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# The Extract of *Portulaca oleracea* and Its Constituent, Alpha Linolenic Acid Affects Serum Oxidant Levels and Inflammatory Cells in Sensitized Rats

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## ABSTRACT

The effects of *Portulaca oleracea* and its constituent, alpha linolenic acid on serum oxidant levels and inflammatory cells in sensitized rats were examined.

Eight groups of rats including control, sensitized, sensitized rats treated with 1, 2 and 4 mg/mL extract of *P. oleracea*, 0.2 and 0.4 mg/mL alpha linolenic acid (ALA) and 1.25 µg/mL dexamethaswere studied serum levels of superoxide dismutase (SOD), catalase (CAT, thiol groups, $NO_2$, $NO_3$, and Malondialdehyde (MDA) as well as total and differential WBC in blood were measured.

Serum concentrations of SOD, CAT and thiol were significantly decreased but $NO_2$, $NO_3$ and MDA as well as total WBC number and percentages of eosinophil and neutrophil were increased in sensitized group ($p<0.001$ for all cases).Treatment of sensitized animals with dexamethasone, high concentrations of the extract and ALA improved all measured variables except monocyte for all three treatment groups and eosinophil for dexamethasone treatment ($p<0.01$ to $p<0.001$). In addition, treatment with low and medium extract and low ALA concentrations improved serum levels of $NO_2$, $NO_3$ and total WBC count ($p<0.001$ for all cases). Neutrophil and lymphocyte percentages and serum level of thiol also improved due to treatment with medium extract and low ALA concentration ($p<0.01$ to $p<0.001$). Medium extract and low ALA treatment also caused improvement of serum level of CAT and eosinophil percentage as well as SOD level respectively ($p<0.01$ to $p<0.001$).

The effect of the extract of *P. oleracea* and ALA on serum oxidants and inflammatory cells were demonstrated in sensitized rats, which was comparable with dexamethasone effects at used concentrations.

**Keywords:** Alpha linolenic acid; Oxidant and antioxidant levels; *Portulaca oleracea;* Sensitized rats; White blood cell

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## INTRODUCTION

Bronchial asthma is a chronic inflammatory illness, in which various cells including mast cells, eosinophils,

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and T lymphocytes, and other cellular components contribute.[1] These inflammatory cells produce several mediators including a range of toxic reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide, hypochlorous acid, and hydroxyl radical that modulate the inflammatory response.[2-5] There are several antioxidants in the lung and blood including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase, glutathione, vitamin E, and vitamin C[6-9], which prevent oxidant-mediated toxicity. The oxidative stress in asthma was shown by increased production of lipid peroxidation products and protein carbonyls in plasma,[10] increased plasma isoprostanes[11], enhanced generation of ROS by blood monocytes, neutrophils, and eosinophils,[12, 13] increased oxidized glutathione in bronchoalveolar lavage (BAL) fluid[14] and increased production of nitric oxide (NO) in exhaled air.[15] Physiological antioxidant system is also impaired in asthma, possibly because of inflammation. Thus, the imbalance between oxidants and antioxidants that is called oxidative stress is critical in asthma pathogenesis.[16] Oxidative stress and inflammatory mediators are contributing mechanisms in pulmonary diseases such as asthma. These two mechanisms alters the production of the oxidants and cause structural damage of the lung.[17]

*Portulaca oleracea* (*P. oleracea*) L. is a warm-climate and annual plant which belongs to the Portulacaceae family.[18] This plant exhibits a wide range of pharmacological effects, including anti-inflammatory[19], antioxidant[20], and wound-healing[21] properties. Moreover, skeletal muscle relaxant effect of this plant has been reported.[22] The relaxant effects of the plant on tracheal smooth muscle[23, 24], potent bronchodilatory effects in the airways of asthmatic patients[25] and its antitussive effect[26] have been studied previously. The effect of *P. oleracea* on Th1/Th2 balances was also demonstrated, which may suggest its therapeutic value in inflammatory disease associated with decreased Th1/Th2 balance such as asthma.[27] *P. oleracea* also provides a source of nutritional benefits owing to its rich omega-3 fatty acids and antioxidant properties.[28]

The plant was studied for its ability to reduce oxidative stress induced by vitamin A deficiency.[29] It was demonstrated that phenolic alkaloids of the plant served as a new class of antioxidant agents.[30] The majors constituents of the plant are flavonoids, alkaloids, coumarins, monoterpene glycoside and fatty acids as well as alpha-linolenic acid (omega-3).[31,32] Alpha linolenic acid (ALA) is an omega-3 fatty acid, which plays an important role in human growth and development and in preventing diseases.[33] ALA can modulate the immune response of the epidermis by influencing T lymphocytes, acting on toll-like receptors (TLRs), and activating caspase cascades that influence many inflammatory dermatoses, including atopic dermatitis, systemic lupus erythematosus, and skin cancer.[34] ALA increases the level of eicosapentaenoic acid (EPA) in the phospholipids of cell membranes and decrease the synthesis of proinflammatory cytokines such as interleukin-1 and TNF-α in mononuclear cells of the human body. These changes resulted in reducing the severity of the response to allergens in asthma disease.[35,36]

With regard to existence of airway inflammation and oxidative stress in asthma and due to anti-inflammatory property of *P.oleracea* and its constituent ALA, in the present study, the effects of the plant and ALA on inflammatory cells and serum oxidant levels in sensitized rats were examined.

## MATERIAL AND METHODS

### Animal Groups and Animal Sensitization

Eight groups of rats were studied in random order including: control or non-sensitized animals (group C), untreated asthma animals (group S), S groups treated with 1, 2 and 4 mg/mL extract of *P. oleracea* (groups P1, P2 and P4, respectively), S groups treated with 0.2 and 0.4 mg/mL ALA (groups A0.2 and A0.4, respectively) and S groups treated with 1.25 μg/mL dexamethasone (group D), (n= 4 for ALA treated groups and n=8 for other groups) (Table 1). The extract, ALA and dexamethasone were administered during sensitization period in animals' drinking water. The animals of C and S groups were given drinking water alone. There was no significant difference in the used drinking water between different groups (each rat used averagely 40 mL/day drinking water).

