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Original Article

In vivo determination of the anti-inflammatory and antioxidant effects of the aqueous extract of *Syzygium aromaticum* (clove) in an asthmatic rat model

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Abstract

Asthma is a chronic inflammatory disease of the airways strongly associated with interleukin-4 (IL-4), a cytokine that mediates and regulates various immune responses, including allergic reactions. This study aimed to evaluate the anti-inflammatory and antioxidant effects of an Aqueous Extract of Clove (AEC) Syzygium aromaticum on the lungs and erythrocytes of an experimental asthma model in Wistar rats. For this purpose, four groups of male rats were examined: control, sensitized with ovalbumin (OVA), treated with AEC, and treated with a combination of OVA/AEC. After treatment, the antioxidant effect was determined by measuring the malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione (GSH), and catalase (CAT) levels. The anti-inflammatory effect was determined by measuring IL-4 levels by performing enzyme-linked immunosorbent assay (ELISA) using serum, lung, and bronchoalveolar lavage fluid (BALF) samples. A significant reduction ($p \leq 0.05$) in the MDA levels and a significant increase ($p \leq 0.05$) in the levels of GPx and CAT were observed in the lungs of rats treated with cloves. However, no statistically significant variation was observed in GSH levels. In erythrocytes, no statistically significant differences were observed between the experimental batches. Regarding the anti-inflammatory effect, the administration of S. aromaticum extract to sensitized rats resulted in a recovery in the levels of total proteins and IL-4 and a decrease in the three compartments studied (lungs, serum, and bronchoalveolar liquid). These results were confirmed by microscopic examination of lung histological sections. Overall, these findings confirmed that the AEC has anti-inflammatory and antioxidant effects.

Keywords: Anti-inflammatory, Antioxidant, Catalase (CAT), Glutathione (GSH), Glutathione peroxidase (GPx), Interleukin-4 (IL-4), Malondialdehyde (MDA), *Syzygium aromaticum.*

1. Introduction

Asthma is a chronic inflammatory disease of the airways characterized by bronchial hyper-reactivity caused by environmental and genetic factors [1] . Air passages in the lungs become narrow due to inflammation and tightening of the muscles around the small airways. The development of inflammation following the action of mediators can lead to an imbalance between the levels of reactive oxygen species (ROS) and antioxidants, resulting in oxidative stress. On the other hand, increased expression and secretion of ROS due to the activation of inflammatory cells may, in turn, lead to the further generation of inflammatory mediators that damage cells. Owing to the chronic nature of asthma and its increasing prevalence, as well as the reputed ineffectiveness of the drugs currently used to treat asthma and the fear of their known side effects, there is a pressing need to find new therapies [2] . Various spices and herbal remedies have been used in traditional medicine and healthcare for the effective prevention of asthma [3]. These natural remedies are a rich source of bioactive compounds and have significant biomedical properties, including antioxidant, antibacterial, anti-inflammatory, antidiabetic, and anticancer properties, which help fight various diseases in humans [3]. Many medicinal plants exhibit interesting biological and pharmacological activities and are used as therapeutic agents. Clove is an example of such a plant, whose dried flower buds are used for therapeutic purposes. This plant, also known as *Syzygium aromaticum,* is a member of the Myrtaceae family that grows in tropical areas [4]. Clove stands out among other spices owing to its powerful therapeutic properties that are useful for pharmaceutical applications, namely, anti-inflammatory and antioxidant properties [5]. Owing to its specific properties, clove is the most commonly used spice in traditional medicine and is closely associated with the reinforcement of the immune system as it increases resistance to diseases [4]. Cloves also play an important role in inhibiting various degenerative diseases. This role is

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attributed to the presence of various chemical constituents with antioxidant activities in high concentrations [5].

In the present study, we analyzed the preventive and ameliorative effects of an aqueous extract of the plant *S. aromaticum* in a rat model of allergic asthma. The effects on the lungs and serum were studied to examine the therapeutic effects (namely, antioxidant and anti-asthmatic) of *S. aromaticum*.

2. Materials and methods

2.1. Plant materials

The flower buds of cloves (*S. aromaticum*; pink in color), previously dried in the sun to a dark color (brown color), were imported from Indonesia. These cloves were purchased from an herbal shop located in the city of Annaba and powdered using an electric grinder.

2.2. Preparation of the Aqueous Extract of Clove (AEC)

The plant was initially placed in water to obtain the AEC following the method described by Rahim & Khan [6], with some modifications. The plant powder (8 g) was suspended in 50 mL of distilled water and mixed using a stirrer with a magnetic bar overnight. The next day, the homogenate was filtered through Whatman No.1 paper and stored at 4 °C for later use.

2.3. Animals

We procured 28 Wistar albino male rats from the Pasteur Institute in Algeria for the experiments. The rats were aged between 6 and 8 weeks and weighed between 150 and 180 g. All protocols used in this study were performed in accordance with the guidelines of the Committee on Use of Laboratory Animals and were approved by the Ethical Committee of the Directorate General for Scientific Research and Technological Development of the Algerian Ministry of Higher Education and Scientific Research (under number's project PRFU:D01N01UN230120200008). The animals were acclimated for 2 weeks under similar laboratory conditions, including similar photoperiod, an average relative humidity of 60 %, and a room temperature of 25 ± 2 °C. Food (standard food, supplied by National Livestock Feed Office of Bejaia, Algeria) and water were available *ad libitum*. The rats in all batches were weighed daily to evaluate variations in body weight during the experimental period.

2.4. Experimental group design

The rats were distributed into four groups of seven (n = 7) males that received the following treatments: the first group (no. 1) served as a control (T), the second group (no. 2) was sensitized to ovalbumin (OVA), the third group (no. 3; AEC) was treated with aqueous clove extract (15 mg/kg body weight/day by gavage) [7] , and the fourth group (no. 4; OVA + AEC) was sensitized to OVA and treated with AEC (15 mg/kg body weight).

