

## Evaluation the effect of clove bud aqueous extract on asthma treatment

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[10.54153/sjpas.2024.v6i1.909](https://doi.org/10.54153/sjpas.2024.v6i1.909)

### Article Information

Received: 24/01/2024

Revised: 26/02/2024

Accepted: 29/02/2024

Published: 30/03/2024

### Keywords:

*Asthma, lipoxygenase,  
histidine decarboxylase,  
eugenol, OVA-induced mice.*

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### Abstract

The aim of this study is to find out how clove buds aqueous extract affected an ovalbumin (OVA)-induced allergic asthma model in mice used as an experimental model. After preparing the clove bud aqueous extract, it is examined as an enzyme inhibitor against 5-lipoxygenase and histidine decarboxylase. Then 30 mice divided into 5 groups to test the effective of clove extract in enhancing the situation of asthma by inhibiting the enzymes. The results showed that the concentration of 50 mg/kg/day gave significant differences versus OVA challenged group and generally using extract gave significant differences. As conclusion of this study we can say that the active constituent of clove extract may act as inhibitor of asthma main enzymes in a synergistic effect comparing of major constituent of extract "eugenol" in case of treating alone.

### Introduction:

Asthma is a global health issue that impacts people of all ages. Coughing, tightness in the chest, wheezing, and shortness of breath are some of the symptoms of chronic asthma, a respiratory condition that causes inflammation and constriction of the airways [1]. In recent times, it has also emerged as one of the most pervasive illnesses. According to World Health Organization statistics, there will be a certain number of asthmatic individuals worldwide in 2021 [2].

Asthma pathophysiology is the product of multiple environmental and genetic factors interacting. Along with exposure to triggers for allergies and respiratory system inflammation, a genetic predisposition to developing asthma and the exacerbation of its symptoms play a major role. The inflammatory response in the airways is mediated by immune cells, cytokines,

and other mediators, which results in bronchoconstriction and increased mucus production [3, 4].

Changes in lifestyle must be coupled with long-term control measures like bronchodilators and inhaled corticosteroids to effectively manage the health of patients with asthma. Acute attacks can also necessitate the prescription of emergency fast-acting medications, like short-acting bronchodilators. Managing asthma is largely dependent on general culture and adherence to health recommendations [5].

There are actual obstacles that stand in the way of achieving optimal control over asthma and its exacerbation, despite the remarkable progress made in managing asthma cases. A deeper comprehension of the fundamental mechanisms underlying asthma allows for the development of more efficient therapeutic approaches, the identification of effective treatments, and the mitigation of treatment side effects. Asthma sufferers' lives will be improved and the global burden of the disease will eventually be lessened by developing strategies based on a precise understanding of disease mechanisms and treatment mechanisms [6]. The *Syzygium Aromaticum* tree yields clove buds, which are prized for their aromatic and therapeutic qualities. Eugenol, eugenyl acetate, beta-caryophyllene, and other volatile compounds are the active ingredients in clove buds. A significant ingredient, eugenol is well-known for its analgesic, antioxidant, and anti-inflammatory qualities [7]. Due to the presence of eugenol as a major constituent, clove buds have a wide range of medicinal uses due to their antioxidant, antimicrobial, antinociceptive, antiviral, and aesthetic properties [8].

The inflammatory mediators known as arachidonic acid (AA) cascade, which are generated by the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, are implicated in numerous human diseases. The second essential biosynthetic pathway, which is triggered by the LOX isozyme 5-lipoxygenase (5-LOX), releases eicosanoids. The 5-LOX pathway terminates in the production of leukotriene B<sub>4</sub>, a mediator of cardiovascular disease, cancer, and atherosclerosis. Asthma is the case that is most directly related to B<sub>4</sub> [9-11].

5-LOX is a non-heme iron atom-containing dioxygenase enzyme in the LT pathway. It adds a molecular oxygen to AA to catalyze the formation of 5(S) hydroperoxyl eicosatetraenoic acid (5-HPETE), which subsequently dehydrates to leukotriene A<sub>4</sub> (LTA<sub>4</sub>). Thus, 5-LOX is thought to be the primary cause of inflammatory diseases such as rheumatoid arthritis, allergic rhinitis, and asthma [12-14].