Animals (Male Wistar rats weighing 220 ± 25 g) were kept at 24 ± 2°C with relative humidity of 44-56 % and sensitized on days 1, 2 and 3 by intraperitoneal injections of 1mg/kg ovalbumin (OVA) + 100 mg $Al(OH)_3$ as adjuvant. OVA and $Al(OH)_3$ were added in 1 mL of 0.9% sterile saline and was shaken vigorously and immediately injected. Animals were exposed to 1% OVA aerosol for 20 min with an air flow of 8 L/min



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**Table 1. Various experimental animal groups used for the evalution of anti-oxidant and anti-inflammatory effect of *Portulaca oleracea***

| Groups | Definition | | Abbreviate name | n |
|---|---|---|---|---|
| Sensitized (asthma) | Non-treated sensitized group | | Group S | 8 |
| Treated groups | Treated with *P. oleracea* extract | 1 mg/mL | Group P1 | 8 |
| | | 2 mg/mL | Group P2 | 8 |
| | | 4 mg/mL | Group P4 | 8 |
| | Treated with alpha linolenic acid (ALA) | 0.2 mg/mL | Group A0.2 | 4 |
| | | 0.4 mg/mL | Group A0.4 | 4 |
| | Treated with Dexamethasone | 1.25 µg/mL | Group D | 8 |
| Control | non-sensitized | | Group C | 8 |

Sensitization of rats were done by intraperitoneal and inhaled ovalbumin. In treated groups, the extract, ALA or dexamethasone were administered in their drinking water during sensitization period.

produced by a DeVilbiss PulmoSonic nebulizer (DeVilbiss Health Care Ltd, Feltham, UK) on days 6, 9, 12, 15, 18 and 21 in a 0.8 $m^3$ chamber, with animal normal-breathing. Rats were housed in a caging system with clean filtered air (Maximiser, Thorens Caging System Inc, Hazleton, PA, USA).[37] Temperature was maintained at 22 ± 2ºC on a 12 h light/dark cycle and water and food available *ad libitum* during experimental period.

**Plant and Extracts**

*P. oleracea* was collected from Sabzevar city, Khorasan Razavi province, Iran, in July. A voucher sample was preserved for reference in the herbarium of the school of pharmacy, Mashhad University of Medical Sciences (Herbarium No: 240-1615-12). The leaves of this plant were came apart and dried in shadow. The extract was obtained by maceration method. For preparation of hydro-ethanolic extract, 100 g of *P. oleracea* powder in 1000 mL ethanol 70% was macerated in laboratory temperature for 72 h. To prepare the dry extract, the solution was dried by rotary evaporator and the solvent was isolated. The yield extract was 17.5%. Finally, the extract concentration was adjusted to 10 mg/mL by adding distilled water to the dried extract.[27]

**Measurement of Serum Antioxidant Levels**

Five mL of blood samples was taken by cardiac puncture immediately after sacrificing and exposing the animal's chest on day 22 study. For measurement of oxidant, antioxidant markers, blood samples were collected into test tube and placed at room temperature for 1 h.The samples were then centrifuged at 3500 g for 10 min. The supernatant was collected and immediately stored at −70 °C until analyzed. For evaluation of total and differential white blood cells, two mL of blood sample was collected into the test tube containing anticoagulant ethylenediaminetetraacetic acid (EDTA).

**Thiol**

Serum thiol concentration was measured using 2, 2´-dinitro-5, 5´-dithiodibenzoic acid (DTNB), which reacts with the thiols. The product has a yellow-colored complex with a peak absorbance at 412 nm. Fifty micro liter serum supernatant was added to 1 mL Tris-EDTA buffer (pH 8.6) in 1 mL cuvettes and sample absorbance was read at 412 nm against Tris-EDTA buffer alone (A1). 20 micro liter DTNB reagents (10 mmol/L in methanol) were then added to the mixture and after 15 min (stored in laboratory temperature) the sample absorbance was read again (A2). As a blank (B), the absorbance of DTNB reagent was also read. Total thiol concentration (mmol/L) was calculated from the following equation: [38]

(A2–A1–B)×1.07/0.05×13.6.

**Superoxide Dismutase (SOD)**

Measurement of SOD activity was performed by colorimetric assay involving generation of superoxide by pyrogallol auto-oxidation and the inhibition of superoxide-dependent reduction of the tetrazolium dye,



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MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to its formazan by SOD at 570 nm.[39] SOD activity of I unit was defined as the amount of enzyme causing 50% inhibition in the MTT reduction rate.

**Catalase (CAT)**

CAT activity was estimated using the principle of the assay, which is based on determination of the rate constant, k (dimension: s-1, k) of hydrogen peroxide decomposition.[40] The decrease in absorbance at 240 nm per minute was measured and the rate constant of the enzyme was determined and activities were expressed as k (rate constant) per liter.

**Measurement of Serum Oxidant Levels**

**Nitric Oxide (NO)**

Serum total stable oxidation products of NO metabolism ($NO_2$-/$NO_3$-) were assessed using a Griess reagent, which consisted of sulfanilamide (SULF) and N-(1-Naphthyl)ethylenediamine dihydrochloride (NEDD).[41] The frozen sample was allowed to reach a temperature of 25˚C followed by being deproteinized by zinc sulfate solution (Sigma, USA). The liquefied serum was then centrifuged at 12000 g for 10 minutes. Aliquots (300 µL) of the clear supernatant was mixed with Griess reagents including 300 µL SULF (2% w/v, Sigma, USA) in 5% HCl and 300 µL NEDD (0.1% w/v, Sigma, USA) in $H_2O$ in a test tube. For the reduction of nitrate to nitrite, 300 µL saturated solutions of vanadium (III) chloride ($VCl_3$; Sigma, USA) in 1 M HCl was added and incubated for 2 h at 30˚C in the dark. The absorbance of samples was measured at 540 nm against a blank containing the same concentrations of ingredients. Linear regression was used to determine NO concentration from standard curve of $NaNO_2$. The final results were expressed as µmol.[41]

**Malondialdehyde (MDA)**

MDA levels, as an index of lipid peroxidation reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS). The resultant product has a red-colored complex, which has peak absorbance at 535 nm. One milliliter serum supernatant was added with 2 mL TBA/trichloroacetic acid (TCA)/HCl reagent and the solution was heated in a water bath for 40 min. The solutions were cooled and centrifuged within 1,000×g for 10 min. The absorbance was measured at 535 nm.[42,43] The MDA concentration was calculated using the equation: C(M)=Absorbance/(1.56×105).