2.5. Sensitization and aerosol exposure

On day 1, the rat groups (no. 2 and 4) were sensitized by administering intraperitoneal injections of 200 µg/mL OVA (grade II; cat. no. A5253-250G; Sigma-Aldrich) that was adsorbed to 10 mg/mL aluminum hydroxide ($Al[OH]_3$) dissolved in 1 mL phosphate-buffered saline (PBS), and the sensitization was boosted on days 2, 3, and 11 [8]. On days 20, 21, and 22, after the initial sensitization, the rat airways were exposed to OVA (2 % w/v, in 0.9 % saline solution) for 20 min using a nebulizer with an unheated compressor (Rossmax) [9]. The last aerosol exposure was performed 24 h before the end of the experiment. The control animal groups (no. 1 and 3) were sensitized and exposed to similar volumes of saline solution (0.9 %) at the same times and under the same conditions (Figure 1).

2.6. Preparation of erythrocytes and lung homogenates

The rats were sacrificed 24 h after the final aerosol challenge. The lungs were quickly removed, washed in 0.9 % NaCl solution, weighed, and then 1 g of the sample was homogenized in 2 mL of TBS (Tris, 50 mM; NaCl, 150 mM; pH 7.4) under ice-cold conditions. The homogenates were centrifuged at 3,500 × g for 35 min at 4 ºC. The hemolysates and tissue supernatants were then aliquoted and stored at -20 ºC for later use [10]. After centrifugation, the blood sediment contained erythrocytes, which were suspended twice in PBS (pH 7.4) and centrifuged at 3,000 × g for 15 min at 4 ºC for the first washing and at 4,000 × g for 30 min at 4 ºC for the second washing. The blood samples were immediately collected in ice-cold polypropylene tubes (tubes with EDTA anticoagulant and dry tubes). The dry tubes were centrifuged first at 3,000 × g for 30 min at 4 ºC. At the end, these tubes contained blood sediments and erythrocytes were present in this sediment after centrifugation. These erythrocytes were suspended twice in PBS (pH 7.4) and centrifuged at 3,000 × g for 15 min at 4 ºC for the first washing and at 4,000 × g for 30 min at 4 ºC for the second washing. The collected serum was used for the determination of total proteins, and the sediment (containing erythrocytes) was stored at -20 °C until it could be used for the determination of oxidative stress parameters. The second tube containing an anticoagulant (EDTA), was used to determine the number of white blood cells in the samples [11].

2.7. Bronchoalveolar lavage

The tracheae were cannulated, and the airway lumina was washed with 2 mL of PBS. This procedure was repeated three times before the lavage fluid was pooled in Eppendorf tubes and centrifuged at 4,000 × g for 10 min.

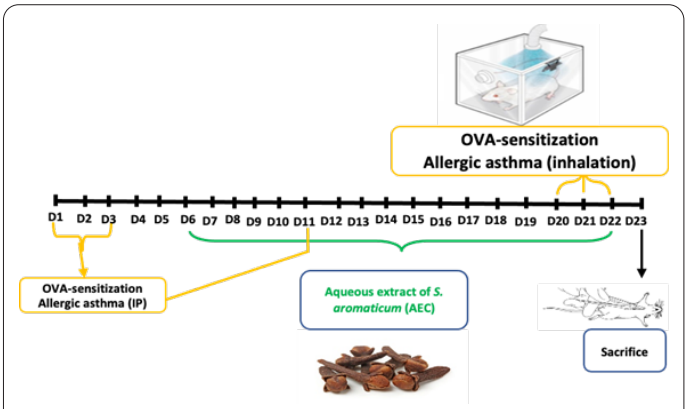

Fig. 1. Representation of the overall experimental protocol showing peritoneal injection and inhalation awareness days (in yellow) and days of force-feeding with the plant extract for 17 days (in green; aqueous plant extract, AEC). Three ovalbumin (OVA) injections (IP) were administered on days 1, 2, 3, and 11. AEC was administered from days 6 to 22 after the third injection. On days 20, 21, and 22, the animals were exposed to aerosolized OVA or saline. After 24 h, the rats were sacrificed.

The Eppendorf tubes containing bronchoalveolar lavage fluid (BALF) samples were stored at -20 °C prior to use for interleukin-4 (IL-4) and total protein measurements [12,13].

2.8. Protein assays

Protein concentrations in the supernatants of the lung, erythrocyte, and BALF samples were determined using the Bradford colorimetric method. Absorbance was measured spectrophotometrically at 595 nm using bovine serum albumin as a standard [12].

2.9. Estimation of lipid peroxidation levels (malondialdehyde)

This assay is based on the condensation of malondialdehyde (MDA) with hot thiobarbituric acid (TBA) in an acidic medium, according to the method described by Esterbauer *et al*. [14]. This reaction results in the formation of a colored product (pink pigment), which can be measured (quantified) by absorption spectrophotometry at 530 nm. The supernatant (375 mL) was homogenized with 150 mL of TBS and 375 µL of trichloroacetic acid–butylhydroxytoluene (TCA-BHT) to precipitate the proteins and then centrifuged (1,000 × g, 10 min, and 4 °C). Subsequently, 400 mL of the obtained supernatant was mixed with 80 µL of HCl (0.6 M) and 320 mL of TBA dissolved in Tris. The mixture was then incubated at 80 °C for 10 min. The absorbance of the resulting supernatant was measured at 530 nm. Next, the amount of thiobarbituric acid reactive substance (TBARS) was calculated using an extinction coefficient of 1.56 Å × 105 M/cm and expressed as nmol MDA/mg of protein. This assay was performed using lung tissues and erythrocytes [15].

