Research Article

Anti-Inflammatory and Antioxidant Effects of Two Extracts from *Pistacia lentiscus* in Liver and Erythrocytes, in an Experimental Model of Asthma

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ABSTRACT

The aim of this work was to study the comparative anti-inflammatory and antioxidant effects of the oil of *Pistacia lentiscus* (H) obtained from the fruit of the tree, and its aqueous leaf extract (EQ), on an experimentally induced asthma. Fifty-four males Wistar rats were sensitized with ovalbumin (OVA) and in parallel with the oil (H) and the aqueous extract (EQ). For measuring the intensity of the airway inflammation, the percentage of inflammatory cells and the level of total proteins in the serum were measured. Histo pathological study in liver sections is performed and oxidative stress parameters are analyzed in erythrocytes and liver. Our results showed that sensitization of the OVA-group, induce an increase in total proteins levels and leukocyte mainly eosinophils. The administration of *Pistacia lentiscus* oil, more than its aqueous extract, reduces significantly those measured parameters. Harmful effects of allergic inflammation caused by OVA sensitization and the treatment with two plant extracts on hepatic tissue architecture revealed a degeneration of liver plates, a slight inflammatory infiltration, an increased sinusoids size and a minor focal necrosis. The results also show that this inflammatory state induce a severe lipid peroxidation, revealed by significant increase in MDA levels and significant activity reduction of various antioxidant systems (GPx, SOD and CAT), both in erythrocytes and in the liver. However, pretreatment with aqueous extract is more effective than oil by restoring the activity of antioxidant enzymes. Conversely, the administration of oil had a better reducing effect that EQ on lipid peroxidation. These results are in favor of anti-inflammatory and antioxidant effects which the extract of *P. lentiscus* are endowed.

Keywords: Experimental asthma, *Pistacia lentiscus*, ovalbumin, oxidative stress, inflammatory cells.

INTRODUCTION

Asthma is an inflammatory pulmonary disease that involves increased oxidative stress. As in any inflammatory condition, the physiological antioxidant system is altered during this disease[1]. Indeed, the infiltrations of leukocyte inflammatory cells release reactive oxygen species (ROS) in the surrounding tissue [2].

Therefore, the development of an experimental asthma protocol in Wistar rats sensitized with ovalbumin (OVA) has all its interest in order to remedy this disease. Owing to the chronic nature and the increasing prevalence of this disease, in addition to the ineffective drugs currently used to permanently cure asthma and fear of their known side effects, there is a pressing need to find new therapies. In this regard, natural products and herbal remedies plants used in traditional medicine were thus the source of many drugs [3, 4, 5].

Many medicinal plants have interesting biological and pharmacological activities and are used as therapeutic agents [6]. *Pistacia lentiscus* known as mastic is an evergreen shrub, belonging to the Anacardiaceae family consists of more than eleven species[7]. It is widely distributed in "extreme" of Mediterranean ecosystems. In Algeria, the tree is widespread in the forest, alone or combined with other tree species (such as oaks, olive trees and carob trees) [8]. Studies on the composition of the leaves and berries *Pistacia lentiscus* reported that this plant contains different types of secondary metabolites, known for their great healing properties [9].

This study helps to spread the disease characteristics induced by ovalbumin in serum and liver and to evaluate the therapeutic effects (antioxidant and anti-asthmatic) of *Pistacia lentiscus*.

MATERIALS AND METHODS

Plant

Mastic, *Pistacia lentiscus*, a shrub of the Anacardiaceae family which was used in this study grows in the region of Annaba and specifically Cheurfa (located east of Algeria). This plant is catalogued in herbarium of Pr. Gérard de Belair (Voucher specimen number: 060_29) and identified by Mr. Pr Azeddine CHEFROUR, botanist at the Biology department (Faculty of Natural Sciences and Life, University of Souk Ahras - Algeria).

Preparation of aqueous extract

The fresh leaves collected in January 2014, are dried for 07-10 days at room temperature, and then crushed and stored until the preparation of the aqueous extract. The aqueous extract was prepared daily from the leaf powder subjected to decoction, by boiling for 15-20 min. The aqueous phase is filtered and the extract is then stored at 4°C.

Extraction of *Pistacia lentiscus* oil

The oil is extracted in a traditional press from ripe fruit (blackberries) mastic collected in December 2013. It was stored in glass bottles protected from light until use.

