



Potential anti-inflammatory and immunomodulatory effects of carvacrol against ovalbumin-induced asthma in rats

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ABSTRACT

Background: Asthma is a complex inflammatory disease which affects multiple individuals worldwide especially pediatric ages.

Aims: This study aimed to assess the possible protective effect of carvacrol, as natural antioxidant anti-inflammatory drug, against bronchial asthma induced experimentally in rats.

Main methods: Rats were randomly allocated into 5 groups; a normal control group, control drug group received only carvacrol, an asthma control group, a standard treatment group receiving dexamethasone (DEXA) and carvacrol treatment group. Bronchial asthma was induced by sensitization with i.p dose followed by challenge with intranasal dose of ovalbumin (OVA). 24 h after the last challenge, absolute eosinophil count (AEC) were determined in bronchoalveolar lavage fluids (BALF). Immunoglobulin E (IgE) was determined in serum. Inflammatory biomarkers like Interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin 13 (IL-13), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were also measured in BALF. Nitrosative stress biomarker namely inducible nitric oxide synthase (iNOS) was determined in BALF as well as oxidative stress biomarkers namely superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) were determined in lung tissue. Additionally, histopathological study, immunohistochemical study of UCN and western blot analysis of SP-D were performed.

Key findings: Carvacrol administration significantly reduced the values of AEC, IgE, IL-4, IL-5, IL-13, TNF-α, IFN-γ, iNOS and MDA, while it significantly increased the values of SOD and GSH as compared to the asthmatic group. Histopathological, immunohistochemical and western blot study reinforced the biochemical results.

Significance: Carvacrol may be a promising protective agent against bronchial asthma induced experimentally in rats.

1. Introduction

Asthma is a chronic life threatening airway disease which is linked with inflammatory disturbances within the lower respiratory tract [1]. About 300 million people are afflicted with asthma globally and this number is rising at an alarming levels reaching asthma-mortality rate over 250,000 people annually [1]. Progression of asthma is accompanied by activation of T helper cells which are considered the key driver of the inflammatory response in asthma, since these cells release various cytokines such as interleukins mainly (IL4, IL5 and IL13) and TNF alpha together with activation of B lymphocytes that produce allergen-specific IgE [2] and release the so called arachidonic acid

pathway metabolites mainly leukotrienes and prostaglandins [3]. This will cause the known hallmarks of asthma: mucus overproduction from goblet cells [4], bronchospasm, reversible airway obstruction and airway hyperresponsiveness with eosinophilic lung [5], besides increasing airway smooth muscle contraction and release histamine from degranulated mast cells [6]. Moreover, these inflammatory cells secrete reactive oxygen species (ROS) that induce oxidative stress and inflammatory processes which are considered to be the main underlying causes of asthma [7]. Many researches notice that ROS level is high in asthmatic people exhalation than in healthy people [8].

Urocortin (URN), a lately identified 40 amino acid peptide, is a member of corticotropin-releasing hormone (CRH) neuropeptide family

Abbreviations: OVA, ovalbumin; AEC, absolute eosinophilic count; IgE, immunoglobulin-E; IL-4, interleukin-4; IL-5, interleukin-5; IL-13, interleukin-13; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; GSH, reduced glutathione; MDA, malondialdehyde; UCN, urocortin; SP-D, surfactant protein-D

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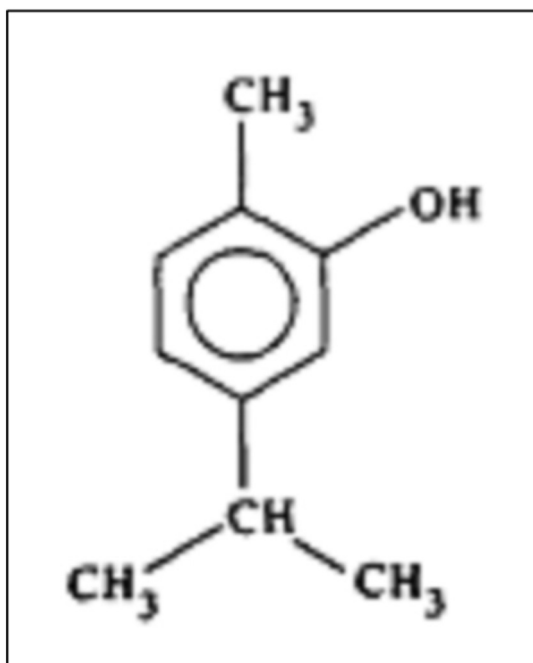


Fig. 1. Chemical structure of carvacrol (C₁₀H₁₄O) [9].

[9]. Accumulated evidences demonstrated that UCN is secreted locally in many inflammatory diseases involving osteoarthritis, rheumatoid arthritis, ulcerative colitis, thyroiditis besides contributing to an increase in the pulmonary vascular permeability through stimulation of CRH receptors [10].

Moreover, surfactant protein D (SP-D), hydrophilic SPs, is a member of collectin proteins family expressed in lungs from goblet cells [11]. It is a part of pulmonary innate immunity involved in host defense mechanisms. It is increased in pulmonary infections playing a key role in orchestrating the phagocytosis of a broad spectrum of pathogens through many steps involving opsonization, aggregation of pathogens and modulation of inflammatory cascade [12].

The currently available asthmatic treatments (glucocorticoids, beta 2 adrenergic agonists, leukotriene modifiers and mast cell stabilizers) depend mainly on relieving the symptoms regardless the underlying causative factor besides their serious adverse risks [13]. Accordingly, the recent studies should be directed towards using natural anti-asthmatic drugs with minimal side effects.

Carvacrol, also called cymophenol (Fig. 1), is a natural monoterpene phenol ingredient of essential oils extracted from different aromatic plants [14]. Multiple studies referred to its great efficacy as anti-inflammatory and antioxidant natural component [15,16], besides its antimicrobial [17], antihepatotoxic [18], antitumor [19,20], neuroprotective [21] and cardioprotective properties [22].

Essential studies demonstrated that peroxisome proliferator activated receptor-alpha (PPAR-α) agonists can suppress airway inflammation in asthma as evidenced by reducing the release of major inflammatory mediators involved in asthma [23]. Therefore, carvacrol, being an agonist on PPAR-α possesses a notable impact on reducing inflammation through decreasing the release of some inflammatory mediators [15,24].

Accordingly, this study discussed the potential protective effect of carvacrol on a rat model of ova-induced bronchial asthma experimentally as compared to the reference standard DEXA.

2. Materials and methods

2.1. Animals

Adult male Wistar albino rats, weighing 140–170 g were purchased from El-Nasr Company, Abo-Zabaal, Egypt and were kept in the Central Animal Housing Facility of Beni-Suef University. Animals were grouped in cages for two weeks prior to the experiment for acclimatization under high human care, provided with food and water ad libitum and survived at 23–25 °C, 40–60% humidity on a 12 h light/dark cycle and were handled according to the recommendations of the National Institutes of Health (NIH) Guide for Care and Use of the Laboratory Animals (Publication No. 8023, revised 1978).