LTB<sub>4</sub> stimulates different leukocytes and induces chemotaxis. As bronchoconstriction and mucus secretion are known to be caused by LTC<sub>4</sub>, D<sub>4</sub>, and E<sub>4</sub>, these factors become important in the development of asthma [15]. The principal enzyme controlling the biosynthesis of histamines is histidine decarboxylase (HDC). Asthma is one of the chronic inflammatory diseases that histamine plays a role in causing [16].

Since 5-lipoxygenase plays a crucial part in asthma episodes, targeting it makes sense. The study's hypothesis was based on the idea that inhibiting the enzyme 5-Lipoxygenase would be a good way to develop asthma treatments and test them in laboratory organisms and humans. This was because the active compounds in clove bud extract are effective at inhibiting the inflammatory mechanism, including the formation of leukotriene (B<sub>4</sub>). Thus, the study's goal

was to find out how clove buds aqueous extract affected an ovalbumin (OVA)-induced allergic asthma model in mice used as an experimental model.

## **Materials and Methods:**

### **Materials:**

The supplier of ovalbumin OVA was SIGMA-ALDRICH CHEMIE GmbH. Diethyl ether is provided by UK-based ROMAN Pure Chemistry. Paraffin oil provided by Germany's Applichem GmbH. enzyme-linked immunosorbent assay (ELIZA) kits for histidine decarboxylase and 5-lipoxygenase.

### **Plant material:**

In May of 2012, clove, *Syzygium aromaticum*, buds were bought from a local market in Ramadi, Iraq. The plant tissues were cleaned, allowed to dry in the shade, ground using a machine, and the resulting aqueous extract was utilized in this investigation as clear below.

### **Extraction procedure:**

Using a grinder, the dried clove bud samples were roughly crushed. The maceration method was used to extract the sample.

A dried clove bud sample was used in this procedure, and it was submerged in 300 ml of distilled water for 12 hours. To get rid of the leftovers, the mixture was filtered (Whatman® No1, Merck KGaA, Darmstadt, Germany). The procedures outlined in earlier works [17] serve as the foundation for the liquid-liquid extraction. In a separatory funnel, the filtrate and diethyl ether (2:1) were added. Until the gas was produced, the stopcock was opened and closed while the funnel was swirled. In order to remove the aqueous layer, the funnel was set aside for a short while until the separated layers were visible. Until the essential oil was the only substance left, the ether layer was kept in a fume hood for several hours. After the extract was weighed, it was sealed in a vial for additional examination. After being weighed, the extracted material was sealed in a vial for additional examination.

### **Quantification of eugenol using HPLC-UV-vis:**

Using an Agilent Technologies 1200 series HPLC with an Eclipse plus C18 (4.6 x 150 mm, 5µm) and a UV-Vis detector, chromatographic analysis was carried out (Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA).

### **In vitro lipoxygenase inhibition activity:**

Using kit of in vitro soybean 5-lipoxygenase inhibition, from cayman chemical, USA, the procedure had been carried out, with Item No. 760700.

### **In vitro Histidine Decarboxylase inhibition activity:**

Using kit of in vitro soybean histidine decarboxylase inhibition, from cayman chemical, USA, the procedure had been carried out, with Item No. Item No. 33828.

## Animals:

Thirty adult male BALB/c albinos, weighing between 20 and 30 g, were obtained and housed in the College of Science/University of Anbar's animal house under special pathogen-free conditions. They were fed and watered freely for twelve hours daily, with a regular room temperature (18–21°C) throughout the study. The University of Anbar's ethical committee approved the use of animals in research, and as such, animals were treated throughout this process in compliance with the Helsinki Declaration.