2.10. Reduced glutathione levels

The reduction in glutathione (GSH) content in the lung and erythrocyte homogenates was determined using a colorimetric technique described by Ellman [16] and modified by Jollow and others [17] , which is based on measurement of the optical absorbance at 412 nm, as it indicates the reduction of DTNB (5,5′-dithiobis-[2-nitrobenzoic acid]) by the -SH groups of glutathione. Total GSH content was expressed as nmol GSH/mg protein [18].

2.11. Estimation of antioxidant enzymes (glutathione peroxidase and catalase) activities

The glutathione peroxidase (GPx; E.C.1.11.1.9) activity in the lung and erythrocyte samples was measured using the method described by Flohé and Günzler [19], which is based on the reduction of hydrogen peroxide (H_2O_2) in the presence of reduced GSH. The absorbance of each sample was recorded at 420 nm. Specific GPx activity was expressed as mmol GSH/mg protein [18].

The catalase (CAT; E.C.1.11.1.6) activity was measured using the Aebi method [20]. This assay is based on the ability of the enzyme to degrade hydrogen peroxide, followed by a decrease in absorbance at 240 nm that is measured using UV/visible light for 1 min. The CAT activity was expressed as µmol H_2O_2 consumed/min/mg protein [18].

2.12. Determination of protein carbonyl groups

The protein carbonyl groups in the lungs were analyzed using the protocol described by Levine *et al*. [21]. This method is based on derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH). This led to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone. Absorbance was measured spectrophotometrically at 370 nm.

2.13. Interleukin-4 (IL-4) measurement

The IL-4 levels in lung, serum, and BALF samples were measured using the Invitrogen Rat IL-4 enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen RET: BMS 628; lot no.:176569000) following the manufacturer's protocol. The optical density was measured at 450 nm (Lecteur Mindray MR-96A, Socimed Sarl, France). The concentration of IL-4 was determined by interpolation from a standard curve, with all data expressed in pg/mL.

2.14. Histopathological examination

The lung samples from each group were fixed in 10 % formaldehyde. They were dehydrated using a series of increasing alcohol concentrations (70–100 %; Leica TP1020 tissue processor, Leica Biosystems, United States) and embedded in paraffin. The lung tissues were cut into 5-µm-thick slices using a microtome, deparaffinized, stained with hematoxylin and eosin, and examined using a Leica DM1000 LED microscope.

2.15. Statistical analysis

The results are expressed in the form of mean plus or minus standard error (mean ± SEM) for seven rats in each group. Statistical evaluation was performed using Student's t-test (Excel 2010), and the statistical significance was set at $p < 0.05$.

3. Results

3.1. Variation in hematological parameters

Regarding white blood cell levels, the results revealed a significant increase ($p \leq 0.01$) in levels in the sensitized rats (OVA) and a highly significant increase ($p \leq 0.001$) in levels in the batch treated with the plant extract (AEC) compared to that in control rats (Table 1); the rates of in-

Table 1. Variation of hematological parameters such as white blood cells in the experimental groups.

Parameters	Control	OVA	AEC	OVA + AEC
WBC (× 10^9)/L	7,44 ± 0,80	8,98 ± 1,47(**)	8,43 ± 0,34(***)	8,20 ± 0,79
Lymphocytes (× 10^9)/L	3,78 ± 0,57	4,50 ± 0,36(**)	3,97 ± 0,49	4,03 ± 1,58
Monocytes (× 10^9)/L	0,48 ± 0,13	1,04 ± 0,11(***)	1,10 ± 0,08(***)	0,73 ± 0,12(*)(##)
Granulocytes (× 10^9)/L	2,66 ± 0,53	3,78 ± 1,53	3,37 ± 0,39	2,70 ± 0,56
RBC (× 10^{12})/L	7,24 ± 0,31	7,07 ± 0,30(*)	7,39 ± 0,60	6,95 ± 0,19

Values are given as means ± standard errors (SEMs) for groups of four animals each. Significant difference: results of aqueous extract (AEC), ovalbumin (OVA), and OVA + AEC groups compared to that of the control group (*$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$), and that of AEC and AEC + OVA groups compared to that of the OVA group (#$p \leq 0.05$; # # $p \leq 0.01$; ### $p \leq 0.001$). WBC= White blood cells. RBC = Red blood cells.

crease were 20.7 and 13.24 %, respectively. Moreover, a significant increase (19.21 %; $p \leq 0.01$) was observed in the levels of lymphocytes in the blood of rats sensitized to OVA compared to that in control rats. The results showed a reduction in the number of white blood cells and lymphocytes in the batch sensitized and treated with the AEC compared with that in the batch sensitized only with OVA. Regarding monocyte levels, we observed a highly significant increase ($p \leq 0.001$) in the batches treated with the plant extract (AEC) and sensitized (OVA), and a significant increase ($p \leq 0.05$) in the batch sensitized with OVA and treated with the plant extract (OVA/AEC) compared to that in the control batch, and a highly significant reduction ($p \leq 0.01$) in the batch sensitized with OVA and treated with the AEC compared to that the batch sensitized with OVA only. Regarding red blood cell levels, a significant decrease ($p \leq 0.05$) was observed in the batch sensitized with OVA compared to that in the control batch. No statistically significant variation in the level of granulocytes was observed among the batches.

3.2. Variation of total proteins in serum and bronchoalveolar lavage fluid

The total protein content in serum and BALF samples of the rats in different experimental groups was determined. Figure 2 shows the results obtained. Regarding total serum protein levels, an increase was observed in rats sensitized to OVA and in healthy rats treated with AEC compared with that in the control group, and a decrease was observed in rats sensitized to OVA and treated with AEC (OVA + AEC). Statistical analysis revealed no significant differences. Regarding total protein levels in BALF samples, a significant increase ($p \leq 0.05$) was observed in rats of the OVA batch compared to that in control rats; in rats from the healthy group treated with AEC, the levels were higher than that in the control group and lower than that in OVA-sensitized rats. Statistical analysis revealed no statistically significant differences.