**White blood Cells Count**

The blood sample prepared for WBC counting was stained with Turk solution consisted of 1 mL of glacial acetic acid, 1 mL of gentian violet solution 1 % and 100 mL distilled water (1:10 dilution), and total white blood cell (WBC) counted in duplicate in a hemocytometer (in a Burker chamber). Differential cell counts were measured on a thin slide, prepared with a smearing blood sample, staining with Wright-Giemsa's. Differential cell analysis was carried out under a light microscope according to staining and morphological criteria, by counting 100 cells, and the percentage of each cell type was calculated.

**Statistical Analysis**

Data were presented as means ± SEM. The data of control, sensitized and treated groups were compared using one way analysis of variance (ANOVA) with Tukey-Kramer's post-test. The results of three concentrations of extract and two concentrations of ALA were also compared using ANOVA with Tukey-Kramer's post-test. The statistical analysis was performed using InStat (GraphPad Software, version 3.06, Inc, La Jolla, USA). *p* values less than 0.05 were considered as statistical significance.

## RESULTS

**Antioxidants Markers**

Serum concentrations of thiol, SOD and CAT in sensitized group were significantly lower than those of control group ($p<0.001$ for all cases; Figure 1).

Treatment of sensitized animals with dexamethasone, two higher concentrations of the extract and both concentration of ALA led to significant increase in serum concentration of thiol, treatment with dexamethasone, the highest concentration of the extract and both concentrations of ALA resulted in significant increase in serum concentration of SOD and treatment with dexamethasone, two higher concentrations of the extract and the highest concentration of ALA led to significant increase in serum concentration of CAT compared to sensitized group ($p<0.01$ to $p<0.001$; Figure 1). However, serum concentrations of thiol,


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SOD and CAT in treated S groups with dexamethasone, all concentrations of extract and ALA were significantly lower compared to control group ($p$<0.001 for all cases; Figure 1).

The effects of highest extract concentration on thiol, SOD and CAT values and the effects of medium concentration on thiol and CAT values were significantly higher than the effect of low extract concentration ($p$<0.05 to $p$<0.001, Table 2). The effect of high ALA concentration on thiol and CAT were also significantly higher than the effect of its low concentration ($p$<0.001 for both cases, Table 2).

The effect of low concentration of the extract (1 mg/mL) on serum concentration of SOD and the highest concentration of extract (4 mg/mL) on serum concentration of CAT was significantly lower than that of the ALA ($p$<0.01 for both cases, Table 2).

The effects of two lower concentrations of the extract (1 and 2 mg/mL) on serum level of thiol and SOD and the effects of its all concentrations on CAT were significantly lower than dexamethasone treatment ($p$<0.01, to $p$<0.001; Figure 1), The effects of low concentration of ALA (0.2 mg/mL) on serum levels of thiol and CAT also were significantly lower than the effect of dexamethasone treatment ($p$<0.001 for both cases; Figure 1a and c).

**Oxidants**

Serum concentrations of $NO_2$, $NO_3$ and MDA in sensitized group were significantly higher than those of control group ($p$<0.001 for all cases; Figure 2).

Treatment of sensitized animals with dexamethasone, all concentrations of the extract and ALA led to significant reduction in serum levels of $NO_2$ and $NO_3$ ($p$<0.001 for all cases; Figure 2 a and b). In addition, treatment with dexamethasone, the highest concentration of extract (4 mg/mL) and ALA (0.4 mg/mL) caused significant reduction in serum concentration of MDA compared to untreated sensitized group ($p$<0.001 for all cases; Figure 2 c). However, serum level of $NO_2$ in treated group with low extract concentration and $NO_3$ in all treated groups were still significantly higher than control group ($p$<0.05 to $p$<0.001). MDA level in treated groups with two lower extract and low ALA concentrations was also significantly higher than control group ($p$<0.001 for all cases; Figure 2).

**Table 2. Values of serum thiol, SOD, CAT, $NO_2$, $NO_3$, and MDA concentration in treated groups with three concentrations of *P. oleracea* (S+P), and two concentrations of alpha linolenic acid in sensitized animals (S+A), (n=4 for ALA-treated groups and n=8 for other group)**

| Groups | Thiol (µM) | SOD (U/mL) | CAT (U/mL) | $NO_2$ (µM) | $NO_3$ (µM) | MDA (nM) |
|---|---|---|---|---|---|---|
| **S + P1** | 0.04±0.005 | 0.008±0.0005 | 0.017±0.001 | 3.72 ± 0.1 | 23.22 ± 0.9 | 1.07 ± 0.14 |
| **S + P2** | 0.08±0.004 ++ | 0.013±0.001 | 0.033±0.01 + | 3.39 ± 0.06 | 18.09 ± 1.24 + | 0.74 ± 0.09 |
| **S + P4** | 0.16±0.01 +++### | 0.024±0.003 +++## | 0.053±0.003 +++## | 2.51 ± 0.14 +++### | 15.51 ± 1.24 +++ | 0.42 ± 0.03 +++ |
| **S + A0.2** | 0.07±0.005 | 0.018±0.002 ** | 0.014±0.002 | 2.92 ± 0.1 *** | 17.48 ±0.52 * | 0.72 ± 0.04 * |
| **S + A0.4** | 0.18±0.01 +++ | 0.02±0.002 | 0.077±0.005 **+++ | 2.07 ± 0.05 +++ | 14.97 ± 0.98 | 0.4 ± 0.03 +++ |

Values are presented as mean + SEM. Three concentrations of *P. oleracea* were 1, 2 and 4 mg/mL, two concentrations of alpha linolenic acid were 0.2 and 0.4 mg/mL. SOD; superoxide dismutase, CAT; catalase, MDA; Malondialdehyde.

Statistical significance for the difference between the data of S + A *vs* S + P: $^{*}p$< 0.05, $^{**}p$< 0.01, $^{***}p$< 0.001.