## Experimental protocol for allergic asthma model:

OVA, the primary protein in egg whites, is used to prepare allergic asthma models. Because it is not fundamentally immunogenic, it needs to be injected systemically. In acute sensitization procedures, several injections of systemic allergen are frequently necessary. administered 1 ml of 10% OVA intraperitoneally on day 1, and exposed to an aerosol containing 1% OVA for 14 days in a row, 30 minutes each day, in an aerosol chamber made specifically for this use out of a portable nebulizer and a plastic food container used as a nebulizing chamber

## Experimental design:

Five groups of six mice each received an equal distribution of the mice. As a control group, Group 1 was kept on the regular diet. Group 2: OVA-challenged asthma model. Oral administration of the clove water extract at 25 and 50 mg/kg/day was administered to groups 3, 4, respectively. Eugenol 15 mg/kg/day was administered orally to Group 5. For two weeks, each mouse received the prescribed oral dose for the experiment. All groups were put to death by human suffocation after two weeks of diethyl ether anaesthesia. Samplings of blood were taken during the slaughter. All mice were examined at necropsy to determine the levels of histidine decarboxylase and 5-lipoxygenase.

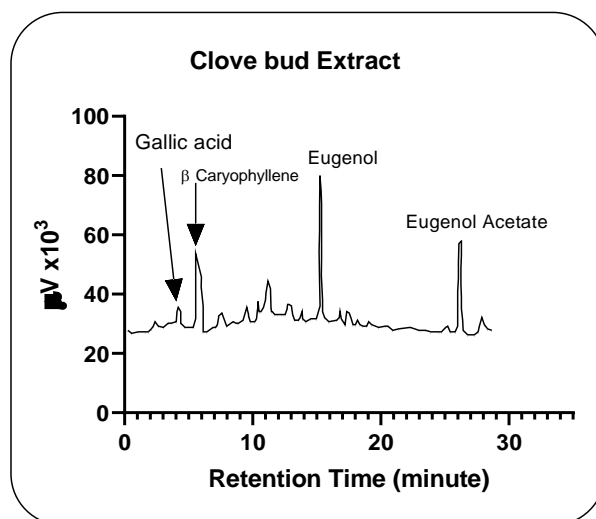
Groups of Study				
Group 1	Group 2	Group 3	Group 4	Group 5
regular Control group	OVA- challenged Asthma group	Oral administration clove extract 25mg/Kg/day	Oral administration clove extract 50mg/Kg/day	Oral administration Eugenol 15mg/Kg/day

## Results and Discussion:

### HPLC of clove extraction:

According to comparing HPLC chromatogram with standard phytochemicals constituents, it clears from figure 1 that clove bud extract frequently contains eugenol, eugenyl acetate,  $\beta$ -caryophyllene, gallic acid and other substances. The column, mobile phase composition, mobile phase flow rate, and detecting wavelength were all examined in order to yield chromatograms with improved resolution of neighbouring peaks in a short amount of time. Following comparison, 0.7 mL/min was determined to be the most appropriate flow rate. This finding,

which corresponds with a study by some researchers who identified and examined the chemicals of clove extract, demonstrates the primary area for eugenol, proving that this active ingredient was the cause of the plant's great activity [18].



**Figure 1:** HPLC chromatograms of water extracts of clove bud extract

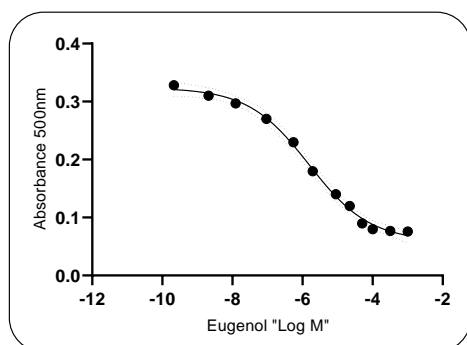
### Inhibition of 5-lipoxygenase:

Utilizing a pre-made diagnostic kit from Cayman Chemical, we investigated the inhibitory effect of the 5-lipoxygenase enzyme in the clove bud aqueous extract. According to the data, the extract had a good dilatation capacity ( $IC_{50} = 70.5$  ppm). When eugenol, eugenol acetate, and beta caryophyllene-the three most significant chemical compounds in the extract-were compared, eugenol's inhibitory potential was clearly visible, and its  $IC_{50}$  value was found to be  $5.4 \mu\text{M}$ , figure 2.