3.3. Variation in interleukin-4 (IL-4) levels

Serum IL-4 levels were higher in the sensitized (OVA) and plant-treated (AEC) groups than those in the control group (Figure 3). However, administration of AEC led to an increase in IL-4 levels in the group sensitized to OVA. Statistical analysis revealed no statistically significant differences. The levels of IL-4 in the lungs showed a significant increase ($p \leq 0.05$) in the batches of rats treated with the plant (AEC) and those sensitized to OVA compared to that in the control batch, whereas a significant decrease ($p \leq 0.05$) in IL-4 levels was observed in rats sensitized to OVA and treated with AEC (OVA + AEC) compared with that in rats sensitized to OVA only. IL-4 levels in the BALF samples increased in the OVA and AEC groups

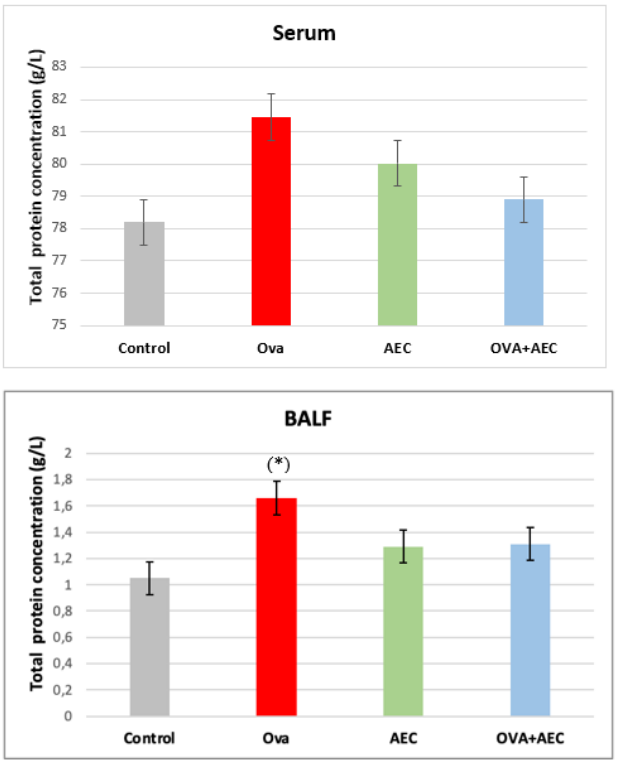

Fig. 2. Variation in total protein levels in serum and bronchoalveolar lavage fluid (BALF) in control and OVA- or AEC-treated rats. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ compared to the results for control rats; #$p \leq 0.05$, ##$p \leq 0.01$, and ###$p \leq 0.001$ compared to the results for ovalbumin-sensitized rats.

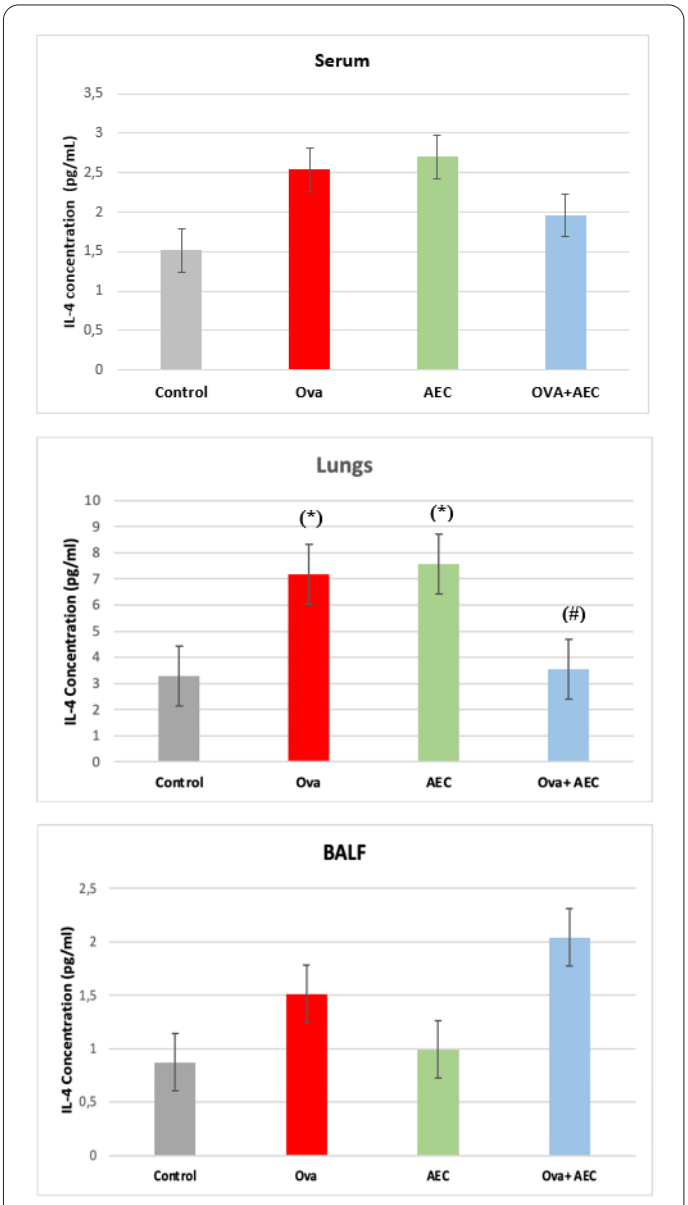

Fig. 3. Variation in interleukin-4 (IL-4) levels in serum, lung, and BALF samples from rats in the four batches. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ compared to the results for control rats; #$p \leq 0.05$, ##$p \leq 0.01$, and ###$p \leq 0.001$ compared to the results for ovalbumin-sensitized rats.

compared to that in the control group, and the levels also increased in the OVA + AEC group compared to that in the OVA group. Statistical analysis revealed no statistically significant differences.