2.2. Chemicals, drugs and reagent kits

Ovalbumin (OVA; grade II), aluminum hydroxide (Al (OH)₃) and Carvacrol were obtained from Sigma Aldrich Chemical Co., USA. DEXA was obtained from El-Kahira Pharmaceuticals & Chemical Industries Co. Egypt. Test reagent kits: IgE ELISA kit was purchased from Express Biotech, USA. IL-4, IL-5, IL-13 kits were obtained from CUSABIO Biotech Co., LTD, China. TNF-α ELISA kit was purchased from Glory Science Company (St. Del Rio, TX). IFN-γ ELISA kit was purchased from Sigma Aldrich Chemical Company, USA. SOD colorimetric kit was purchased from Sigma Aldrich Chemical Industries Co. Egypt, GSH colorimetric kit was purchased from Biodiagnostic Company (Giza, Egypt) and MDA colorimetric kit was purchased from Clinilab Company (Cairo, Egypt). All other used chemicals and reagents were of analytical grade.

2.3. Apparatus

Homogenizer (yellow line, DI18 basic, Germany), Cooling centrifuge (Sigma 3-30K, USA), UV-vis spectrophotometer (Shimadzu, Japan), chemiluminescence western blotting substrate (Chemiluminescent Sensitive Plus HRP Microwell and/or Membrane Substrate; SurModics, Inc., Eden Prairie, MN, USA) (Clarity™ Western ECL substrate - BIO-RAD, USA cat#170-5060) and Deep freezer (−80 °C freezer, Als Angelantoni Life Science, Italy) were used in this study.

2.4. Experimental design

Rats were randomly divided into five main groups in cages each of 10 rats. Group A acted as a vehicle control model, group B acted as control drug model received only carvacrol (15 mg/kg, p.o. for 17 consecutive days), group C acted as OVA-induced asthma model, group D acted as a reference model receiving DEXA drug (1 mg/kg, p.o. for 17 consecutive days), group E acted as treated model receiving carvacrol (15 mg/kg, p.o. for 17 consecutive days). The duration of 17 consecutive days comprises 14 days before challenge and through the 3 days of challenge. All groups except control groups were exposed to ovalbumin i.p sensitization dose.

2.5. Methods

2.5.1. Induction of bronchial asthma

2.5.1.1. Sensitization by ovalbumin. Ovalbumin and aluminum hydroxide (adjuvant) were prepared in concentrations 400 µg/ml and 20 mg/ml, respectively in ratio 1:1. All groups except control groups were exposed to ovalbumin i.p sensitization dose (1 ml normal saline suspension containing 200 µg ova and 10 mg Al (OH)₃) at days 1,2,3 and 11.

2.5.1.2. Intranasal challenge with OVA. Under anesthesia, rats were received 300 µl saline containing 1.5 mg ova at day 20, 21 and 22.

OVA was dripped into both nostrils to reach the airways of the nasal cavity according to the dose described above.

2.5.2. Preparation of blood samples

At day 23, blood samples were collected from retro orbital venous plexus in non-heparinized tubes under light anesthesia and left at room temperature (25 °C) for coagulation then centrifuged at 1500 r.p.m. for 10 min. Supernatant serum layer was carefully withdrawn and stored at –20 °C for estimation of IgE level by biochemical analysis.

2.5.3. Bronchoalveolar lavage fluid (BALF) collection

BALF was collected by incising the pleural space of the chest by cannulating the trachea to gain access to the thoracic organs (lungs) after sacrificing rats by cervical dislocation and bloodletting. The two lungs were washed twice using 5 ml normal saline for each time wash through the cannula with gentle massage. Actual volume of fluid (8 ml) was pulled out and centrifuged using cooling centrifuge for 10 min at 400 r.p.m. The obtained pellets were resuspended in 200 µl phosphate buffer saline [25] for eosinophils counting.

2.5.4. Preparation of tissue sample

After the midline thoracotomy, the two lungs were anatomized, cleaned out from blood by ice cooled saline and blotted with filter paper. The right lung was separated from the left lung then the right lung was used for histopathological, immunohistochemical examination and western blot analysis while the left lung was homogenized and centrifuged at 3000 r.p.m. for 20 min at 4 °C then the supernatant was withdrawn and stored at –80 °C till assaying of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione reduced (GSH).

2.5.5. Biochemical measurements

The absolute eosinophil count (AEC) was determined in pellets within 1 h after collecting of BALF using hemocytometer by introducing freshly prepared Dunge's diluting fluid (0.5 g Eosin Yellow, 0.5 ml of 95% acetone, 0.5 ml of 40% formalin and 99 ml of distilled water) in neubauer's chamber that gives clear esinophilic granules with distinct stain while lysing the RBCs and other WBCs [26]. IgE was assessed in serum by ELISA kit according to the manufacturer's instructions [27]. IL4 [28], IL5 [29], IL13 [30], TNF-α [31], IFN-γ [32] were determined in BALF using ELISA kits according to the manufacturer's instructions. The oxidative stress biomarkers MDA [33], SOD [34] and GSH [35] were measured in lung homogenates using U-V colorimetric method described above.

2.5.6. Detection of iNOS gene expression by quantitative real time PCR (qRT-PCR)

2.5.6.1. RNA isolation and reverse transcriptase. BALF samples were collected and centrifuged at 8000 r.p.m. Pellets was washed and stored at –80 °C till RNA extraction. Total RNA was extracted from BALF using Qiagen extraction kit (Qiagen, USA), according to the manufacturer's instructions, the purity (A260/A280 ratio) and the RNA concentration was obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). RNA samples were stored in aliquots at –80 °C till use. 0.5–2 µg total RNA was reversely transcribed into cDNA using high capacity Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase kit (Fermentas thermoscientific, USA).

2.5.6.2. Quantitative real time PCR (qRT-PCR). Real time PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The used reagents of the reaction Master Mix (25 µl) were shown in the (Table 1). The primer pairs sequences used are shown in the (Table 2). Results were analyzed and quantified; the resulted values of the targeted gene (iNOS) were normalized to the values of the endogenous control gene (β-actin). The relative fold expression

Table 1

Preparation of the reaction master mix components for RT-PCR.

PCR reaction mix component	Volume
Forward primer	1 µl
Reverse primer	1 µl
Syber green mix	12.5 µl
Cdna template	5 µl
RNase free water	5.5 µl
Total volume	25 µl

Table 2

Primer sequences used for the studied gene in RT-PCR.

	Primer sequence
iNOS	Forward primer: 5'-GACCAGAAACTGTCTCACCTG-3' Reverse primer: 5'-CGAACATCGAACGCTCACA-3'
B-actin	Forward primer: 5'-GGTCGGTGTGAACGGATTGG-3' Reverse primer: 5'-ATGTAGGCCATGAGGTCCACC-3'

(relative quantification) was calculated by the delta delta comparative threshold ($\Delta\Delta$ -CT) method according to Applied Biosystem Software. CT of each sample was calculated by the Applied Biosystem Software. Then subtracting CT of the targeted gene (iNOS) from the CT of the control gene (β-actin) to get Δ -CT, after that subtracting Δ -CT of the sample from Δ -CT of the control to get $\Delta\Delta$ -CT, finally the relative quantification was calculated by $2^{-\Delta\Delta CT}$ [36].