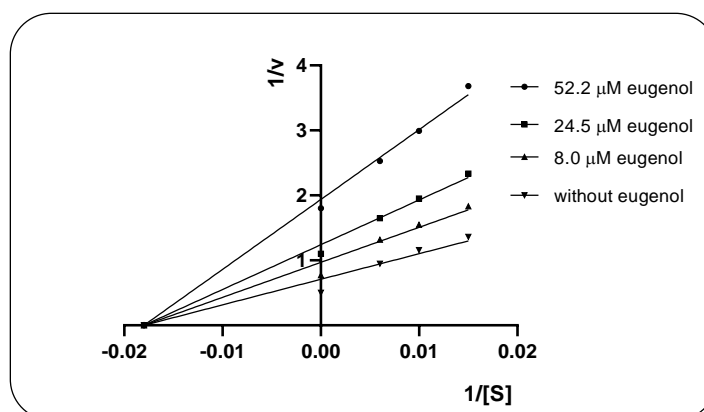
To comprehend the mechanism underlying eugenol's inhibition of 5-lipoxygenase, enzyme kinetics studies were conducted. Various concentrations of arachidonic acid ( $45\text{--}205 \mu\text{M}$ ) and different concentrations of eugenol ( $8.0$ ,  $24.5$ , and  $52.5 \mu\text{M}$ ) were used in substrate-dependent inhibitory studies. Figure 3 shows a double reciprocal plot of substrate versus enzyme velocity when eugenol is present. The maximum velocity of enzyme activity, or  $V_{\text{max}}$ , of 15-HETE/min/mg protein was reduced by eugenol treatment ( $0$ ,  $8.0$ ,  $24.5$ , and  $52.5 \mu\text{M}$ ) from  $4.39$  to  $4.1$ ,  $2.78$ , and  $1.68 \mu\text{mol}$ , respectively, without significantly changing the  $K_m$  values of  $115$ ,  $121$ ,  $117$ , and  $114 \mu\text{M}$  (Table 1). Eugenol is a non-competitive, reversible inhibitor of 5-lipoxygenase, according to the enzyme kinetic data. It is worth noting that 15-HETE means 15-Hydroxyeicosatetraenoic Acid., the product of lipoxygenase from the substrate arachidonic acid AA.

**Table 1:** Effect of eugenol on  $V_{\text{max}}$  and  $K_m$  values of 5-lipoxygenase

Main clove constituents	Concentration ( $\mu\text{M}$ )	$K_m$ ( $\mu\text{M}$ )	$V_{\text{max}}$ (mmoles HETE/min/mg protein)
Eugenol	0	115	4.39
	8.0	121	4.1
	24.5	117	2.78
	52.5	114	1.68



**Figure 2:** Inhibition of soybean 5-lipoxygenase by eugenol,  $IC_{50}=5.4 \mu\text{M}$ . Arachidonic acid was used as a substrate in this experiment.



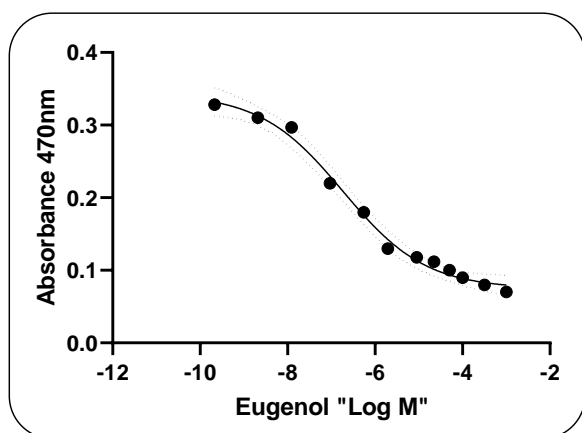
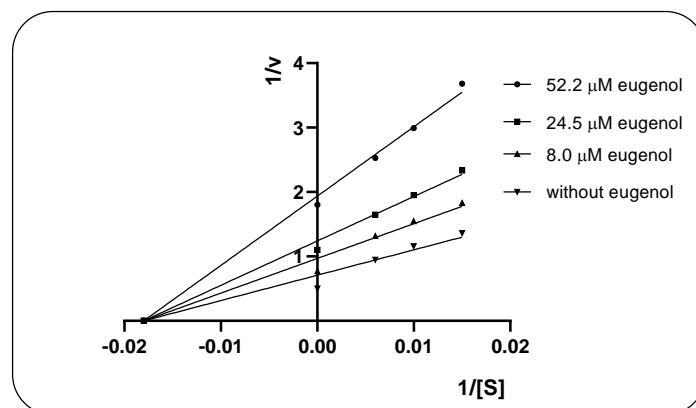
**Figure 3:** A double reciprocal diagram illustrating the substrate-dependent enzyme kinetics and how eugenol inhibits 5-lipoxygenase activity. The enzyme 5-lipoxygenase was incubated with different eugenol concentrations while being exposed to diverse Arachidonic acid conc. When varying eugenol concentrations are present, no change in  $K_m$  values is seen.