Animals

Fifty-four Wistar albino male rats weighing between 280 and 320g, obtained from Pasteur institute (Algiers, Algeria) were used for the experimental procedures. All protocols used in this study were used in accordance with the guidelines of the Committee on Use of Laboratory Animals and approved by the Ethical Committee of Directorate General for Scientific Research

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and Technological Development at Algerian Ministry of Higher Education and Scientific Research. Animals were acclimated for 2 weeks under the same laboratory conditions of photoperiod, an average relative humidity of 60 % and room temperature of 20±2 °C. Food (standard food, supplied by the "ONAB, Bejaia", Algeria) and water were available ad libitum.

Experimental group design

The rats were distributed in six groups of nine (n=9) male receiving the following treatments: the first group served as a control (T). The second group (EQ) was treated with aqueous extract of mastic (200 ml / kg body weight / day by gavage), while the third group (H), was treated with mastic oil (3.3 ml / kg body weight / day by gavage) [6, 10]. The fourth group was sensitized to ovalbumin (OVA). The fifth group (OVA + EQ) was sensitized and treated with aqueous extract of mastic (200 ml / kg). The sixth group (OVA + H) was sensitized and treated with mastic oil (3.3 ml / kg).

Sensitization and airway challenge

Rats groups (N°4, 5, 6) were sensitized to OVA (grade II, Item A5253-250G, Sigma. Aldrich) in a manner causing acute inflammation with allergic asthma phenotype, validated in the literature. Thus, the sensitization is carried with an intraperitoneal injection of 1 mg/ml of OVA, combined with an aluminum hydroxide adjuvant (Al (OH_3) (Alum, Sigma Aldrich) dissolved in an amount of 1mg/ml in a saline solution (9 ‰) on days 0 and 13. Rats were exposed to the airway with OVA (1%, w/v, in saline solution (9 ‰)) for 30 min using a nebulizer unheated compressor (OMRON NE-C29-E) on days 21, 22 and 23 after initial sensitization [11].Control animals of groups (N°1, 2, 3) were sensitized and exposed to similar volumes of saline solution (9 ‰) at the same times and in the same conditions [12].

Samples Preparation

Blood Collection

Blood samples were immediately collected into two groups of ice-cold polypropylene tubes. The first one was dry, which has been centrifuged at 3,000×g for 15 min. The collected serum used for the determination of total proteins; as to the sediment (containing erythrocytes) was stored at –20 °C until to be used for the determination of oxidative stress parameters. The second group contained anticoagulant (EDTA) was used for determination of the number of white blood cells.

Preparation of Erythrocytes and Liver Homogenates

The sediment containing erythrocytes were twice suspended in phosphate buffer saline [KH_2PO_4 (10 mM), NaCl (150 mM), pH 7.4] and centrifuged at 3,000×g for 15 min at 4°C for the first washing; and at 4,000×g for 30 min at 4°C for the second washing. The hemolysats were then aliquoted and stored at −20°C before use for antioxidant enzyme activities, and the determination of malondialdehyde (MDA).

Livers were quickly removed, washed in 0.9 % NaCl solution and weighed after the careful removal of the surrounding connective tissues, and then, a quantity of 1 g was homogenized in 2 ml of phosphate buffer solution (TBS: Tris 50 mM, NaCl 150 mM, pH 7.4) at 1:2 (w/v), in ice-cold condition. Homogenates were centrifuged at 3,000×g for 35 min at 4°C; the supernatants were divided into aliquots and then stored at −20°C.

Total proteins

The total proteins concentration was determined by a colorimetric method biuret [13]. 20 µl of each sample (serum) were added to 1000 µl of the biuret reagent and mixed then incubated for 5 minutes at 37°C. Thereafter, the spectrophotometric reading (SECOMAM) is carried out at 540 nm.

Determination of leukocyte formula

A blood smear was prepared and then fixed with methanol for 3 minutes and finally stained with May-Grünwald-Giemsa (MGG)[14]. The percentages of the different types of leukocytes were calculated by counting 100 cells under microscope (× 100) (OPTIKA).

