leads to biochemical damage at the level of the body's cells, including damage to the integrity of the cell membrane through the induction of lipid peroxidation³⁵⁻³⁶. Thus, analysis of free radicals in biological membranes was performed using malondialdehyde (MDA), one of the degradation products of cyclic endoperoxides generated during lipid oxidation, determination assay. Malondialdehyde is an early indicator of foreign aggression and is often used as a biomarker of oxidative stress³⁷⁻³⁸.

In this present study, MDA level increased in untreated but OVA-sensitized group, indicating the occurrence of damage to the cell membranes. These results are consistent with those reported by Agbonon et al.¹⁶ and Missembukpo et al.³⁹. Pretreatment of rats with EHEM significantly prevented the increase in MDA content. Our results, suggest that the extract inhibits lipid peroxidation and preserves the integrity of the lungs of the asthmatic rats. Furthermore, these results confirm the effect of the extract on vascular permeability.

It has been reported that the occurrence of oxidative stress is due to the overproduction of nitric oxide (NO). Higher intracellular contents of NO react with other free radicals including the superoxide anion (O_2^-) to form the peroxynitrite anion ($ONOO^-$), a powerful oxidant⁴⁰. Activation of leukocyte cells in inflammatory process induce overproduction of NO under the action of inducible nitric oxide synthase (iNOS)⁴⁰. iNOS-induced overproduction of NO is mostly associated with highly detrimental pro-inflammatory effects⁴¹⁻⁴². In airway inflammation ailments, NO is produced by a variety of cells in the respiratory tract,

including not only inflammatory cells but also epithelial cells⁴³. Nitric oxide therefore plays a crucial role in the pathogenesis of airway inflammation in allergic asthma⁴³. In this study, EHEM inhibited NO production in treated rats. This suggests that the extract inhibited overproduction of NO and MDA and therefore inhibited oxidative stress while avoiding its adverse effects.

CONCLUSION

Ultimately, this study showed that EHEM inhibits: airway infiltration by leukocytes, vascular permeability, histamine release and NO production in bronchoalveolar fluid (BAL) and then MDA production in lung tissue. This suggests that EHEM would inhibit OVA-induced inflammation. What are then the molecules resulting from this combination and which are responsible for this anti-inflammatory activity? Our future perspectives will attempt to answer this question.

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