Statistical significance for the difference between the data of S + P2 and S + P4 *vs* S + P1, and also S + A 0.4 *vs* S + A 0.2: $^{+}p$< 0.05, $^{++}p$< 0.01, $^{+++}p$< 0.001.

Statistical significance for the difference between the data of S + P4 *vs* S + P2: $^{\#\#}p$< 0.01, $^{\#\#\#}p$< 0.001.

The statistical comparisons were made using Tukey–Kramer multiple pot test.



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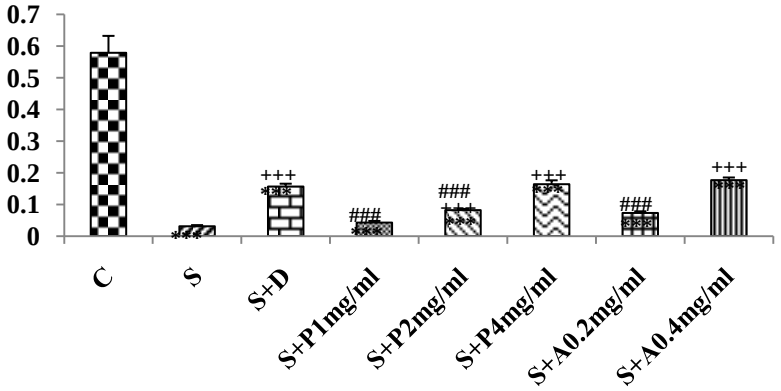
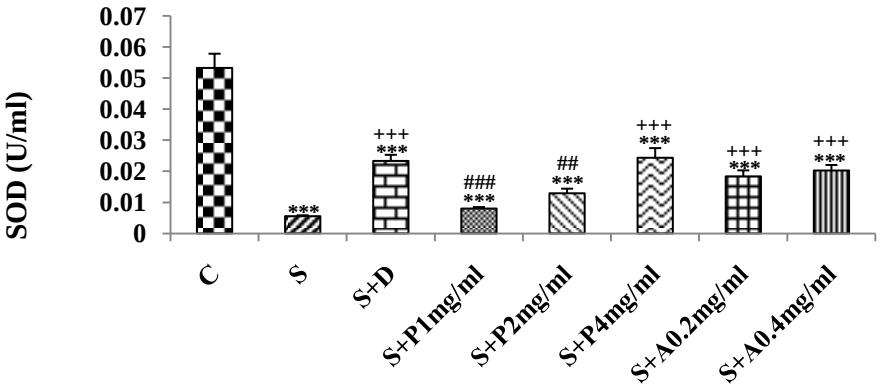



**(A)**

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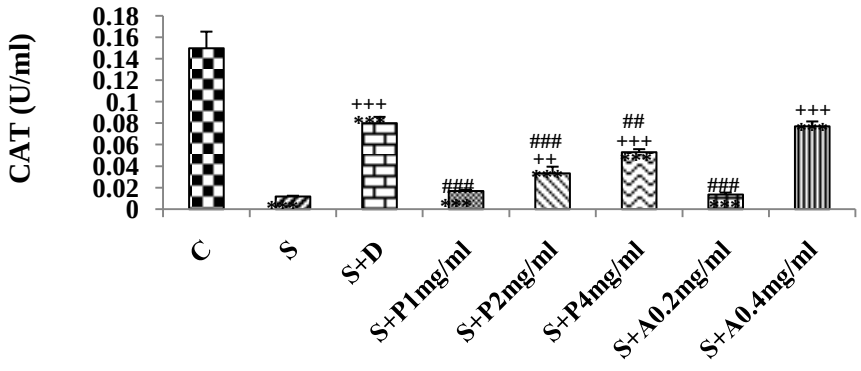


**(C)**

**Figure 1. The levels (mean±SEM) of serum (a) thiol, (b) superoxide dismutase (SOD) and (c) catalase (CAT ) in control rats (C), sensitized animals (S), S groups treated with dexamethasone (S+D), three concentrations of *P. oleracea* (S+P) and two concentrations of alpha linolenic acid (S+A), (n=4 for ALA treated groups and n=8 for other group).**

**Statistical significance for the difference between the data of S, S + D, S + P and S + A *vs* C: $^{***}$ $p<0.001$**

**Statistical significance for the difference between the data of S + D, S+P and S + A *vs* S:**

**$^{++}$ $p<0.01$, $^{+++}$ $p<0.001$**

**Statistical significance for the difference between the data of versus S+P and S + A *vs* S+D: $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$. The statistical comparisons were made using Tukey–Kramer multiple pot test.**



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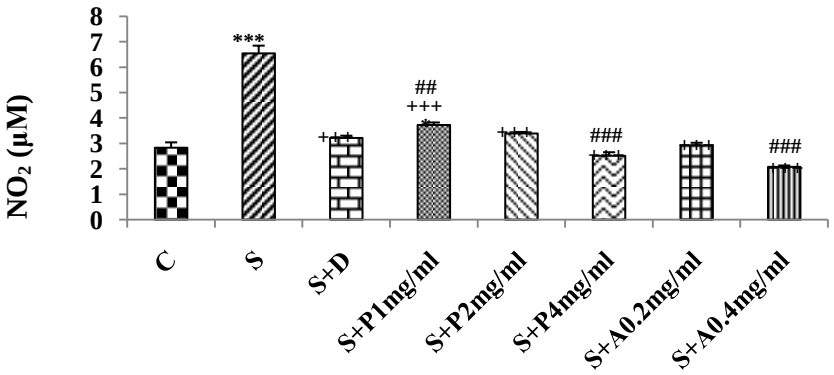
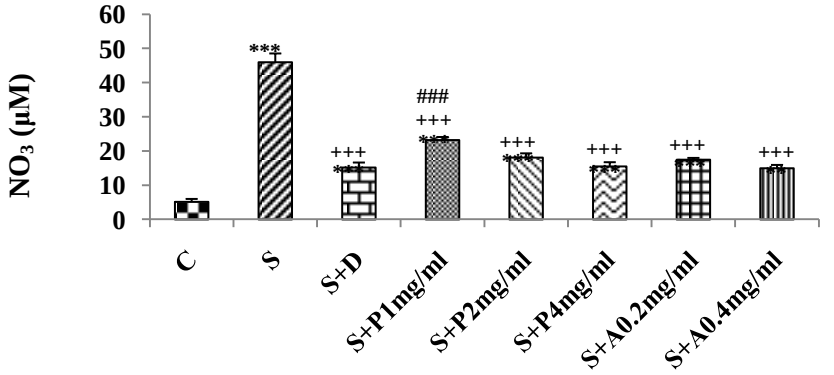
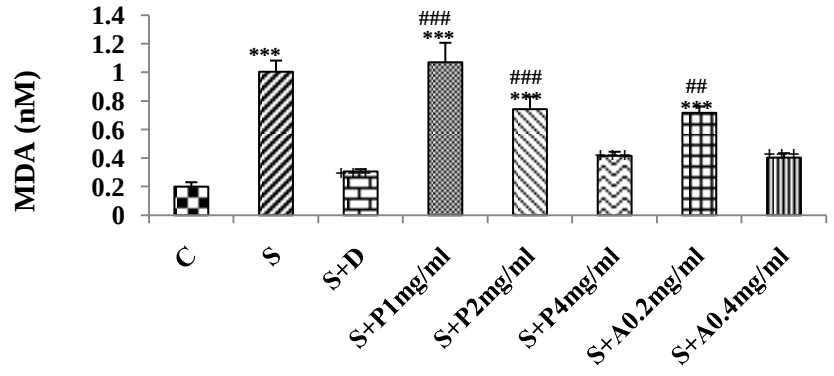