Determination of oxidative stress parameters

Activities of glutathione peroxidase (GPx, E.C. 1.11.1.9), superoxide dismutase (SOD, E.C.1.15.1.1) and catalase (CAT, E.C.1.11.1.6) in erythrocytes and liver homogenates were done using reported protocols[15-17]. Further the extent of lipid peroxidation and total protein were estimated in erythrocytes and liver homogenates by the formation of malondialdehyde (MDA) using thiobarbituric acid (TBA) according respectively to the methods [18, 19].

Histo pathological Examination

Liver was dissected and immediately fixed in formol solution to 10% for 24 h, processed by using a graded ethanol series, and then embedded in paraffin (increased to 56/58°C). The paraffin sections were cut into5-µm thick slices using a microtome (Leica RM2125RT), followed by a staining with hematoxylin and eosin (Leica ST4040) and a mounting EUKITT (kindler GmbH co lot No: c70)[20]. Finally, the sections were observed and analyzed under an optical microscope (Leica DM LB 2) and then photographed.

Statistical Analysis

All data are expressed as mean±SD for nine rats of each group. These calculations were performed using Microsoft Excel (2010). Significant differences between the group's means were determined by Student's t test. The statistical signification of difference was taken as $p \leq 0.05$.

RESULTS

Effects of treatments on body weight, relative and absolute liver weight

Changes in body weight and the relative and absolute liver weight are presented in **Table 1**. A highly significant decrease in body weight in rats groups EQ, OVA and O/EQ (respectively, 5.57%, 6.09%and 5.15%) were registered. Administration of the aqueous extract in rats groups (EQ and O/EQ) induced a significant and highly significant reduction in the relative and absolute liver (respectively, 8.50% 7.43% and 14.51%; 12.90%) compared with control rats. However, mastic oil induces a better boost in body weight compared to OVA group.

Effect on leukocyte population

Changes in leukocyte counts in the blood smear are presented in **Fig. 1**. Sensitization to OVA caused an increase in total leukocyte cells and monocytes (respectively, 141.62%, 53.23%). The increase in the leukocyte cell count is highly significant for both eosinophils and lymphocytes; on the other hand, it is significant for basophils. However, treatment with the aqueous extract as well as mastic oil allows recovery of these values, in sensitized rats, which was expressed by a very significant decrease in eosinophils and lymphocytes (78.95%, 78.70% and 31.45%, 33.45%). It has been demonstrated that the administration of more mastic oil than its aqueous extract (p =

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0.06) reduced significantly basophils rates of 59.56%.In the treated groups with both extracts (EQ and H), there was a significant increase in the distribution of leukocyte cells relative to group T.

Table 1: Changes in Body Weight and Absolute and Relative Liver Weight of Control and Treated Rats.

Parameters	Experimental groups					
	T	EQ	H	OVA	O/EQ	O/H
Body weight (g)	317,166 ± 4,015	299,5 ± 3,344**	306,5 ± 4,808	297,833 ± 3,674**	300,833 ± 2,892**	306,5 ± 2,125
Absolute liver weight (g)	10,333 ± 0,333	8,833± 0,307**###	9,833 ± 0,307	10,5 ± 0,223	9 ± 0,258**###	9,833 ± 0,166#
Relative liver weight (g/100g de PC)	3,221 ±0,062	2,947 ±0,088*	3,206 ±0,073	3,279 ±0,142	2,982 ±0,058**	3,246 ±0,07

Values are given as mean±SD. Significant difference: all treated groups compared to the control one (*$p \leq 0.05$, **$p \leq 0.01$), all treated groups compared to the OVA treated one (# $p \leq 0.05$, ### $p \leq 0.001$).