3.4. Variation in oxidative stress parameters of lungs

The MDA level was determined as the first parameter. A significant increase ($p \leq 0.001$) in MDA levels in the lungs was observed in rats sensitized to OVA compared to that in control rats. On the other hand, a significant reduction ($p \leq 0.05$) in MDA levels was observed in rats treated with a combination of ovalbumin plus *S. aromaticum* extract (OVA + AEC) compared to that in control rats, indicating recovery of the OVA + AEC-treated rats (Table 2). Significant and highly significant variations in MDA levels were also observed in the batch treated with *S. aromaticum* extract (AEC) compared to those in the control and OVA-sensitized rats, respectively.

Treatment of rats with OVA led to a significant decrease ($p \leq 0.05$) in the cellular content of reduced glutathione (GSH) in the lungs compared to that in control rats; the GSH levels also decreased in rats treated with *S. aromaticum* extract (AEC) compared to that in control rats (Table 2). However, no statistically significant variation was observed in the GSH levels in the lungs of rats treated with the combination of ovalbumin and *S. aromaticum* extract (OVA + AEC) compared to that in rats treated with OVA alone (Table 2).

Regarding GPx activity, sensitization of rats to OVA caused a significant decrease ($p \leq 0.05$) in the GPx activity in the lungs of these rats. Recovery was characterized by a statistically significant increase in the GPx activity in the lungs after treatment with *S. aromaticum* extract (AEC) (Table 2).

Regarding the CAT activity, the results obtained show that the CAT activity significantly decreased ($p \leq 0.05$) in the lungs of rats treated with OVA compared to that in control rats. No statistically significant change in CAT activity was observed in the lungs of *S. aromaticum* extract (AEC)-treated rats compared to that in control rats. On the other hand, a significant increase ($p \leq 0.05$) in CAT activity was observed in the lungs of rats treated with the combination of ovalbumin and *S. aromaticum* extract (OVA + AEC) compared to that in the group treated with OVA alone, indicating recovery of CAT activity in OVA + AEC-treated rats.

In the lungs, protein carbonyl levels (PC) showed a significant increase ($p \leq 0.05$) in the OVA- and AEC-treated groups compared to those in the control group (Table 2). However, the administration of AEC significantly decreased ($p \leq 0.05$) the PC levels in the OVA + AEC group compared to that in the OVA group, suggesting an improvement in the OVA + AEC group. The levels of PC in the lungs showed a significant decrease ($p \leq 0.05$) in the AEC group compared to that in the OVA group.

3.5. Variation of oxidative stress parameters in erythrocytes

A highly significant increase ($p \leq 0.01$) in the MDA levels was observed in the OVA + AEC-treated rats compared with that in the rats sensitized to OVA. Moreover, the MDA levels in the OVA batch were higher than those in the control batch. The AEC-treated batch produced the highest dosage of MDA compared with that produced by the other batches.

The GSH levels significantly decreased in rats sensitized to OVA and healthy rats treated with AEC only compared with that observed in the control group. Unlike in the batches of rats sensitized to OVA and then treated with AEC, a considerable increase in GSH levels was observed in the OVA+AEC group compared to that in the control group (Table 3). Regarding GPx activity in erythrocytes, the level of GPx activity decreased in the OVA-sensitized batch compared to that in the control batch (Table 3). A significant increase in the enzymatic activity of GPx was also observed in the healthy group treated only with AEC compared to that in the group sensitized to ovalbumin. The batch of rats sensitized to OVA and then treated with the plant extract (OVA + AEC) also showed an increase in the level of GPx activity compared with that in the batch of

Table 2. Effects of treatments on certain antioxidative defence status parameters of lungs, in the experimental groups.

Parameters	Control	OVA	AEC	OVA + AEC
MDA (nmol/mg prot)	0.20 ± 0.11	0.59 ± 0.08 (***)	0.55 ± 0.15 (**) (#)	0.49 ± 0.18 (*)
GSH (µmol/mg prot)	0.25 ± 0.17	0.19 ± 0.06	0.10 ± 0.07	0.17 ± 0.08
GPx (µmol GSH/mg prot)	0.47 ± 0.01	0.4 ± 0.28	0.11 ± 0.08	0.21 ± 0.17
CAT (µmol H_2O_2/mg prot)	126.60	80.83	37.84	88.29
Protein carbonyl groups (µmol/mg prot)	32.36 ± 7.96	46.99 ± 3.41 (*)	41.30 ± 0.73 (#)	34.62 ± 8.90 (#)

Values are given as mean ± SEM for groups of four animals each. Significant difference: results of AEC, OVA, and OVA + AEC groups compared to those of the control group (*$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$), and results of AEC and AEC + OVA groups compared to those of the OVA group (#$p \leq 0.05$; ##$p \leq 0.01$; ###$p \leq 0.001$).

Table 3. Effect of treatments on certain antioxidative defence status parameters of erythrocytes, in the experimental groups.

Parameters	Control	OVA	AEC	OVA + AEC
MDA (nmol/mg prot)	0.04 ± 0.03	0.07 ± 0.03	0.06 ± 0.02	0.05 ± 0.02 (##)
GSH (µmol/mg prot)	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.03
GPx (µmol GSH/mg prot)	3.21 ± 2.83	3.78 ± 1.51	1.05 ± 0.55	3.46 ± 3.58
CAT (µmol H_2O_2/mg prot)	39.55	52.20	16.33	35.14

Values are given as mean ± SEM for groups of four animals each. Significant difference: results of AEC, OVA, and OVA + AEC groups compared to those of the control group (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$), and results of AEC and AEC + OVA groups compared to those of the OVA group (#$p \leq 0.05$; ##$p \leq 0.01$; ###$p \leq 0.001$).

rats sensitized to ovalbumin.