**(A)**




**(B)**




**(C)**

**Figure 2. The serum levels (mean±SEM) of (a) $NO_2$, (b) $NO_3$ and (c) malondialdehyde (MDA) in control rats (C), sensitized animals (S), S groups treated with dexamethasone (S + D), three concentrations of *P. oleracea* (S + P) and two concentrations of alpha linolenic acid (S+A), (n=4 for ALA treated groups and n=8 for other group)**

**Statistical significance for the difference between the data of S, S+D, S+P and S+A *vs* C: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.**

**Statistical significance for the difference between the data of S+D, S+P and S + A *vs* S: $^{+++}p<0.001$**

**Statistical significance for the difference between the data of S+P and S+A *vs* S+D : $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.**

**The statistical comparisons were made using TukeyKramer multiple pot test.**



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The effect of highest extract concentration (4 mg/mL) on serum $NO_2$, $NO_3$ and MDA values ($p$<0.001 for all cases) and its medium concentration (2 mg/mL) on $NO_3$ level ($p$<0.05) were significantly higher than the effect of its low concentration (1 mg/mL), (Table 2). There was significantly higher reduction in $NO_2$ level due to treatment with highest compared to medium concentration of the extract ($p$<0.001, Table 1). The effects of high ALA treatment on $NO_2$ and MDA values were also significantly higher compared to its low concentration ($p$<0.001 for both cases, Table 2).

The effects of low concentration of extract (1 mg/mL) on serum level of $NO_2$ ($p$<0.001), $NO_3$ and MDA ($p$<0.05 for both case) were significantly lower than that of the effects of corresponding concentrations of ALA (Table 2).

The effects of treatment with low extract concentration on $NO_2$ and $NO_3$ and the effect of its two lower concentrations (1 and 2 mg/mL) and low ALA (0.2 mg/mL) concentration on MDA values were lower than the effect of dexamethasone treatment ($p$<0.01, p<0.001; Figure 2). However, the effects of high extract (4 mg/mL) and ALA (0.4 mg/mL) on serum level of $NO_2$ were higher than dexamethasone treatment ($p$<0.001 for both cases; Figure 2 a).

**Total WBC and Differential WBC**

Total WBC number and percentages of eosinophil and neutrophil were significantly increased but lymphocyte percentages was decreased in S group compared to control animals ($p$<0.001 for all cases; Figures 3 and 4).

Total WBC number in blood of all treated groups and percentages of eosinophil in treated groups with two higher concentrations of the extract (2 and 4 mg/mL) and the highest concentration of ALA (0.4 mg/mL) were significantly decreased compared to sensitized group ($p$<0.01 to $p$<0.001). Percentages of neutrophil in treated groups with dexamethasone, two higher concentrations of the extract and all concentrations of ALA were significantly decreased but percentages of lymphocyte were significantly increased compared to sensitized group ($p$<0.001 for all case; Figures 3 and 4).

However, the percentages of eosinophil in treated groups with dexamethasone, low concentrations of the extract (1mg/mL) and ALA, the percentages of neutrophil in treated groups with dexamethasone and two lower concentrations of the extract were higher, but the percentages of lymphocyte in treated groups with low concentration of the extract was significantly lower than those of control group ($p$<0.05 to $p$<0.001; Figures 3 and 4).

**Table 3. Values of blood total and differential WBC count in three concentrations of *P. oleracea* (S+P) and two concentrations of alpha linolenic acid (S+A), (n=4 for ALA treated groups and n=8 for other group)**

| Groups | Total WBC | Eosinophil | Monocyte | Neutrophil | Lymphocyte |
|---|---|---|---|---|---|
| **S + P1** | 8312.5±303.37 | 4.37±0.37 | 4. 5±0.32 | 31.87±1.0 | 59.25±0.59 |
| **S + P2** | 7893.75±325.20 | 1.37±0.32 +++ | 4.25±0.31 | 23.25±0.99 +++ | 71.12±0.61 +++ |
| **S + P4** | 7425±575 | 1.25±0.31 +++ | 3.75±0.36 | 20.87±0.47 +++ | 74.12±0.97 +++# |
| **S + A0.2** | 8075±1026.4 | 3±0.40 | 3.75±0.47 | 21.25±0.62 *** | 72±0.81 *** |
| **S + A0.4** | 7875±426.96 | 1±0.4 + | 2.25±0.62 | 18.75±0.62 + | 78±0.7 *++ |

Values are presented as mean + SEM. The data of WBC is their count in 1 mL blood and those of each type of WBC is the percentage of total WBC. Three concentrations of *P. oleracea* (P) were 1, 2 and 4 mg/mL, two concentrations of alpha linolenic acid were 0.2 and 0.4 mg/mL and that of dexamethasone was 1.25 µg/ mL.