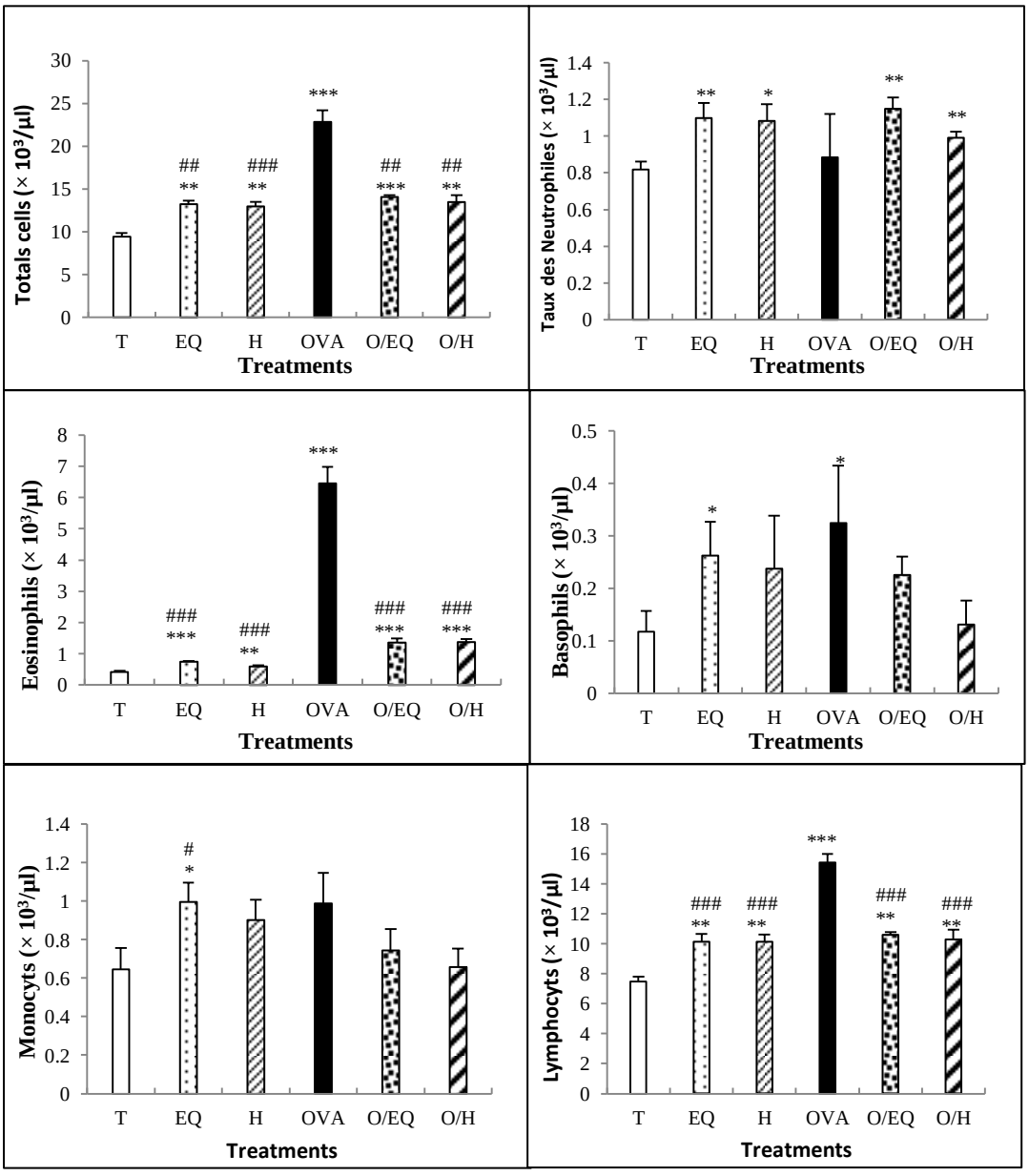

Figure 1: Distribution Of Leukocytes In The Blood Smear Of Different Experimental Groups.

Values are given as mean ± SD. Significant difference: all treated groups compared to the control one (*$p \leq 0.05$, **$p \leq 0.01$,***$p \leq 0.001$), all treated groups compared to the OVA treated one (# $p \leq 0.05$, ##$p \leq 0.01$, ### $p \leq 0.001$).

Effect on the total proteins

The results show that the OVA sensitization causes a significant increase of the total protein of 4.96% compared to the control group **(Fig. 2)**. However the administration of mastic oil (O/H and H) more than its aqueous extract revealed a highly significant decrease in serum proteins 9.46% and 8.22% respectively compared

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to sensitized group (OVA) and control group (T). As for the group treated with the aqueous extract (EQ), it shows no significant change compared to group T.