The results in Table 3 show no statistically significant differences between the results obtained for different experimental batches. Nevertheless, the most significant variations were characterized, as for the other parameters of oxidative stress, by a reduction in the levels of CAT in rats sensitized to ovalbumin compared to that in the control group. An increase in CAT levels was observed in rats sensitized to OVA and treated with the plant extract (OVA + AEC), as well as in rats treated only with AEC, compared to that in the control group.

3.6. Effect on histological changes of lungs

Microscopic observation of sections of the lung samples of control rats showed normal lung architecture. As shown in Figure 4-C, alveolar bags (red arrow) were observed, and the walls of the bronchioles (blue arrow) possessed normal epithelium. Similar results were observed using the histological sections of lungs of the batch treated with AEC only. However, in the OVA batch suffering from asthma, significant cellular infiltration (red arrow) was observed, as illustrated in Figure 4-OVA (A). In addition, edema and a thick epithelium (black arrow) with a few centers of hemorrhage were observed, and mucus and inflammatory cells were present in the lumen of bronchioles (white arrow; Figure 4-OVA (B)).

Regarding the observations of the histological sections of lungs of rats sensitized to OVA and treated with the AEC (Figure 4-OVA + AEC), we observed signs of histological correction with minimal infiltration, a reduction or absence of inflammatory cells in the lumen of the bronchioles (red arrow), and a reduction in the thickness of the epithelium (blue arrow).

4. Discussion

Asthma is a public health issue that affects more than 300 million people worldwide. Its pathophysiology is based on chronic inflammation, leading to airflow limitation and airway remodeling, resulting in specific symptoms (dyspnea, cough, and chest discomfort) [22]. It manifests as a severe airway disorder caused by various triggers (such as allergies, smoking, and air pollution). This disorder begins with airway obstruction, airway muscle spasms, inflammatory wheezing, and dyspnea [23]. In the absence of a curative treatment, the long-term objective of the therapeutic strategy for asthma is to control various symptoms. Traditional remedies serve as the basis for modern medicine because of their effectiveness, low cost, and fewer side effects than those of drugs [24].

Some compounds are capable of modulating oxidative stress and contributing to the reduction of inflammatory mediators as anti-inflammatory agents [25]. Examples of these bioactive components include the main constituents of cloves (*S. aromaticum*), namely, flavonoids, β-caryophyllene, and essential oils (eugenol) [26]. The extract of *S. aromaticum* is used in the pharmaceutical field owing to its analgesic, antimicrobial, antitumor, anti-inflammatory, antioxidant, and cytotoxic properties [26]. The highly antioxidant and antimicrobial properties of the AEC (*S. aromaticum*) were elucidated in our previous study [27], where high values were obtained for the antioxidant activity (2,2-diphenyl-1-picrylhydazyl [DPPH], 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid [ABTS], and β-carotene tests). Antimicrobial tests conducted using the AEC have shown zones of remarkably high degree of inhibition and sensitivity for gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli)* and gram-positive strains of bacteria *(Staphylococcus aureus*) [27].

Although asthma is a heterogeneous disease comprising various phenotypes, approximately half of the patients with asthma develop type 2 inflammation, characterized on the one hand, by the presence of eosinophilic granulocytes in the airways, and on the other hand, by high levels of immunoglobulin E (IgE) class antibodies (produced by plasma cells) and Th2-type cytokines, such as IL-13, IL-5, and IL-4 [22]. IL-4 is considered to play a key role in asthma because it is associated with the differentiation of immune cells during allergic inflammation [23]. First, among these important roles, IL-4 allows the regulation of the expression of certain molecules present in mast cells, basophils, monocytes, macrophages, and B cells. Examples of these molecules are high-affinity IgE receptor (FcεRI) and major histocompatibility complex (MHC) class II molecules. Second, the IL-4 cytokine makes it possible to generate other pro-allergic cytokines, such as IL-5 and IL-13, via Th2 cells in patients with allergic diseases. Finally, IL-4 allows the migration of Th2 cells and eosinophils toward the inflamed site. It also allows the development of myeloid dendritic cells (mDCs) [23].

With regard to the variations in the levels of IL-4 in the different groups in our study, our results indicated an increase in the levels of IL-4 in the AEC group compared to that in the control group. This can be attributed to the toxicity of eugenol, which is widely known for its antiseptic, anti-inflammatory, and antitumor properties. However, some degree of toxicity may be present depending on the concentrations used and the histology of the organ explored [28]. Eugenol is considered as an allergenic component by some authors [29]. Eugenol is known for its allergenic properties, particularly in the field of cosmetics. As such,

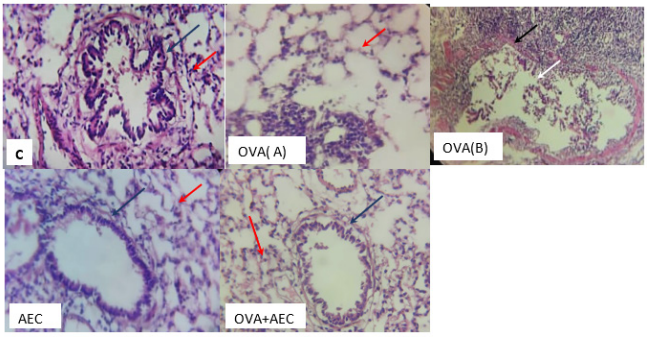

Fig. 4. Microphotographies of histological sections of lung tissue stained with hematoxylin-eosin (× 40). **(C)** Lung of a control rat representing a normal structure of the lungs (alveolar bags : red arrow ; bronchioles : blue arrow (possessed normal epithelium)) ; **(OVA)** Lung of a rat sensitized to ovalbumin, showing the inflammatory infiltration in the form of significant cellular infiltration (red arrow) **(OVA-A)**, which results in the apparent accumulation of eosinophils and other lymphocytes in the lumen of the bronchiole (black arrow) **(OVA-B); (AEC)** Normal lung of a rat treated with clove extract (normal alveolar bags : red arrow ; normal bronchioles :blue arrow); **(OVA + AEC)** Lung of a rat sensitized to ovalbumin and treated with AEC shows signs of histological correction with minimal infiltration, a reduction or absence of inflammatory cells in the lumen of the bronchioles (red arrow), and a reduction in the thickness of the epithelium (blue arrow).