2.5.7. Histopathological study

After cleaning out the lungs from blood as described above, the right lung of each rat was fixed in 10% formalin in saline for 72 h and embedded in paraffin. 5 µm paraffin sections were taken and stained with hematoxylin & eosin (H&E) as described earlier by Bancroft and Steven for evaluation of the degrees of inflammation [37]. The degree of inflammation was assessed for peribronchiolar inflammatory cells infiltration, alveolar inflammatory cells infiltration, thickened alveolar wall with narrow alveoli and macrophages infiltration. Inflammation was scored on the basis of the following: (–) refers to no change, (+) refers to mild change, (++) refers to moderate change and (+++) refers to severe change.

2.5.8. Immunohistochemical study

Paraffin sections (3 µm) were immunohistochemically stained using avidin-bioin complex technique. These paraffin sections were deparaffinized and immersed in 3% hydrogen peroxide for 10 min to consume hydrogen peroxidase activity. Non-specific binding sites were blocked by 0.2% bovine serum albumin (BSA), UCN antibody (Chongqing Biospes Co., Ltd) [38] was applied to lung sections and incubated at 37° for 60 min. Finally, lung tissues were incubated with streptavidin biotinylated peroxidase complex for 45 min and washed with phosphate-buffered saline (PBS) for estimation of UCN expression [10,39]. The average brown color intensity of 3–5 microscopic fields for each tissue section was measured and analyzed using Image-J software (National Institute of Health “NIH”, USA).

2.5.9. Western blot analysis

Using western blot analysis, surfactant protein D (SP-D) can be detected in lung tissues. Lung sections were homogenized with RIPA lysis buffer containing equal volumes of protease and phosphatase inhibitor enzymes (protein lysates) then centrifuged at 4 °C at 16,000 ×g for 20 min and equal concentration of proteins in each sample were determined using Bradford assay kit (Bio-Rad, USA). Western blot technique was done through few steps. Briefly, 20 µg of each sample protein lysate were fractioned on 8% SDS-PAGE and electrophoretically transferred into nitrocellulose membrane (Bio-Rad laboratories) which contains protein plots that blocked with 5% (w/v) skimmed milk and

incubated overnight at 4 °C with primary antibody (anti SP-D) thereafter for 1 h with secondary antibody. Endogenous control beta actin antibody was used to calculate the density of SP-D protein bands relatively to beta actin protein bands to normalize the data, protein bands were visualized using enhanced chemiluminescence western blotting substrate [40].

2.6. Statistical analysis

Data of all measured parameters were expressed as mean $\pm$ SEM. ANOVA test was performed to determine the statistically significant difference between the experimental groups using SPSS (version 22) program followed by post hoc analysis using Bonferroni test, considering the p value < 0.05 for significance.

3. Results

3.1. Effect of carvacrol on AEC in BALF pellets

AEC was investigated as marker of lung eosinophilia. The value of normal control group regarding AEC in BALF was 3.87 ± 0.32 . Intranasal OVA challenge induction caused a significant elevation in AEC reaching to 2197% compared to the normal control. DEXA caused a significant decrease in AEC reaching about 9.5% compared to the asthma control group. Similarly, pre-treatment with carvacrol caused a significant decrease in AEC reaching about 10% compared to the asthma control group. Carvacrol control group exhibited no significant difference as compared to the normal control group (Table 3).

3.2. Effect of serum on IgE

IgE was investigated as a marker of allergic reactions. The values of normal control group regarding IgE in serum was 16.0 ± 1.5 . Intranasal OVA challenge significantly increased IgE serum level nearly by 2979% in comparison with the normal control group. DEXA significantly decreased IgE serum level nearly by 17% compared to the asthma control group. Similarly, pre-treatment with carvacrol significantly decreased IgE serum level nearly by 18.5% compared to the asthma control group. Carvacrol control group exhibited no significant difference as compared to the normal control group (Table 3). These data indicated that carvacrol decrease allergic reactions in ova-induced bronchial asthma.

3.3. Effect of carvacrol on IL-4, IL-5 and IL-13 levels in BALF

IL-4, IL-5 and IL-13 were investigated as markers of inflammation. The BALF values of normal control group regarding IL-4, IL-5 and IL-13 were 17.26 ± 0.89 , 14.1 ± 0.74 and 31.76 ± 1.93 respectively. OVA challenge caused a significant increase in IL-4, IL-5 and IL-13

Table 3

Effect of Carvacrol on AEC in BALF and serum IgE level in OVA-induced bronchial asthma in rats.

Groups	BALF AEC count/mm ³	Serum IgE ng/ml
Normal control	3.87 ± 0.32	16.00 ± 1.5
Carvacrol (15 mg/kg)	4.26 ± 0.32	6.74 ± 0.350
Asthmatic group (OVA)	85.16 ± 7.5^a	476.6 ± 46.78^a
Dexamethasone (1 mg/kg)	8.05 ± 0.76^b	85.71 ± 7.89^b
Carvacrol in asthmatic rats	8.7 ± 0.83^b	88.41 ± 5.6^b

Statistical analysis was carried out by one way ANOVA followed by Tukey–Kramer Multiple Comparison Test.

Each value represents the mean of 8–10 rats $\pm$ SEM.

^a Significantly different from normal control group at $p < 0.05$.

^b Significantly different from asthmatic group at $p < 0.05$.

Table 4

Effect of carvacrol on BALF level of cytokines (IL-4, IL-5 and IL-13) in OVA-induced bronchial asthma in rats.

Groups	BALF IL-4 pg/ml	BALF IL-5 pg/ml	BALF IL-13 pg/ml
Normal control	17.26 ± 0.89	14.1 ± 0.74	31.76 ± 1.93
Carvacrol (15 mg/kg)	14.94 ± 0.91	19.53 ± 0.45	25.25 ± 1.85
Asthmatic group (OVA)	161.39 ± 16.46^a	147.04 ± 14.74^a	242.06 ± 14.87^a
Dexamethasone (1 mg/kg)	$76.36 \pm 6.9^{a,b}$	$61.91 \pm 3.71^{a,b}$	$73.94 \pm 5.27^{a,b}$
Carvacrol in asthmatic rats	$79.24 \pm 7.02^{a,b}$	$76.65 \pm 5.29^{a,b}$	$67.81 \pm 5.33^{a,b}$

Statistical analysis was carried out by one way ANOVA followed by Tukey–Kramer Multiple Comparison Test.

Each value represents the mean of 8–10 rats $\pm$ SEM.

^a Significantly different from normal control group at $p < 0.05$.