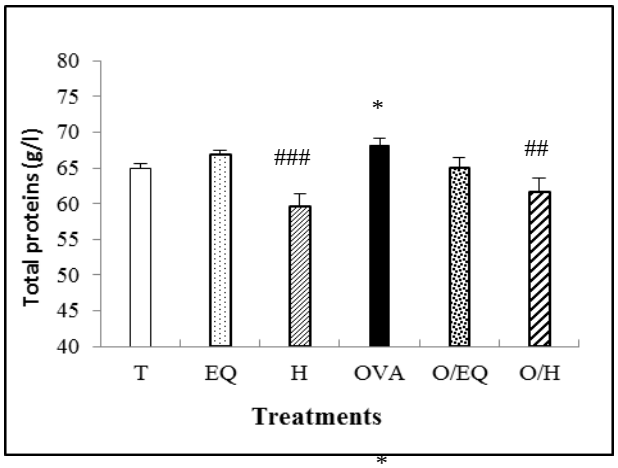

Figure 2 : Changes in Total Proteins Concentration of Control and Treated Rats.

Values are given as mean ± SD. Significant difference: all treated groups compared to the control one (*$p \leq 0.05$, **$p \leq 0.01$), all treated groups compared to the OVA treated one (## $p \leq 0.01$, ### $p \leq 0.001$).

Study of oxidative stress parameters

Data concerning liver antioxidant enzyme activities (GPx, SOD and CAT) are presented in **Fig.3**. In OVA group, GPx, SOD and CAT activities were decreased significantly compared to the control (group T). Indeed, administration of oil and aqueous extract of *P. lentiscus* showed an improvement of enzyme activities in OVA+H and OVA+EQ groups compared to the OVA group.

Results of various antioxidant enzyme activities measured in erythrocytes are presented in **Fig. 4**. In OVA-group, GPx, SOD and CAT activities were declined compared to the control, but the supplementation of oil and aqueous extract of mastic has diminished. In addition, the administration of the aqueous extract was more effective (p = 0.05) than the mastic oil with a significant increase in the levels of CAT.

The results show that the OVA sensitization causes a lipid peroxidation revealed by significant increase malondialdehyde (MDA) contents in liver **(Fig. 3)** and erythrocyte **(Fig. 4)** compared to control groups. However, the administrations of two mastic extracts have allowed a significant reduction of the MDA levels. It was shown that mastic oil is more effective than its aqueous extract with a better reduction of lipid peroxidation.

Histopathological Results

Microscopic observation of rat liver control shows organization into lobules wherein the hepatocytes are separated by adjacent sinusoids **(Fig. 5-A)**. The livers of treated rats with the aqueous extract **(Fig. 5-B)** and mastic oil **(Fig. 5-C)** shows a sinusoidal enlargement leading to the decrease of hepatocyte diameter (DH). As for the section of the liver of treated rats with ovalbumin, it reveals a prominent cell change, which results in hyperplasia, a burst of hepatocytes and infiltration of inflammatory cells **(Fig. 5-D)**. For liver sections of rats sensitized with ovalbumin and treated with either the aqueous extract **(Fig. 5-E)** is in the mastic oil **(Fig. 5-F)**, they respectively show the appearance of the necrosis, an increase in the size of the sinusoids.