Statistical significance for the difference between the data of S + A *vs* S+P: $^{*}$ $p$<0.05, $^{***}$ $p$< 0.001

Statistical significance for the difference between the data of S + P2 and S+P4 *vs* S + P1, and also S+A 0.4 *vs* S + A 0.2: $^{+}$ $p$< 0.05, $^{++}$ $p$<0.01, $^{+++}$ $p$< 0.001

Statistical significance for the difference between the data of S+P4 *vs* S + P2: $^{\#}p$< 0.05

The statistical comparisons were made using Tukey–Kramer multiple pot test.



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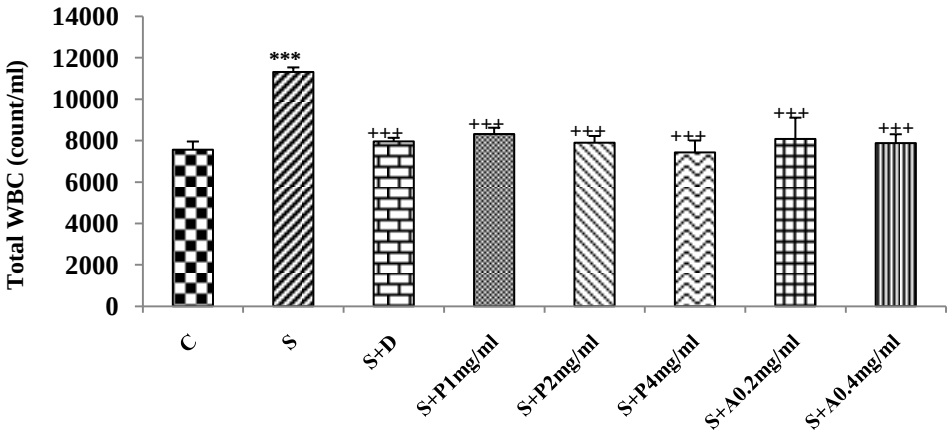
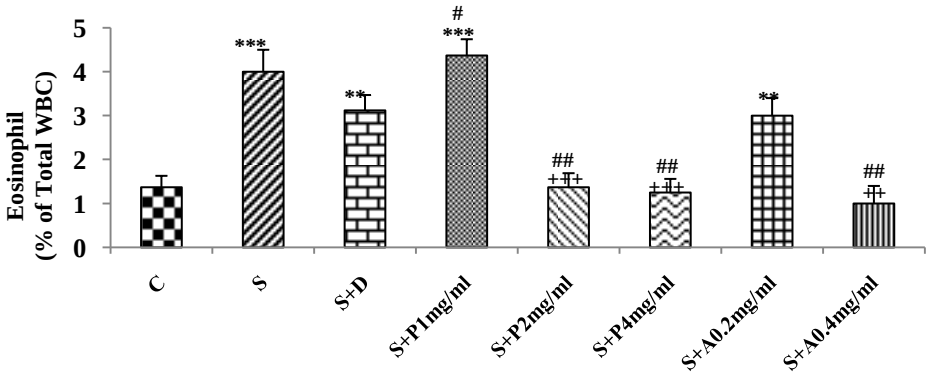



**(A)**

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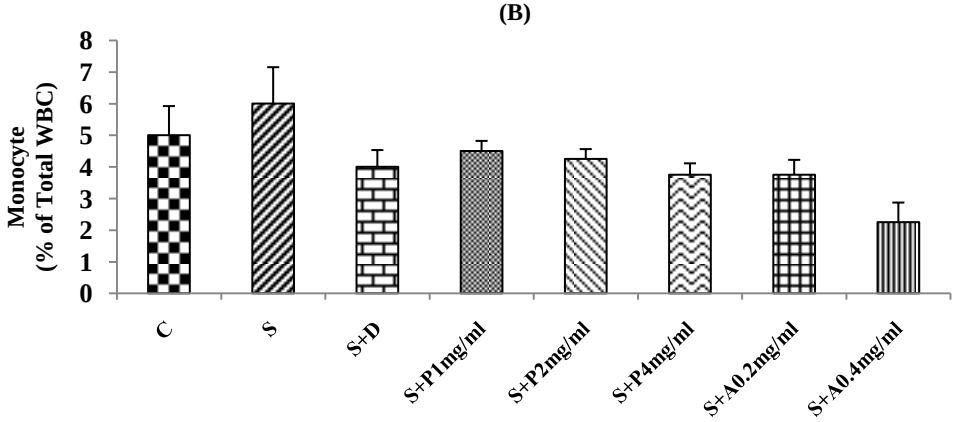


**(C)**

**Figure 3. Values (mean±SEM) of total WBC number in one mL blood (a) and percentages of eosinophil (b) and monocyte (c): in control rats (C), sensitized animals (S), S groups treated with dexamethasone (S + D), three concentrations of *P .oleracea* (S + P) and two concentrations of alpha linolenic acid (S+A), (n=4 for ALA treated groups and n=8 for other group)**
**Statistical significance for the difference between the data of S, S+D, S+P and S+A *vs* C: $^{**}p<0.01$, $^{***}p<0.001$**
**Statistical significance for the difference between the data of S + D, S + P and S+A *vs* S: $^{++}p<0.01$, $^{+++}p<0.001$**
**Statistical significance for the difference between the data of S + P and S + A *vs* S+D: $^{\#}p<0.05$, $^{\#\#}p<0.01$**
**The statistical comparisons were made using Tukey–Kramer multiple pot test.**