it is on the list of 26 substances included in Annex III of the European Directive 76/768/EEC1 because of its sensitizing potential in humans (AFSSAPS, 2010). In addition, in this study, an increase in IL-4 levels was observed in the three compartments studied, which was also observed in the OVA-sensitized batch. In the lungs, the increase in IL-4 levels can be explained by an increase in the number of Th2 lymphocytes activated in the pulmonary alveoli [30, 31].

Several studies have shown that Th2 lymphocytes play an important role in the onset and progression of allergic diseases, including asthma, by releasing IL-4 [32, 33]. In contrast, treatment of sensitized rats with the AEC reduced the concentration of IL-4 in the serum and lungs. Our results corroborate those of the study by Pan and Dong [34], who used an experimental model of allergic asthma induced by ovalbumin (OVA) to demonstrate that the administration of eugenol inhibits eosinophilia induced by OVA in the lung tissue, prevents increases in IL-4 and IL-5 levels, and inhibits the nuclear factor kappa B (NF-κB) signalling pathway. According to these authors, a reduction in the inflammatory response plays a central role in the anti-asthmatic effects of eugenol [30, 34].

In patients with asthma, airway inflammation develops because of the action of inflammatory mediators, which can lead to an imbalance between ROS and antioxidants, leading to oxidative stress. However, increased expression and secretion of ROS due to the activation of inflammatory cells may, in turn, lead to the further generation of inflammatory mediators, which damage epithelial cells and increase bronchial hyperreactivity [2]. Oxidative stress reflects an imbalance between defensive and aggressive biological systems mediated by the excessive production of reactive oxygen, oxygen species (ROS), hydroxyl radicals (OH), and H_2O_2 (hydrogen peroxide). Under these conditions, the organism loses its antioxidant ability, which is neutralization of the excessive production of ROS [35]. This failure results in toxic effects and alterations in the normal redox state, which are associated with cellular damage and lipid peroxidation [25]. With regard to the phenomenon of inflammation, during these processes, a large amount of ROS is produced in the damaged inflammatory tissues. In these damaged tissues, the signaling pathway for the production of inflammatory mediators, such as proinflammatory cytokines and chemokines, is stimulated, resulting in inflammatory cell migration [36]. In fact, in order to counter the dysregulation induced by oxidative stress, the body has a whole arsenal of enzymes such as superoxide dismutase (SOD), CAT, and GPx and endogenous antioxidants (GSH) that help fight against this aggression [18].These are considered the first line of defence against oxidative damage and the compounds eugenol, eugenyl acetate, and β-caryophyllene appear to play a key role in this process [37]. The antioxidant activity of these compounds is attributed to their capacity to protect cells from free-radical oxidation. Clove possesses radical scavenging activity and inhibits lipid peroxidation. Indeed, this capacity comes from the presence of eugenol, which contains a hydroxyl group (responsible for its antioxidant activity). The presence of phenolic compounds (such as eugenol) allows the transfer of electrons or hydrogen atoms and neutralization of free radicals, resulting in blockage of the oxidative process [37].

Regarding the biochemical profile of eugenol, a previous study confirmed that its antioxidant activity is associated with its anti-inflammatory activity [25]. Another study [38] showed that pre-treatment of rats with eugenol for 15 days resulted in a decrease in the lipid peroxidation (LPO) indices, protein oxidation, and inflammatory marker levels (reduction in the expression levels of cyclooxygenase-2 [COX-2], tumor necrosis factor-alpha [TNF-α], and IL-6) and improved the antioxidant status by maintaining the levels of antioxidants such as GPx and CAT. These results concerning the antioxidant effects are consistent with those observed in our study. Indeed, the levels of GPx and CAT decreased in the lungs and erythrocytes of the batch of asthmatic rats treated with the plant extract compared with those in the untreated OVA batch. Kaur *et al*. [39] confirmed the antioxidant and anti-inflammatory effects of eugenol in albino mice after pretreatment. In fact, eugenol pretreatment is not only effective in reducing inflammation caused by lung exposure to lipopolysaccharides (LPSs), but also enhances the levels of SOD, CAT, GPx, and GST. Eugenol can be used as an anti-inflammatory agent to protect against the damage caused by oxidative stress [40].

Oxidative damage to DNA, lipids, and proteins induced by pesticides or inflammatory diseases is a consequence of ROS production [18]. ROS are free radicals produced by mitochondria during normal aerobic metabolism [41]. However, excessive production of ROS and/or a decrease in the cellular capacity to promote an effective antioxidant leads to the initiation of oxidative stress [42]. In this study, the effect of ROS on biological membranes was analyzed by measuring the level of MDA using spectrophotometry. MDA is a biomarker of oxidative stress that can be used to determine the degree of lipid peroxidation LPO in cell membranes following a foreign attack [43,44]. Indeed, as shown by our results regarding the amounts of MDA and GSH (Table 2), in the batch treated with the AEC, we observed a significant reduction ($p \leq 0.01$) in MDA levels and a significant increase ($p \leq 0.05$) in GSH levels. These results can be explained by the potential ameliorative effects of eugenol on organ structure and function [45]. Thus, eugenol reduces organ damage by reducing the levels of inflammatory mediators and modulating the redox status [45].