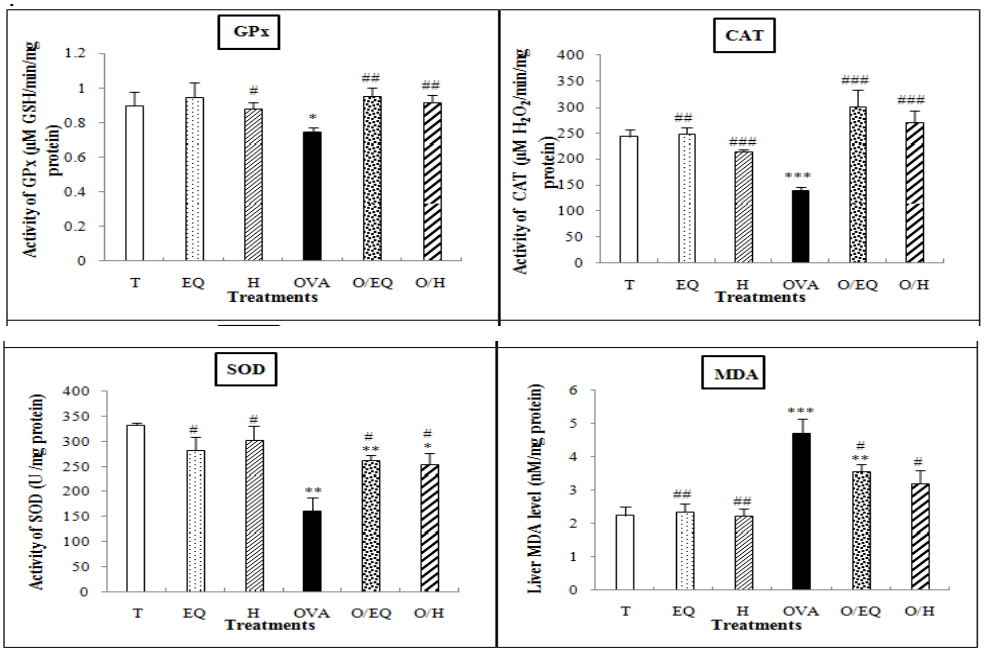

Figure 3: Antioxidant Enzyme activities and Malondialdehyde levels in liver of control and treated rats.

Values are given as mean ± SD. Significant difference: all treated groups compared to the control one (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$), all treated groups compared to the OVA treated one (# $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$).

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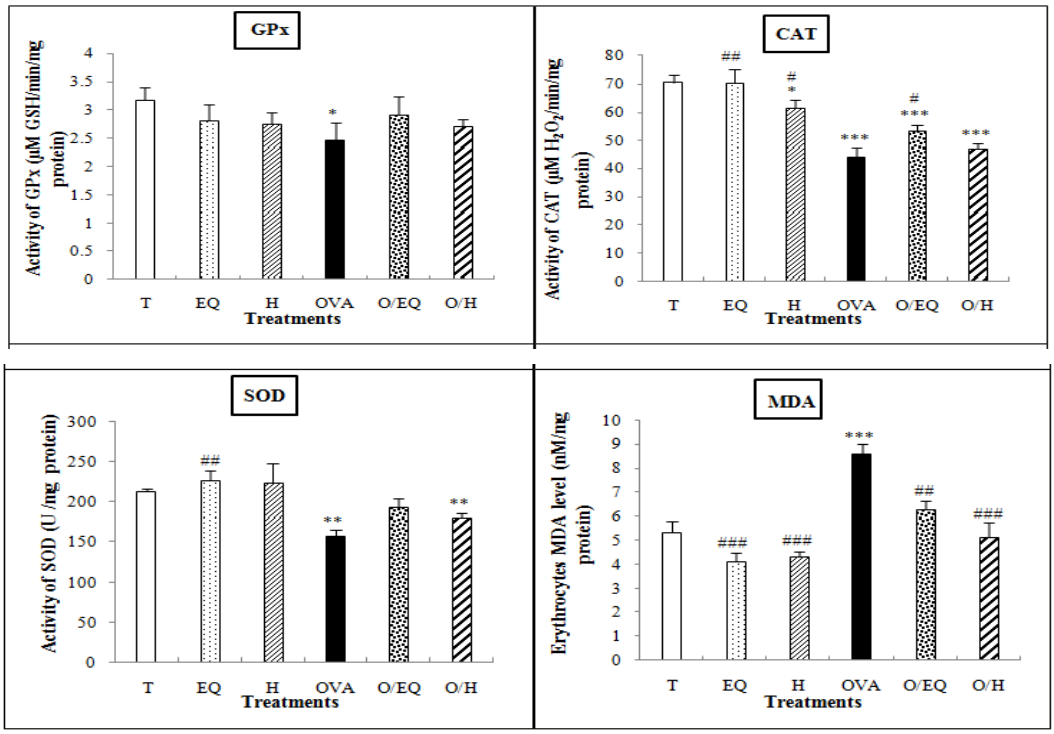

Figure 4: Antioxidant Enzyme activities and Malondialdehyde levels in Erythrocytes of control and treated rats.

Values are given as mean ± SD. Significant difference: all treated groups compared to the control one (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$), all treated groups compared to the OVA treated one (# $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$).