### Inhibition of Histidine Decarboxylase:

Reducing the potentially harmful histamine-related local immune response in allergic diseases is the theory behind the use of histidine decarboxylase inhibitors, which are agents that block the conversion of histidine to histamine. The combination of a plant-derived histidine decarboxylase inhibitor and an H1 antihistaminic medication, which has good oral efficacy, low CNS side effects, and high selectivity, is thought to offer a significant therapeutic improvement in the treatment of allergic diseases, especially considering the shortcomings of the synthetic antihistaminic drugs currently on the market [19, 20]. Same concentration of eugenol test to inhibit 5-lipoxygenase had been repeated with histidine decarboxylase. results show that eugenol has good activity to inhibit histidine decarboxylase and  $IC_{50}$  was  $7.2 \mu\text{M}$  figure 4. Examining types of inhibition then carried out using different concentration of inhibitor and it appeared that the inhibition is noncompetitive. Eugenol treatment as figure 5 illustrates (0, 8.0, 24.5, and  $52.5 \mu\text{M}$ ) decreased the maximum velocity of enzyme activity, or  $V_{\text{max}}$ , of histamine/min/mg protein from 5.86 to 3.81, 2.88, and  $2.19 \mu\text{mol}$ , respectively, without significantly altering the  $K_m$  values of 55.5, 56.7, 55.2, and  $57.2 \mu\text{M}$  (Table 2). The enzyme kinetic data indicates that eugenol is a reversible, non-competitive inhibitor of histidine decarboxylase.

**Table 2:** Effect of eugenol on Vmax and Km values of histidine decarboxylase

Main clove constituents	Concentration ( $\mu\text{M}$ )	Km ( $\mu\text{M}$ )	Vmax (mmoles histamine/min/mg protein)
	0	55.5	5.86
Eugenol	8.0	56.7	3.81
	24.5	55.2	2.88
	52.5	57.2	2.19

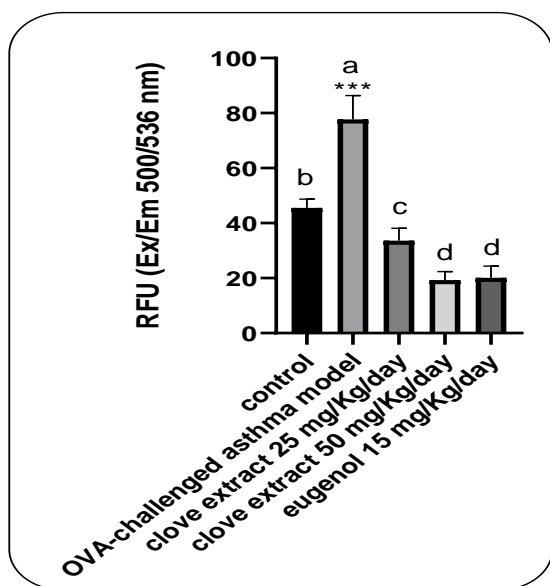
**Figure 4:** Inhibition of histidine decarboxylase by eugenol,  $\text{IC}_{50}=7.2 \mu\text{M}$ . Arachidonic acid was used as a substrate in this experiment.**Figure 5:** A double reciprocal diagram illustrating the substrate-dependent enzyme kinetics and how eugenol inhibits histidine decarboxylase activity. The enzyme histidine decarboxylase was incubated with different eugenol concentrations. When varying eugenol concentrations are present, no change in Km values is seen.