^b Significantly different from asthmatic group at $p < 0.05$.

nearly by 935%, 1043% and 762%, respectively, compared to the normal control group. DEXA significantly decreased IL-4, IL-5 and IL-13 nearly by 47%, 42% and 30.5%, respectively, compared to the asthma control group. Similarly, pre-treatment with carvacrol significantly decreased IL-4, IL-5 and IL-13 nearly by 49%, 52% and 28%, respectively, compared to the asthma control group. Carvacrol control group exhibited no significant difference as compared to the normal control group (Table 4). These data indicated that carvacrol improved inflammation in ova-induced bronchial asthma.

3.4. Effect of carvacrol on TNF- α , IFN- γ and iNOS in BALF

TNF- α , IFN- γ were investigated as markers of inflammation and iNOS as a marker of nitrosative stress. The BALF values of normal control group regarding TNF- α , IFN- γ and iNOS were 24.04 ± 1.03 , 28.35 ± 0.81 and 1.17 ± 0.13 , respectively. Intranasal OVA challenge caused significant increase in TNF- α , IFN- γ and iNOS nearly by 533%, 496% and 1505% respectively compared to the normal control group. DEXA significantly decreased TNF- α , IFN- γ and iNOS BALF levels nearly by 50%, 49.5% and 28.5%, respectively compared to the asthma control group. Similarly, pre-treatment with carvacrol significantly decreased TNF- α , IFN- γ and iNOS BALF level nearly by 53.5%, 46.5% and 17.5% respectively compared to the asthma control group. Carvacrol control group exhibited no significant difference as compared to the normal control group (Fig. 2).

3.5. Effect of carvacrol on tissue SOD activity and GSH and MDA contents

SOD, GSH and MDA were investigated as markers of oxidative stress. The values of normal control group regarding SOD, GSH and MDA in lung tissues were 3.24 ± 0.10 , 64.70 ± 1.70 and 5.30 ± 0.44 , respectively. Intranasal OVA challenge caused a significant drop in SOD activity and GSH tissue content to reach 17% and 37.5%, respectively, and a significant increase in MDA tissue level to reach 2000% compared to the normal control group. DEXA significantly increase SOD activity and GSH tissue content to reach about 296% and 200% with a significant decrease observed in MDA tissue content to reach about 52% with compared to the asthma control group. Similarly, pre-treatment of carvacrol significantly increased SOD activity and GSH tissue content to reach about 269% and 176.5%, respectively, while it significantly decreased MDA tissue content to reach about 24% compared to the asthma control group. Carvacrol control group exhibited no significant difference as compared to the normal control group (Fig. 3). These data indicated that carvacrol ameliorated oxidative stress in bronchial asthma.

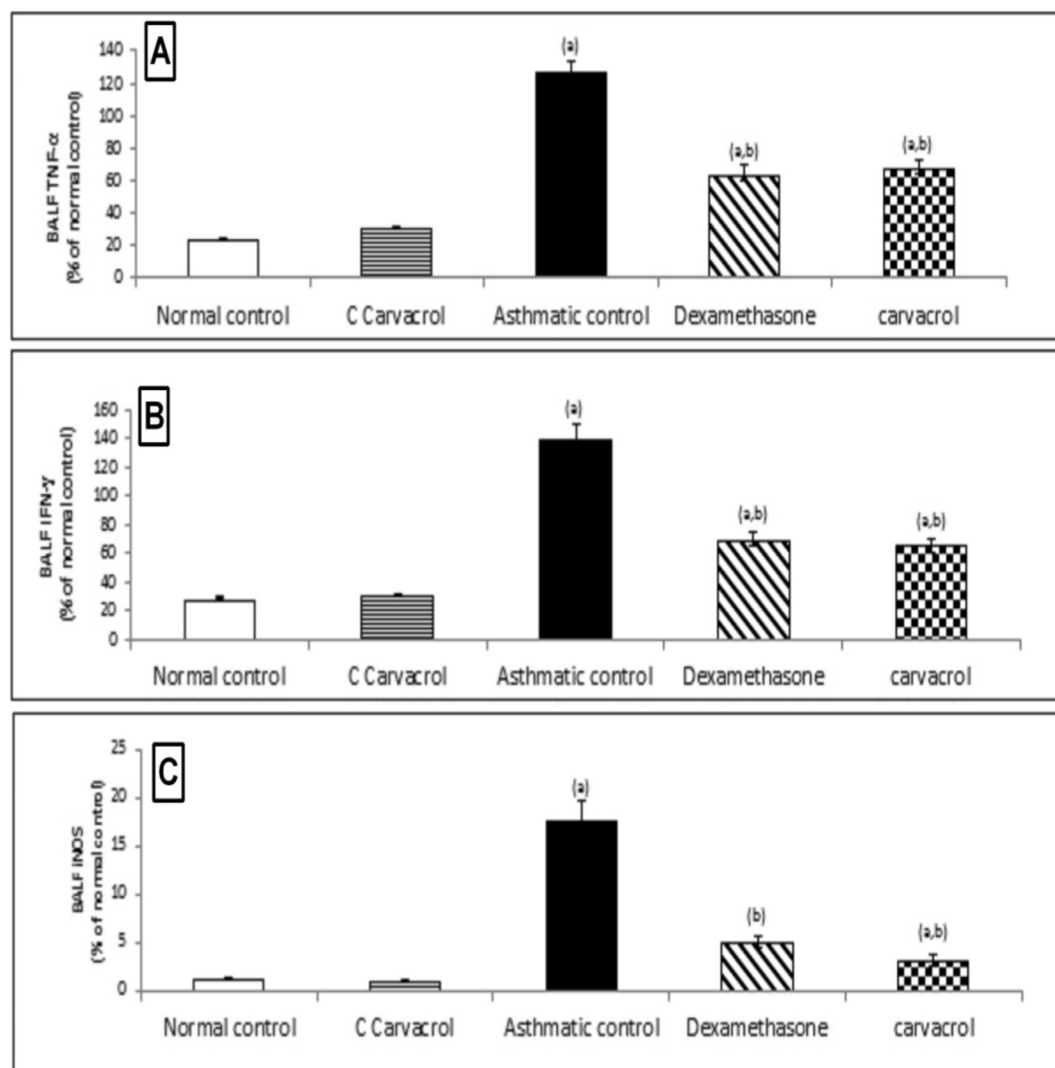


Fig. 2. Effect of carvacrol (15 mg/kg) on (A): Tumor Necrosis Factor-alpha (TNF- α), (B): Interferon-gamma (IFN- γ), (C): Inducible Nitric Oxide Synthase (iNOS) in bronchoalveolar fluid (BALF) in asthmatic rats. Asthma was induced by intraperitoneal administration of ovalbumin (OVA; 200 μ g/(OH) $_3$; 10 mg) at days 1,2,3 and 11 then challenged intranasally by 1.5 mg/300 μ l saline OVA. BALF samples were collected 24 h after the last challenge. Data were expressed as mean $\pm$ SEM (n = 8–10 rats).