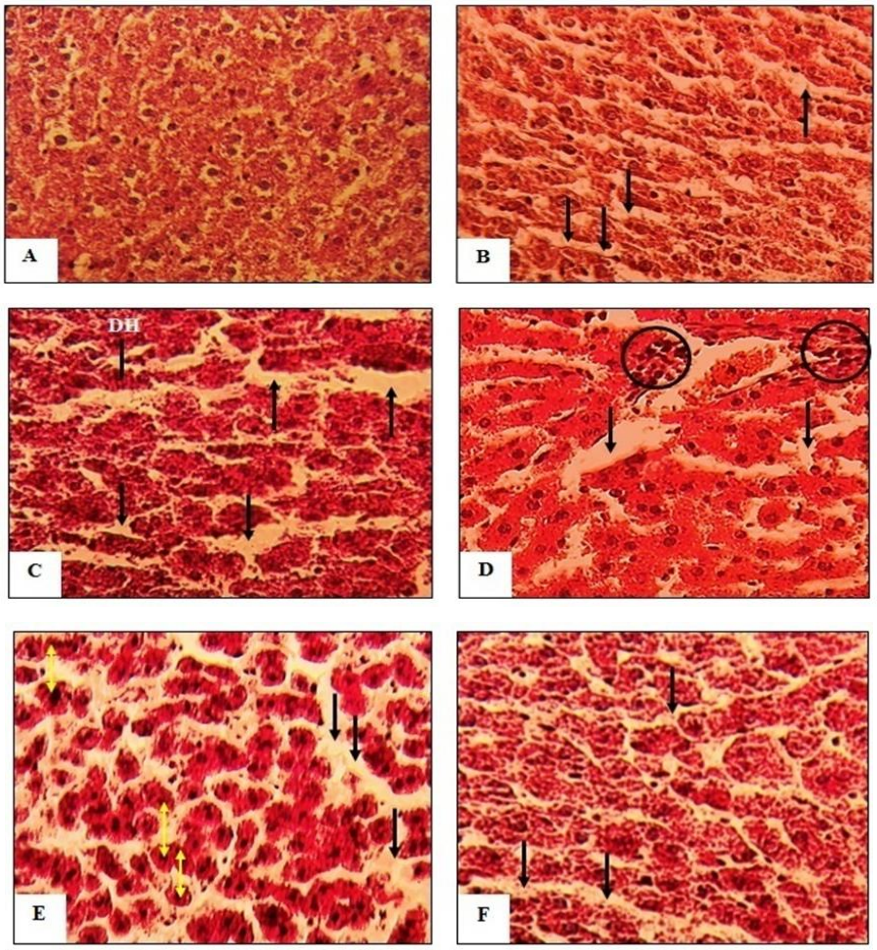

Figure 5: Histological sections of the Liver in rats of different Experimental groups (X40).

(A) Liver of a control rat, with normal architecture and no pathological abnormalities; (B) liver of a treated rat with aqueous extract showing sinusoidal enlargements (arrows); (C) liver of a treated rat with mastic oil, showing a smaller diameter hepatocytes (DH) and an increase in the size of sinusoids

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(arrow); (D) liver of a treated rat with ovalbumin, showing an increase in the size of sinusoids (arrow) and an inflammatory cell infiltration (circle); (E) liver of a rat sensitized to ovalbumin and treated with aqueous extract; showing necrosis in hepatocytes (double arrow direction) and an increase in the size of sinusoids (arrow); (F) liver of a rat sensitized to ovalbumin and treated with mastic oil showing sinusoidal enlargements (arrows).

DISCUSSION

In the present study, the effect of *Pistacia lentiscus* (under two comparative forms: oil and aqueous extract) on hepatic alterations in a murine allergic asthma model was investigated.

In our experiment, we first found a reduction in body weight basically treated groups aqueous extract (EQ; O/EQ) and sensitized to OVA. The relative and absolute liver weight in rats treated with the aqueous extract is also reduced (Table 1). These changes in weight are probably due to both, the toxicity of ovalbumin, but also gallic acid and tannins contained in *Pistacia lentiscus* whose are responsible for the decrease in the amount of food ingested, in the rate of growth, in metabolic energy and digestive proteins [21].

The subsequent administration of the allergen by inhalation induced inflammatory airway response as has been shown in many studies [22, 23, 24]. Many molecules involved in inflammation are proteins synthesized by the liver in response to foreign aggression, here; in this case, it is the allergen consisting of ovalbumin and adjuvant, aluminum hydroxide. Indeed, our results showed in Fig. 1 an increased leukocyte recruitment of total cells in rats sensitized with OVA, mainly eosinophils and lymphocytes which are a main feature of asthma and a significant increase in serum total protein (Fig. 2).

However, pretreatment of the rats, whether with the aqueous extract of the leaves or oil of mastic attenuate significantly the rate increases previously reported and it's probably due to their richness in secondary metabolites, such as phenolic acids, flavonoids, tannins, terpenoids and phyto sterols found in fruits [6, 25].