### Lipoxygenase activity in ovalbumin-induced asthma mice:

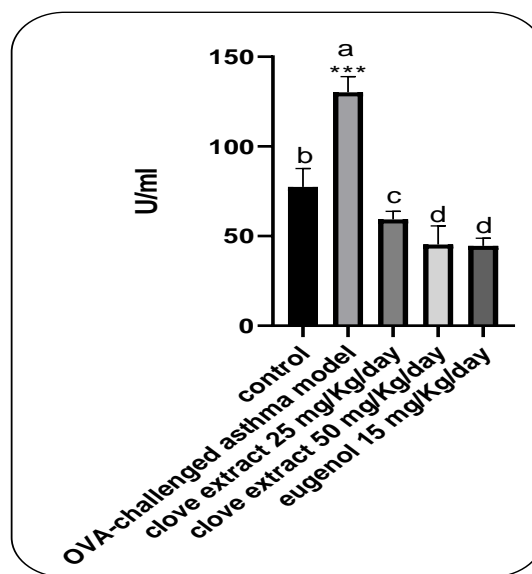
Under the direction of LTA4 hydrolase or LTC4 synthase, respectively, unstable LTA4 is converted to LTB4 or LTC4. Type 2 (MGST2) and type 3 (MGST3) microsomal glutathione S-transferases are isoenzymes that possess LTC4 synthase activity [21, 22]. LTB4 activates different leukocytes and induces chemotaxis. Because LTC4, D4, and E4 are known to induce bronchoconstriction and mucus secretion, they become important in understanding the genesis of asthma [23, 24]. Figure 6 illustrates that using clove extract 50 mg/Kg/day inhibits 5-lipoxygenase clearly in a manner mimic of eugenol 15 mg/Kg/day and there is no significant differences while both whole extract and isolated eugenol have significant differences ( $p < 0.01$ ) compared to control group. It is worth mentioning that the lipoxygenase activity can be calculated from recording fluorescence (RFU), so that one unit of lipoxygenase is the amount of enzyme that will cause oxidation of 1  $\mu\text{mol}$  of the lipoxygenase probe per one minute at pH 7.4.

In laboratory rodents, ovalbumin (OVA), an allergen commonly used, causes severe allergic lung inflammation. OVA is derived from chicken egg white [25]. The aetiology of asthmatic disorders is complex. Cytokines are crucial for the immune system and inflammatory responses in asthma. Evidence-based studies of natural medicinal herbs in the treatment of asthma suggest that a range of natural compounds have immunomodulatory qualities, such as regulating inflammatory cell activity and affecting the expression of inflammatory cytokines [26]. From this study the results may indicate that in comparison to the control group, OVA

caused an allergic reaction in the lungs of mice that resembled allergic asthma and was characterized by increased levels of lipoxygenase and histidine decarboxylase, as seen in Figures 6 and 7. Unit of histidine decarboxylase can be defined as: One unit will release 1.0  $\mu$ mole of CO<sub>2</sub> from L-histidine per minute at pH 4.5 at 37°C. Figure 7 shows that there is significant differences of using clove extract and isolated eugenol compared to control group while there is no significant differences between using clove extract 50 mg/Kg/day and eugenol 15 mg/Kg/day groups.



**Figure 6:** comparison between traits of lipoxygenase inhibition. The best result is using crude extract 50 mg/Kg/day and eugenol 15mg/Kg/day



**Figure 7:** comparison between traits of histidine decarboxylase inhibition. The best result is using crude extract 50 mg/Kg/day and eugenol 15mg/Kg/day

Leukotrienes, which are produced by lipoxygenase activity, are important in the pathophysiology of asthma because they promote mucus production, airway inflammation, and bronchoconstriction. Therefore, this study focused on the isozyme 5-lipoxygenase, that is the responsible of initial steps in leukotriene synthesis [27]. Asthma-related inflammatory processes are linked to leukotrienes, specifically leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and cysteinyl leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>). They add to the typical symptoms of asthma by drawing in and stimulating inflammatory cells, causing bronchoconstriction, and secreting more mucus [28]. By inhibiting lipoxygenase, leukotriene production can be decreased, which in turn can lessen inflammatory reactions in the airways [29]. Its anti-inflammatory properties are essential for managing asthma symptoms and averting flare-ups. Although lipoxygenase inhibitors have great potential for treating asthma, more research is necessary to fully understand their safety, effectiveness, and best use in various asthma phenotypes. For individualized medical advice and treatment decisions, always seek the advice of healthcare professionals [30].