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by post hoc using Bonferroni test to compare between multiple means at p level < 0.05.

^aSignificantly different from normal control at p level < 0.05.

^bSignificantly different from asthmatic group.

3.6. Effects of carvacrol on histopathological study and airway remodeling

Under a light microscope, representative sections of each group were examined to assess any morphological changes in the lungs. Lung sections taken from normal rats exhibited normal histological structure of the bronchioles, including normal epithelial lining and thin smooth muscle layer, surrounded by expanded alveoli with thin interalveolar septa which illustrate healthy lungs. Similarly, lung sections taken from carvacrol control group exhibited normal histological structure of lung tissues including bronchioles and alveoli with absence of any pathologic lesions. On contrast, lung sections taken from the asthma control group exhibited distortion of the normal bronchiolar architecture combined with peribronchiolar inflammatory cellular infiltrates and intrabronchiolar macrophages. In addition, the alveolar spaces were collapsed with thick interalveolar septa besides massive perivascular mononuclear inflammatory cells infiltration in the interalveolar septa with numerous alveolar macrophages infiltration which underline severe inflammatory reactions, also dilated congested pulmonary blood

vessel was noticed. Lung sections taken from the group pretreated with DEXA exhibited remarkable improvement in inflammatory reactions with normalized lungs to a great extent except presence of few alveolar macrophages. In addition, lung sections taken from the group pretreated with carvacrol exhibited extreme improvement in the inflammatory reactions and returned the bronchiolar and alveolar architecture to the almost normal pattern. Only dilated pulmonary blood vessel was noticed (Fig. 4). Inflammation scores were shown in (Table 5).

3.7. Effects of carvacrol on immunohistochemistry study

UCN expression in lung tissues was estimated by immunostaining of lung sections with UCN antibody. Immunohistochemical staining of UCN in lung tissues of normal group and the carvacrol control group showed basal expression of UCN. On the other hand, immunohistochemical staining of UCN in the asthma control group significantly increased the expression of UCN as compared to the normal

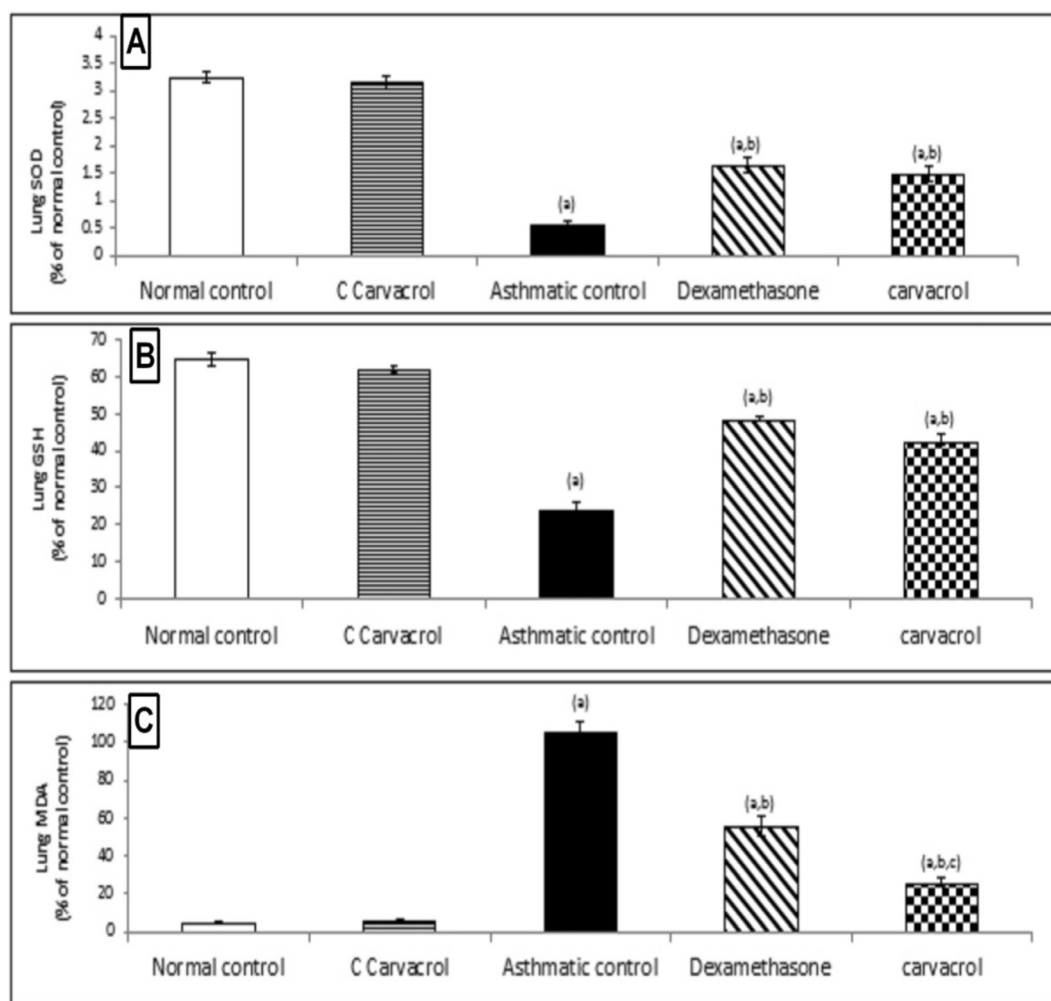


Fig. 3. Effect of carvacrol (15 mg/kg) on lung tissue (A): Superoxide Dismutase (SOD), (B): Glutathione (GSH), (C): Malondialdehyde (MDA) in asthmatic rats. Asthma was induced by intraperitoneal administration of ovalbumin (OVA; 200 μ g/(OH)₃; 10 mg) at days 1,2,3 and 11 then challenged intranasally by 1.5 mg/300 μ l saline OVA. Lung samples were collected 24 h after the last challenge. Data were expressed as mean $\pm$ SEM (n = 8–10 rats). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by post hoc using Bonferroni test to compare between multiple means at p level < 0.05.

^aSignificantly different from normal control at p level < 0.05.

^bSignificantly different from asthmatic group.

control group in the bronchial epithelium and alveolar epithelium identified by strong brown color in these regions. Immunohistochemical staining of UCN in lung tissues of the group pretreated with DEXA significantly decreased the expression of UCN as compared to the asthma control group. Similarly, the group pretreated with carvacrol significantly decreased the expression of UCN as compared to the asthma control group (Fig. 5A, B).

3.8. Effect of carvacrol on expression of SP-D in lung tissues

Western blot analysis was performed to study the effect of carvacrol on the expression of SP-D in lung tissue. It was shown that protein lysates isolated from the lung tissues of the normal control group and the control carvacrol group exhibited no expression of SP-D, while protein lysates isolated from the lung tissues of the asthma control group showed a significant increase in SP-D expression to 535% as compared to the normal control group. Protein lysates isolated from lung tissue of the group pretreated with DEXA registered a significant decrease in SP-D expression to 58% as compared to the asthma control group. Similarly, protein lysates isolated from lung tissues of the group pretreated group with carvacrol recorded a significant decrease in SP-D

expression to 48% as compared to the asthma control group (Fig. 6).