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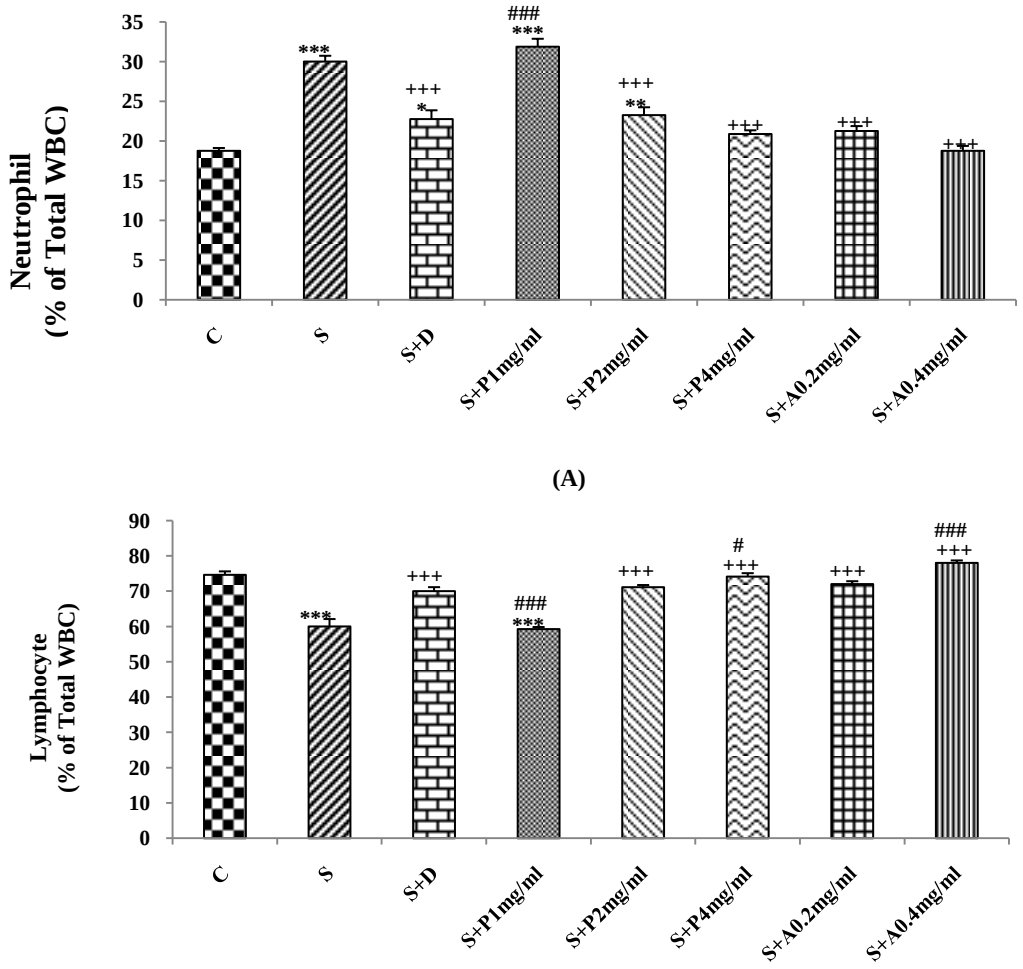


**Figure 4. Percentages (mean±SEM) of neutrophil (a) and lymphocyte (b): in control rats (C), sensitized animals (S), S groups treated with dexamethasone (S+D), three concentrations of *P. oleracea* (S+P) and two concentrations of alpha linolenic acid (S+A), (for each group, n=8).**

**Statistical significance for the difference between the data of S, S+D, S+P and S+A *vs* C: $^{*}p<0.05$, $^{**}p< 0.01$, $^{***}p<0.001$**

**Statistical significance for the difference between the data of S+D, S+P and S + A *vs* S: $^{+++}p<0.001$**

**Statistical significance for the difference between the data of S+P and S + A *vs* S+D: $^{\#}p<0.05$, $^{\#\#\#}p< 0.001$**

**The statistical comparisons were made using Tukey–Kramer multiple pot test.**

The effects of highest (4 mg/mL) and medium (2 mg/mL) concentrations of the extract on percentages of eosinophil, neutrophil and lymphocyte were significantly higher than its low concentration (1 mg/mL), ($p$<0.001 for all cases, Table 3). The effect of highest concentration of the extract on percentages of lymphocyte was also significantly higher than its medium concentration ($p$<0.05, Table 2). In addition, the effects of high (0.4 mg/mL) concentration of ALA on percentages of eosinophil, neutrophil and lymphocyte were significantly higher than its low concentration (0.2 mg/mL), ($p$<0.05 to $p$<0.01, Table 3).

The effects of low concentration of the extract (1 mg/mL) on percentages of neutrophil and lymphocyte ($p$<0.001 for both cases) and the effect of its highest concentration (4 mg/mL) on percentages of lymphocyte ($p$<0.05) were significantly lower than that of the ALA (Table 3).

The effects of low concentration of the extract



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treatment on percentages of eosinophil ($p$<0.05), neutrophil and lymphocyte ($p$<0.001 for both cases) were significantly lower than the effect dexamethasone (Figures 3 and 4). However, The effects of treatment with two higher concentrations of the extract and high concentration of ALA on percentages of eosinophil and the effects of high extract and ALA concentrations on lymphocyte percentages were significantly higher than dexamethasone treatment ($p$<0.05 to $p$<0.001; Figures 3b and 4b).

## DISCUSSION

The anti-inflammatory and antioxidant effect of *P. oleracea* and its constituent, ALA was reported previously. Therefore, the effect of the extract of this plant and ALA on inflammatory cells and serum oxidant levels in sensitized rats were examined in the present study. Serum concentrations of $NO_2$, $NO_3$ and MDA, total WBC number, and percentages of eosinophil and neutrophil in sensitized animals were significantly higher compared to those of control group. However, thiol, SOD and CAT levels in sensitized group were significantly lower than those of control group.

Increased NO level in exhaled air in asthma and chronic obstructive pulmonary disease was reported previously.[44] NO is an important mediator in the lung, which may contribute to the inflammatory response in asthma[45] and is a possible index of oxidative stress in the airway diseases.[46] It was shown that NO is able to increase the number of Th2 cells and its cytokines, IL-4 and IL-5, which can augment the inflammatory response.[47] Increase MDA and protein carbonyls in BAL fluid and peripheral blood accompanied with oxidative stress, in asthma have been reported.[48] In addition, increased MDA level in patients with different airway diseases including asthma, COPD, and bronchiectasis has been reported.[49] Increased total WBC and eosinophil count have been observed in asthmatic group compared to healthy controls in human.[50] Increased total and differential WBC count but decreased lymphocytes percentage in sensitized animals were also reported.[51]