According to KIM et al., gallic acid and its derivatives cause inhibition of the activation of p38 MAPK, and the inhibition of binding of NF-κB essential for the expression of pro-inflammatory cytokines such as histamine, TNF-α and IL-6[26]. Also, XAGORARIet al. identify the luteolin, present in the leaves, as the most powerful flavonoid tested in the inhibition of TNF-α, activation of NF-κB induced by lipopolysaccharide (LPS), and activation of AP-1[27]. In addition, the ability of tannins to inhibit phospholipase A2 is already established which will participate in the inhibition of prostaglandins and leukotrienes[28].

Inflammatory cells recruited to asthmatic airways have exceptional ROS-producing capability. Moreover, many human studies have reported the role of eosinophils in allergic inflammation; up to consider that the evaluation of the serological levels of ECP (eosinophil cationic protein) seems to be a good biological marker of asthma [29]. The production of ROS is essential for the inflammatory response by activating redox transcription factors and pro-inflammatory signaling pathways. At the same time, the endogenous antioxidant mechanisms that are present to reduce the imbalance between these two opposite mechanisms could lead to chronic illness and more severe inflammatory condition [30].

Consistent with thesis findings, our data show evaluation of certain parameters of oxidative stress in liver and erythrocytes. The elevated level of MDA in the OVA challenged group could be linked to the peroxidation damages of biological membranes, caused by an increased reactive Fe^{+2} and/or inactivation of enzymes involved in antioxidant defense. A decreased GPx, SOD and CAT activities showed in these results confirmed this theory (Fig. 3, Fig. 4).

Our results confirm those of TIWARI et al.[31], which showed experimental work in its oxidative inactivation of SOD, catalase, glutathione reductase, glutathione peroxidase and the decrease in MDA formation in Ova Treated asthmatic rats. Moreover, this antioxidant enzyme activities increase in substantially is suppressed by extracts *P.lentiscus*. This antioxidant stimulation is more pronounced when treated with aqueous extract of mastic. However, mastic oil has a better reduction of lipid peroxidation.

This rebalancing of the balance is due to the high antioxidant potential of the plant to study because of the power associated with these secondary metabolites (flavonoids, phenolic acids and polyphenols) [32, 33]. They are able to scavenge hydroxyl radicals (OH •), superoxide anion (O2.-) and peroxylipidic radicals [34]. Indeed, gallic acid and flavonoids inactivate and stabilize free radicals thanks to its hydroxyl group (C3-OH) highly reactive. They are also able to chelate metal ions (dropped from their mounting or transport proteins) that can strengthen these deleterious effects by producing hydroxyl radicals (OH •) [35, 36].

In an effort to secure the above results, histopathological analysis was performed in the liver tissue. Several features were observed. Increased cellular levels of lipid peroxidation (MDA), the increase of free radicals and decrease the enzymatic activity of GPx, catalase and SOD are many factors responsible for the tissue damage observed and materialized the bursting of hepatocytes in rats sensitized to ovalbumin. Indeed, there are similar results in the literature suggesting intracellular changes of hepatocytes characterized by cytolysis and visible hyperplasia in batches treated with agents causing oxidative stress such as arsenic, cadmium and thyroxine[37, 38, 39]. Moreover, an infiltration of inflammatory cells in the OVA Challenged group was observed.

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Also in this study, histological Findings Increase in indicated essentially year sinusoids size and necrosis in the liver of the rats exposed to *P. lentiscus*. However, aqueous extract was more devastating (Fig. 5). Indeed, we know the leaves of this plantcontain condensed tannins are proven that hepatotoxins[40, 41].

Focal necrosis of the liver tissue observed in OVA+EQ group could be driven from the animal's excessive activity to get rid of the toxicant from its body during the process of detoxification. Also, the liver incapability in regenerating new cells may lead to necrosis [42].

CONCLUSION

The results revealed that the sensitization to OVA induced disruption of antioxidant defense system with a significant state of inflammation. However, extracts *Pistacia lentiscus* allowed through secondary metabolites decreased significantly this inflammation and have an additive effect on the activity of protective antioxidant enzymes.

Moreover, the aqueous extract of mastic proved with antioxidant properties relatively higher than its oil. Furthermore, the data obtained from the study, showed that the leaf of this plant can be hepatotoxic.

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