4. Discussion

OVA was considered as a key reference protein allergen used universally in asthma models because of its responsibility on T cells immune response alternations [41].

The present investigation showed that OVA challenge significantly increased AEC in pellets and eosinophilic infiltration in asthmatic rats as compared to the normal control group. This finding reinforced the previous report that ova induced pulmonary eosinophilia-driven inflammation [41]. This is implicated in the pathogenesis of asthma, since OVA allergic challenge causes eosinophilic recruitment and transmigration to the site of injury under the effect of eotaxin and IL-5 [42]. Eotaxin which is a potent specific chemoattractant of eosinophils that is accumulated in the respiratory epithelium after exposure to allergen in sensitized animals inducing pulmonary eosinophilic chemotactic effects and lung eosinophilia [43]. DEXA significantly decreased AEC in pellets as compared to the asthmatic group which can be explained by its ability to reduce some cytokines like TNF- α and IL1 β leading to prevention of eotaxin protein production in the airway [43]. Similarly,

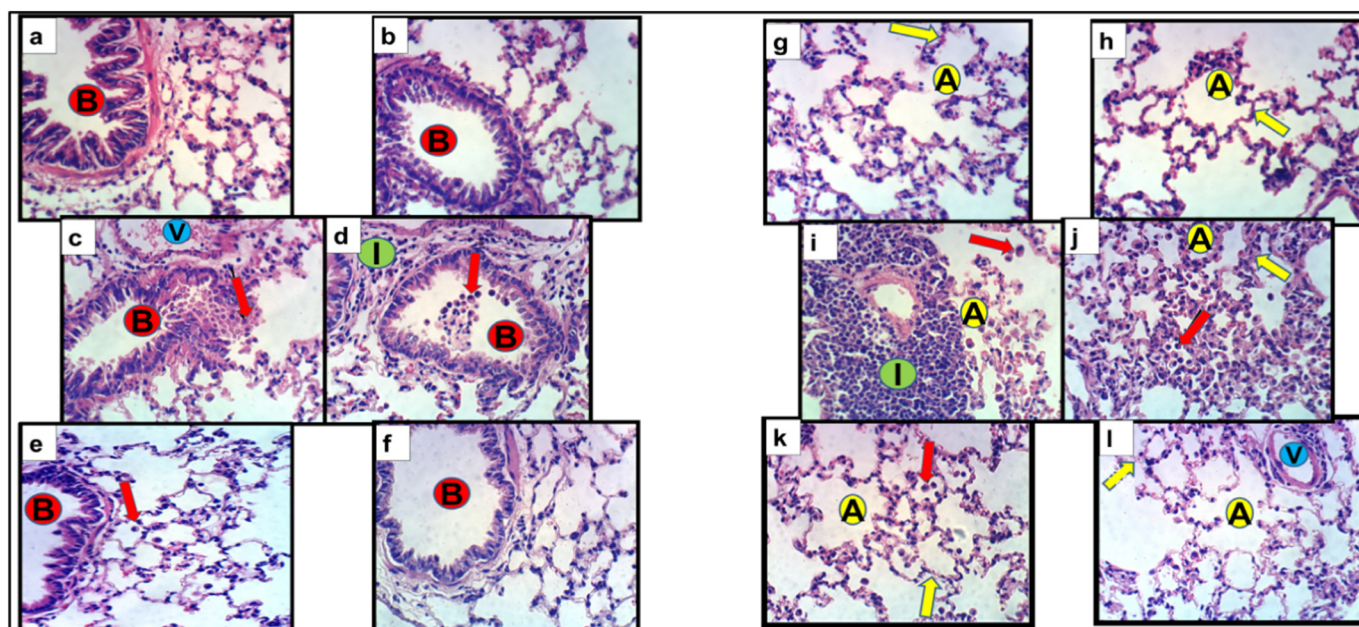


Fig. 4. Effect of carvacrol (15 mg/kg) pretreatment on histological changes in the lung tissues of OVA induced asthma in rats.

Photomicrographs of lung sections from experimental groups (H&E $\times 400$). [a, g]: Normal control group shows normal bronchiolar architecture including normal epithelial lining and thin smooth muscle layer (letter B), normal expanded alveoli (letter A) with thin interalveolar septa (yellow arrow), [b, h]: Carvacrol control group shows normal bronchiolar architecture including normal epithelial lining and thin smooth muscle layer (letter B), normal expanded alveoli (letter A) with thin interalveolar septa (yellow arrow), [c, d, i, j]: Asthmatic control group shows distortion of the normal bronchiolar architecture (letter B), peribronchiolar and alveolar inflammatory cellular infiltrates (letter I), intrabronchiolar and alveolar macrophages (red arrow), collapsed alveoli (letter A) with thick interalveolar septa (yellow arrow), dilated congested pulmonary blood vessel (letter V), [e, k]: Asthmatic rats pretreated with dexamethasone shows normal bronchiolar architecture (letter B), normal expanded alveoli (letter A) with thin interalveolar septa (yellow arrow), few alveolar macrophages (red arrow). [f, l]: Asthmatic rats pretreated with carvacrol shows normal bronchiolar architecture (letter B), normal expanded alveoli (letter A) with thin interalveolar septa (yellow arrow), dilated pulmonary blood vessel (letter V). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

carvacrol limited the lung eosinophilia as compared to the asthmatic group. This is compatible with a guinea-pig model of COPD [16]. This can be explained by the ability of carvacrol to suppress IL-5, as seen in the present study, which has been previously reported to play a crucial role in the lung eosinophilia [44].

IgE produced by B cells is the main mediator released in allergic asthma that initiates inflammatory response by binding to specific receptors for IgE (Fc ϵ RI) on mast cells stimulating mast cells degranulation and release of allergic bronchoconstrictive factors involving leukotrienes, prostaglandins and histamine [45]. In agreement, results of the present investigation revealed that OVA challenge recorded a significant increase in serum IgE level as compared to the normal control group. DEXA significantly decreased IgE serum level. This was returned to its immunosuppressive effect on B lymphocytes [46]. Carvacrol significantly decreased serum IgE level as compared to the asthmatic group. This indicated that carvacrol has immunomodulatory effect which can be useful in the case of asthma.