An essential enzyme in the production of histamine, a biogenic amine involved in several physiological processes like inflammation and allergic reactions, is histidine decarboxylase.



Histidine decarboxylase is responsible for converting histidine into histamine. The activity of this enzyme influences the levels of histamine available for release in response to allergic stimuli or other triggers.

In response to allergens, mast cells and basophils release histamine, which sets off inflammatory reactions. Histamine plays a role in asthma by increasing mucus production, bronchoconstriction, and airway hyperresponsiveness, which result in symptoms like wheezing and dyspnea [31]. Since histamine plays a major role in asthma, it has been investigated as a possible treatment strategy to target histidine decarboxylase or histamine receptors. Antihistamine drugs, which bind to histamine receptors, are frequently prescribed to treat allergic reactions, such as asthma [32]. The active constituents in clove bud extract showed great synergistic effect mainly by eugenol via targeting the two enzymes and inhibited them in markedly way.

**Conclusion:** It can be concluded that the whole extract may play important role in treating asthma and reducing asthmatic symptoms via its active ingredient mainly, eugenol and by less important the other high percent constituent eugenol acetate and beta caryophyllene. Also, the result reached to the fact of inhibition of eugenol of both 5-lipoxygenase and histidine decarboxylase enzymes as reversible noncompetitive inhibitor. Thirdly, using clove extract play important role to treat OVA induced asthma model mice.

**Acknowledgments:** We thank the staff of department of Chemistry, Education College for Women, University of Anbar for supporting and present their laboratory and all facilitations. Grateful thanks to College of Science, University of Anbar for their support. Funding by the Turkish Ministry of Higher Education and Scientific Research is gratefully acknowledged.

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## تقييم تأثير المستخلص المائي لبراعم القرنفل في علاج الربو

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### الخلاصة:

تهدف الدراسة الى معرفة تأثير مستخلص براعم القرنفل المائي على الفئران المصابة بالربو المستحث باليومين البيض. بعد إتمام استخلاص القرنفل تم استخدامه كمشتب لانزيمات أساسية في الربو وهي انزيم اللايبواوكسيجينيز وانزيم النازع للكربوكسيل من الهستيدين او ما يسمى الهستيدين ديكاربوكسيليز. تم تقسيم الثلاثين فأراً المستخدمة للدراسة الى خمس مجموعات احداها مجموعة السيطرة من الفئران السليمة بينما المجموعة الثانية كانت تمثل مجموعة الفئران التي استحثت للربو ولم تتلق أي علاج. المجموعتين الثالثة والرابعة اشتملت على الفئران التي تلقت العلاج متمثلاً بالمستخلص لكن بتركيزين مختلفين بينما المجموعة الخامسة فعوضاً عن المستخلص فقد تم تجريعها باليوجينول المعزل من المستخلص. تشير النتائج الى فاعلية المستخلص وبتركيز ٥٠ ملغم لكل كغم من وزن الفأر يومياً أظهرت تأثيراً معنوياً وبالمقارنة مع تركيز اليوجينول الأقل فكانت تأثيراته مشابهة لمجموعة ٥٠ ملغم. يمكن القول من النتائج المستحصلة ان المكونات الفعالة لبراعم القرنفل لها دور تثبيطي للانزيمات الأساسية المسببة لنوبة الربو ولها تأثير متداوب ولليوجينول الدور الأهم في تثبيط الانزيمات.

### معلومات البحث:

تاريخ الاستلام: ٢٠٢٤/١/٢٤

تاريخ التعديل : ٢٠٢٤/٢/٢٦

تاريخ القبول: ٢٠٢٤/٢/٢٩

تاريخ النشر: ٢٠٢٤/٣/٣٠

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