Increased nitrite, nitrate[52, 53], MDA[54] concentrations and decreased levels of antioxidant parameters (such as SOD, CAT and Thiol)[55] in asthmatic patients, indicate the role of these agents in pathophysiology of asthma. Physiological antioxidant system is impaired in asthma, possibly because of inflammation. Thus, the imbalance between oxidants and antioxidants, which is called oxidative stress, is critical to asthma pathogenesis.[16] Increased eosinophil can release a variety of preformed mediators including eosinophil cationic protein, major basic protein, eosinophil derived neurotoxin, eosinophil peroxidase, superoxide ion and lipoxin A, which can contribute in the airway inflammation in asthma.[56] Increased lymphocytes can also recognize antigen through specific receptors and thereby initiate an inflammatory response by releasing cytokines.[57]

One important component of airway inflammation in asthma is oxidative stress. The production of several oxidazing agents such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH) in macrophages, eosinophils, neutrophils and antigen presenting cells occur in the lung of asthmatic patients. Increased ROS production in asthma is also indicated that can result in lung structural cellular damage through the oxidation of proteins, lipids, and DNA. Increased exhaled markers of oxidative stress such as NO, CO which can lead to cellular injury and pathological changes of the lung is also shown in asthma.[58] All the above studies support the findings of the present study regarding the process of sensitization of rat (induction of animal model of asthma in rat).

Dexamethasone, a known anti-inflammatory drug, used in the present study as a positive control caused reduction of serum level of $NO_2$, $NO_3$ and MDA, as well as total WBC number and percentages of eosinophil and neutrophil but increased serum values of thiol, SOD and CAT. The inhibitory effect of dexamethasone on airway inflammation and its effect on lymphocytes in asthmatic mice have been reported, which supports the results of our study.[59]

Similar to the effect of dexamethasone, treatment of sensitized animals with hydro-ethanolic extract of *P. oleracea* also showed reduction of serum level of $NO_2$, $NO_3$ and MDA, as well as total WBC number and percentages of eosinophil and neutrophil but increased serum values of thiol, SOD and CAT specially for two higher concentrations of the extract. Treatment of sensitized rats with two concentrations of alpha linolenic acid (ALA), which is one of the main constituent of *P. oleracea* also caused reduction of serum level of $NO_2$, $NO_3$ and MDA, as well as total WBC number and percentages of eosinophil and neutrophil but increased serum values of thiol, SOD and CAT. These results indicate anti-inflammatory



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effects of the plant and its constituent, ALA.

Concentration-dependent effects of the extract and ALA on all measured variables were observed in the current study. The effect of highest concentration of the extract on all measured variables except for total WBC and monocyte percentage and the effects of its medium concentration on most measured variables were significantly higher than its low concentration. The effects of highest concentration of the extract also on some variables were significantly higher than its medium concentration. In addition, the effects of the high concentration of ALA on most variables were also higher than the effects of its low concentration. The concentration dependency effect of the extract and ALA on antioxidant and oxidant biomarkers as well as on total and differential WBC also support their anti-inflammatory and antioxidant effects on rat animal model of asthma.

The results of the present study showed increased lymphocyte percent in sensitized and its reduction in treated groups. However, this finding is perhaps due to increased total WBC in sensitized animals and reduction of total WBC in treated groups. In fact, increased number of the lymphocyte using similar method of animal sensitization was reported previously.[60]

In the previous studies also, anti-inflammatory and anti-oxidant effects of *P. oleracea*[19,20] and alpha linolenic acid[33] , were reported, which support the observed effects of the plant and its constituent on sensitized rats in the present study. Potent antioxidant effect of purslane by decreasing enzyme activities and its effect against oxidative stress have been reported.[61] Ethanolic extract of the *P. oleracea*, showed significant analgesic and anti-inflammatory activities, when compared with synthetic drugs.[19] Increased levels of GPx, GR, GST, CAT and SOD, were all found to correlate with elevated glutathione level and decreased MDA and NO in rats due to *P. oleracea* and ALA treatment were also shown.[62] ALA treatment also lead to reduction of TNF-α and IL-6/IL-8, which are involved in the inflammation process.[63] Our previous study also indicated reduction in total and differential WBC count in sensitized guinea pigs treated with an anti-inflammatory drug[60,64], which supports the results of the present study. Therefore, these results suggest that the extract of *P. oleracea* and ALA may have preventive effects on asthma disease by reduction of oxidative stress, inflammatory cells and airway inflammation.

The effects of low concentration of ALA on several measured parameters were significantly higher than the effects of corresponding concentrations of the extract. However, the effect of high concentration of ALA only on serum level of CAT and lymphocyte percentage was higher than the effects of corresponding extract concentrations. The studied concentrations of ALA in the present study were one tenth (10%) of those of the extract of *P. oleracea* (0.2 and 0.4 mg/mL for ALA and 1 and 4 mg/mL for low and high concentrations of the extract). It was shown that the amount of ALA in 100 grams of fresh dry plant is about 300-400 mg or about 0.4%[65], which is much lower than the studied concentrations of ALA used in the present study. These results showed that ALA is only partially responsible for anti-inflammatory and antioxidant effects of the extract of *P. oleracea* in sensitized rats.

The effect of low concentration of the extract and ALA on most measured variables were lower than the effect of dexamethasone treatment. However, the effects of high concentration of the extract and ALA were not statistically different from the effect of dexamethasone or even were significantly higher than dexamethasone treatment group (serum $NO_2$ level, eosinophil and lymphocyte percentage). These antioxidant and anti-inflammatory effect of the extract and its constituent ALA in sensitized rats, which is comparableto the effect of dexamethasone also support these effectiveness of the plant and ALA.

The present study as well as other similar studies [19,20,30,34,36] indicate anti-inflammatory and antioxidant effects of *P. oleracea* and alpha linolenic acid, which could have a therapeutic effect on inflammatory diseases such as asthma by reduction of lung inflammation. However, further studies are needed to evaluate the effect of this plant and its constituent ALA as well as the effect of other constituents of the plant on asthmatic patients.

In conclusion, the results of this study showed a preventive effect of the extract of *P. oleracea* and its constituent alpha linolenic acid on inflammatory cells and serum oxidant, antioxidant levels in sensitized rats comparable to the effect of dexamethasone at used concentrations. The results also suggest that the effect of the plant is perhaps due to its constituent, ALA.



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