Moreover, OVA challenge showed a significant increase in T helper 2 (Th2) cytokines including IL-4, IL-5 and IL-13 as compared to the normal control group. These cytokines play a pivotal role in amplifying the immune hyper-response and airway pathological alternations [47]. The OVA-induced release of Th2 cytokines is mediated through

activation and translocation of phospho-Nuclear Factor kappa-light-chain-enhancer of activated B cells (p-NF- κ B) unit p65 from cytoplasm to nucleus, since NF- κ B is an important transcription factor that exerts its immune function through upregulation of different genes involved in the inflammatory pathway [48]. DEXA significantly decreased IL-4, IL-5 and IL-13 as compared to the asthmatic group which is in agreement with a previous study of [49] which attributed that to the ability of DEXA to attenuate the OVA-induced overexpression of toll like receptor 4 and its subsequent induction of NF- κ B [49]. Furthermore, DEXA inhibits GATA-3 expression (Th2 transcription factor) that modulate Th2 cytokines [50]. This study revealed that, carvacrol limited inflammation as evidenced by the significant decrease observed in BALF level of IL-4, IL-5 and IL-13 as compared to the asthmatic group. This inhibitory effect of carvacrol was explained by a previous study on OVA sensitized guinea pigs which demonstrated that carvacrol regulates the balance between Th1/Th2 due to its excitatory effect on Th1 and inhibitory effect on Th2 which secretes these cytokines. [51]. Moreover, carvacrol strongly suppresses eosinophils which are considered an important source of IL-5 [52].

In addition, T helper 1 cytokines involving TNF- α and IFN- γ play a predominant role in many inflammatory diseases and immune disorders [53]. TNF- α is a pleiotropic mediator that promotes a cascade of

Table 5

Scoring system for assessing inflammation in OVA-induced bronchial asthma in rats.

Histopathological lesion	Normal control	Control carvacrol	Asthmatic group	DEXA treatment	Carvacrol treatment
Peribronchial inflammatory cells infiltration	—	—	+++	—	—
Alveolar inflammatory cells infiltration	—	—	+++	—	—
thickened alveolar wall with narrow alveoli	—	—	+++	—	—
Macrophages infiltration	—	—	++	+	—

(—) no change, (+) mild change, (++) moderate change, (+++) severe change.

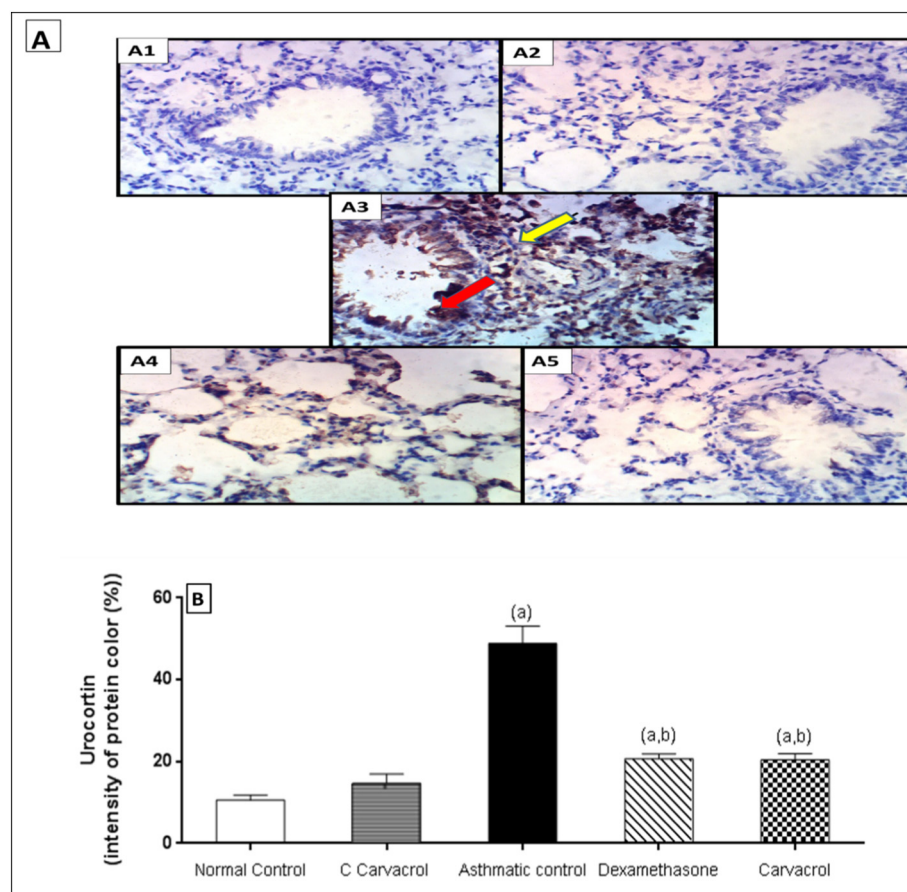


Fig. 5. Effect of carvacrol (15 mg/kg) on expression of urocortin in lung tissue.

Photomicrographs of immunohistochemical stained lung sections (original magnification $\times 400$). [A1]: Normal control group showed basal expression of UCN, [A2]: Carvacrol control group showed normal basal expression of UCN, [A3]: Asthmatic control group showed strong positive expression of UCN (as seen as deep brown color) in the bronchiolar epithelium (red arrow) and alveolar epithelium (yellow arrow), [A4]: Asthmatic rats pretreated with dexamethasone shows weak expression of UCN, [A5]: Asthmatic group pretreated with carvacrol showed very weak expression of UCN.

(B) Quantitative image analysis of immunohistochemical staining expressed as brown color intensity using Image-J software. Data are expressed as mean $\pm$ SEM using one-way analysis of variance (ANOVA) followed by post hoc using Bonferroni test to compare between multiple means at p level < 0.05 .

^aSignificantly different from normal control at p level < 0.05 .

^bSignificantly different from asthmatic group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

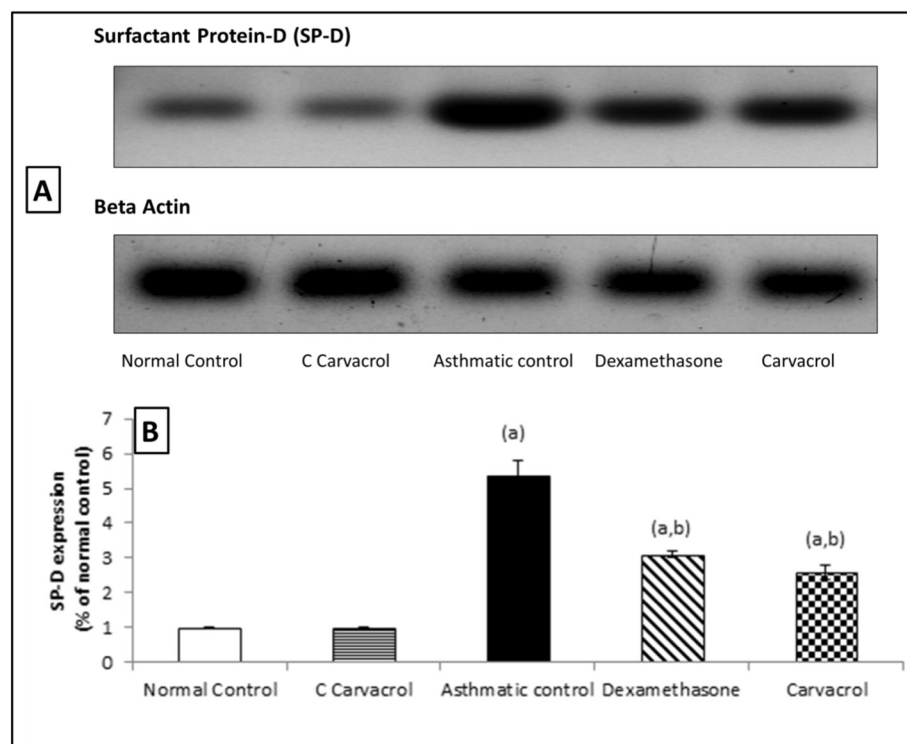


Fig. 6. Effects of carvacrol on surfactant protein D (SP-D) expression in lung tissue.