Flavonoids, the second most essential component of the plant *S. aromaticum*, belong to the non-enzymatic antioxidant system because they confine ROS [46]. This process protects against oxidative injury, particularly because of the structural properties of some secondary flavonoid metabolites. Among these structural properties, plant stress tolerance is important [47].

An additional parameter used to determine the antioxidant effect of the plant is protein carbonyl group content. The protein carbonyl group is more stable in the form of oxidized proteins than lipid peroxidation products [48]. Protein carbonyl content is the most common indicator and most commonly used marker of protein oxidation [49], and the accumulation of these compounds has been found in several human diseases (such as airway inflammation, oxidative lung injury, and Alzheimer's disease) [12]. The protein carbonyl levels in the lungs showed a significant decrease ($p \leq 0.05$) in the OVA + AEC group compared to that in the OVA group, whereas the levels showed a significant increase ($p \leq 0.05$) in the OVA + AEC group compared to that in the control group. This result

shows that the AEC prevented oxidation-induced protein damage by decreasing protein carbonyl formation and protecting against the loss of protein thiol groups [50]. These results prove that the polyphenols found in the AEC (one of the components of the plant that confers antioxidant ability) were capable of inhibiting protein oxidation [50]. Regarding variations in the inflammatory response triggered by OVA treatment, a significant increase in total leukocyte counts was observed in OVA-sensitized rats. Therefore, the involvement of leukocytes in the local and general inflammatory processes is confirmed. Indeed, an intense recruitment of cells from the bloodstream occurs during the inflammatory process. This "homing" phenomenon involves the expression of ligands on the surface of vascular endothelial cells and on circulating leukocytes. They are then attracted to the inflammatory site by chemokines and cytokines released in the vicinity of the inflamed tissue and enter the site by diapedesis [51, 52].

The white blood cell and lymphocyte counts in the OVA + AEC groups increased compared with those in the OVA group. The anti-inflammatory effect of eugenol, the main bioactive molecule in cloves, on the migration of leukocytes has been studied using different stimuli such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), leukotriene B4 (LTB4), and carrageenan. The polymorphonuclear (PMN) cell recruitment to the inflammatory site occurs as a function of a complex response involving interactions between endothelium and leukocytes [53]. Estevão-Silva and colleagues [54] demonstrated that eugenol significantly decreases leukocyte migration in vitro and in vivo in response to chemotactic factors by modulating rolling and adhesion of leukocytes to the perivascular tissue.

In the present study, a significant decrease ($p \leq 0.05$) in the number of red blood cells was observed in the batch sensitized to ovalbumin. These results also seem to be in agreement with the findings of Suzuki *et al*. [55], who observed an alteration in iron levels (iron is used in the synthesis of hemoglobin) under conditions of acute inflammation and a decrease in the level of blood hemoglobin.

Biochemical analysis of the levels of total proteins in the serum and BALF showed that the batches treated with the aqueous extract of cloves did not show any significant variation in levels compared to that in the control batch. The results (Figure 2) showed an increase in the concentrations of total proteins in the serum and BALF in the OVA group. In the OVA + AEC group (sensitized to ovalbumin and treated with the aqueous extract), a decrease in the concentrations of total proteins in the serum and BALF was observed, and the values reached close to those of the control group. These results agree with those obtained in the study by Haro-Gonzalez *et al*. [37], which showed that cloves exerted a protective effect against biochemical changes in proteins.

Considering that proteins are organic compounds distributed widely in the body and function as structural and transport elements [56],the increase in total protein levels reflects the increase in levels of inflammatory mediators and is, therefore, considered a biomarker of pulmonary inflammation caused by OVA [56]. However, administration of the aqueous extract of cloves considerably reduced the levels of inflammatory mediators. This may have been due to the inhibitory effects of eugenol. Indeed, Pan and Dong [34] demonstrated that eugenol has the ability to decrease the levels of the following proteins: IL-4, IL-5, inhibitor of nuclear factor kappa B alpha (P-IκBα), and p-NF-κBP65. Thus, studies have shown that eugenol can negatively regulate inflammation by inhibiting the release of inflammatory mediators (proteins in nature) by macrophages [57].

All the previously obtained results corroborate those of the present histopathological study of the lungs. Indeed, in asthmatic rats (OVA batch), infiltration of granulocytes, neutrophils, and macrophages was associated with the occurrence of perivascular and peribronchial edema, as shown in Figure 4 (black, white, and red arrows) [58]. Most of these inflammatory cells are composed of lymphocytes and eosinophils and are mainly located around the small bronchi, bronchioles, and blood vessels. In addition to the infiltration of these cells, edema is also present [13]. However, treatment with the aqueous extracts of the *S. aromaticum* plant helped in reducing alveolar edema by decreasing pulmonary inflammation and reducing the concentrations of inflammatory cells and their infiltration into the lumen of the bronchioles [58]. In this regard, it is necessary to measure the effects of other medicinal plants [59-61].

5. Conclusion

Two important elements were validated through these results: the first concerns the relationship and link between allergic asthma (via ovalbumin sensitization) and the increase in the levels of IL-4 and oxidative stress parameters in lungs. The second point involved confirmation of the anti-inflammatory and antioxidant benefits of cloves *(S. aromaticum)* against asthmatic pathology.

Conflict of interest

Authors have declared that no competing interests exist.

Consent for publication

The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed consent

The authors declare that no patients were used in this study.

Availability of data and materials

All data generated during this study are included in this published article.

Author's contribution

Boumendjel Amel and Messarah Mahfoud contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ounaceur Lynda Sabrina, Djaber Nesrine, Latifa Atoui, Khadidja Belkacem Djeffel, Sonia Boudjil, Zohir Garri, Hanene Ghadab and Djihane Touaibia. The first draft of the manuscript was written by Ounaceur Lynda Sabrina and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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