(A) Protein blotting and immunodetection of expressed SP-D and β -actin in rats' lung tissue, (B) protein density of SP-D expression in different groups. Values were expressed as mean $\pm$ SEM ($n = 8-10$) at $p < 0.05$.

^aSignificantly different from normal control group at $p < 0.05$.

^bSignificantly different from asthmatic group at $p < 0.05$.

inflammatory events through induction of inflammatory cytokines, cell adhesion molecules and cell apoptosis [54]. The present study reported that OVA challenge significantly increased BALF level of TNF- α and IFN- γ as compared to the normal control group. This is in agreement with the results of a previous study of [55]. DEXA significantly decreased BALF level of TNF- α and IFN- γ as compared to the asthmatic group which could be explained by the suppressive effect of DEXA on T-bet (Th1 transcription factor) that modulate Th1 cytokines [50] besides the ability of DEXA to inhibit toll like receptor-4 (TLR4) expression [56]. Similarly, carvacrol significantly decreased BALF level of TNF- α and IFN- γ as compared to the asthmatic group. This result is compatible with the previous study of [54] which showed that carvacrol minimized mRNA and protein expression of TNF- α . In addition, [50] explained this by the suppressive effect of carvacrol on T-bet expression. Amirghofran et al. [57] illustrated that carvacrol has immunoregulatory effect and could suppress the mitogenic and allogenic T cells responses inhibiting IFN- γ release.

ROS and RNS are the most common sources of reactive species that cause oxidative stress contributing to cellular damage [58]. The current results showed that OVA challenge significantly increased BALF level of the nitrosative biomarker (iNOS) as compared to the asthmatic group. This is in accordance with the previous study of [59]. iNOS is the main source of NO which plays a crucial role in the pathophysiology of asthma involving induction of pulmonary vascular permeability, damage of epithelial lining, mucus hyperproduction, infiltration of inflammatory cell and Th2-mediated airway [60]. DEXA significantly reduced iNOS BALF level protecting the lung from nitrate/nitrite-derived oxidative stress, severe hypoxia and bronchoconstriction. This is in full agreement with the previous study [61]. Additionally, carvacrol showed negligible iNOS BALF level as compared to the asthmatic group which affirms a previous evidence of [62]. Aristatile et al. concluded that carvacrol-induced suppression of iNOS may be related to its limitation of NF- κ B expression which plays fundamental role in the inflammatory cascade through controlling the expression of iNOS gene [54].

Reactive oxygen metabolites released by the cascade of inflammatory mediators can modify the airway smooth muscle contractility causing airway obstruction [60]. Eosinophils are the main cells to be infiltrated through the epithelium of the airway producing endogenous oxidants; these oxidants increase lung vascular permeability, airway epithelial damage and bronchial hyperresponsiveness contributing to exacerbation of asthma [63]. Concerning this study, it was shown that OVA challenge produced severe oxidative stress as evidenced by its decrease in SOD activity and GSH tissue content and a significant increase in MDA tissue content. This is compatible with a previous study of a mouse model of chronic asthma [64]. DEXA significantly abolished this oxidative stress. [65]. [66] referred this to the ability of DEXA to decrease the activity of lung xanthine oxidase which plays obvious role in acute lung injury due to production of XO-derived superoxide radicals, exacerbating the oxidative status. Carvacrol also alleviated the oxidative stress induced by OVA. This result is supported by the previous work of [22]. Being an essential oil, carvacrol may have antioxidant activity due to the phenolic moiety in its chemical structure which could trap the free radicals and increase thiol group and consequently it could inhibit oxidative stress and lipid peroxidation [16].

Concerning histopathological examinations, the asthma control group showed severe inflammatory reactions, distortion of bronchiolar and alveolar architecture with massive inflammatory cellular infiltrates and numerous macrophages which complies with the previous study of [67]. The group pre-treated with DEXA showed remarkable improvement in the pathologic changes with normalized lungs to a great extent except presence of few alveolar macrophages which is in agreement with the study of [13]. The group treated with carvacrol showed a significant reduction in the inflammatory reactions with restoring the lungs to almost normal pattern which strongly affirms the biochemical results.

With regard to the immunohistochemical study, The present study showed that, OVA challenge significantly increased UCR expression in lung tissues. This result came in accordance with previous studies of [9,10] which illustrated that OVA allergen activates the release of the inflammatory cells such as lymphocytes, mast cells and macrophages that increase the expression of UCN. UCN is considered to be an activator of these inflammatory cells and mediators stimulating T and B lymphocyte proliferation, mast cell degranulation and macrophage infiltration exaggerating the inflammatory cascade [9]. This high expression of UCN was limited by DEXA as a strong anti-inflammatory drug. This is an expected result and compatible with this of [9]. The present investigation is novel in exhibiting that the protein expression in carvacrol pre-treated group was significantly diminished as compared to the asthmatic group which needs further in-depth investigations.

Respecting the western blot analysis of SP-D, the current study showed that OVA challenge significantly increased SP-D expression as a compensatory mechanism in asthmatic rats. This increase in SP-D expression was previously rationalized as that SP-D consists of a multi-valent homooligomeric protein so can bind with high affinity to oligosaccharides of most pathogenic microorganisms including bronchial pathogens and can acts as antigen-presenting cells to enhance phagocytosis [68,69]. This elevation in SP-D expression was significantly restricted by DEXA as previously reported by [69]. Interestingly, this investigation is unique to exhibit the ability of carvacrol to reduce SP-D expression as compared to the asthmatic group.

5. Conclusion

These data (The biochemical findings together with those of airway tissue sections), collectively, highlighted on that Carvacrol may have a precise role in limiting the inflammation in bronchial asthma and therefore it could be considered a promising natural anti-asthmatic drug.

Declaration of competing interest

The authors declare that there are no conflicts of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Statement on the contribution of authors

Yousra M. Ezz El-Din, Marwa M. Khalaf, Ali A. Aboseif conceived and designed the experiment, Yousra M. Ezz El-Din conducted the experiments, Yousra M. Ezz El-Din, Marwa M. Khalaf contributed in statistical data analysis, Yousra M. Ezz El-Din, Marwa M. Khalaf wrote the manuscript, Yousra M. Ezz El-Din, Ali A. Aboseif revised the manuscript. All authors read and approved the manuscript for publication.

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