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RESEARCH ARTICLE

Anti-allergic Effect of *Thymus serpyllum* on Ova Albumin-Induced Asthma in Syrian Hamsters

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ABSTRACT

Herbalism has a long tradition of use outside of conventional medicine even before recorded history. It is becoming more mainstream as improvements in analysis and quality control along with advances in clinical research show the value of herbal medicine in the treating and preventing inflammatory and other diseases. This focuses the attention on the possibility of the presence of anti-allergic compounds in the extracts of some plants that may help control common allergic diseases including asthma. To test the possible anti-allergic and prophylactic effects of the aqueous extract of *Thymus serpyllum* L. (*Ts*) on ovalbumin-induced asthma clinical and hematological parameters in Syrian hamster models. The antigen-induced asthmatic response and subsequent airways reactivity have been associated with increased airways inflammation. Employing animal models of ovalbumin-induced asthma in Syrian hamsters previously developed in our laboratory, 3 phases of experiment (sensitization, challenging and treatment) were performed to investigate the effect of the aqueous extract of *Thymus serpyllum* on asthma clinical manifestations and white blood cells count and differential. *Thymus serpyllum* showed significant effect in improving nasal symptoms ($p< 0.001$) by affecting inflammatory white blood cells count and differential. There was a significant decrease in eosinophils level ($p< 0.001$) which is a potent inflammatory cell in asthma development. Moreover, *Ts* effect extended to be also prophylactic as the significant decrease in symptoms in pre-treated group showed. In regard to histamine, it was found that *Ts* effect is not significant in improving asthma clinically and at white blood cells level; $p< 0.001$ compared with control group.

KEYWORDS

Thymus Serpyllum, Therapy, Asthma, Allergy

INTRODUCTION

Asthma is "a disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person. In an individual, they may occur from hour to hour and day to day"[1]. This condition occurs as a reaction to infections or allergens that cause inflammation of the air passages in the lungs which associated with infiltration of eosinophils, T helper 2 (Th2) and neutrophils from blood to air way results in swelling of the lining of air passages and increasing sensitivity of the nerve endings in the airways causing the airways to be narrow and easily irritated, and leads to reduction of air-flow in and out of the lungs beside the other symptoms of asthma.[2,3] Over the past few decades, the prevalence and severity of asthma have increased dramatically in the industrialized societies and it becomes a

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major public health concern.[4,5] Drug therapy is unable to act at all stages and targets of asthma, it is usually used to control symptoms along with complementary medicine, including herbal treatments.[6]

Many anti-allergy drugs have been developed from several sources including from natural products that contain diverse chemical compounds as sources of therapeutics to treat some diseases. Therefore it is promising to discover new potential anti-allergy drug isolated from natural products.[7]

Anti-allergic effect of any crude extract obtained from *Ts* has never been studied and the overall body of researches into complementary and alternative medicine (CAM) for asthma is still small and of limited quality in Palestine. For that, this research was designed to study the therapeutic and prophylactic effects of the aqueous extract of *Ts* on asthma in animal models looking for a new potent anti-allergic drug to treat allergic asthma.

MATERIALS AND METHOD

Study Design and Study Settings

This study is a randomized control experimental study. It was conducted in the laboratories of Faculty of Medicine and health sciences at An-Najah National University in Nablus and the animal unit of Basha Scientific Center in Jenin.

Study Population and Sample Size of Experimental Animals

Study population consists of Syrian hamsters of sex, (10-12) weeks, and (50-60) g weight.[8]

For statistical reasons, sample of thirty five animals was randomly selected and separated into seven groups (n=5/group).[8]

Experimental Design

Animal Models

The groups were defined as **C group**; negative control group that was treated with phosphate buffered saline (PBS) nasally, **CT group**; positive control group that was given *Ts* aqueous extract orally and treated with *Ts* extract nasally, **OVA group**; negative ova albumin (OVA)-challenged group that was sensitized and challenged with ovalbumin and then treated with PBS nasally, **OVA T group**; positive OVA-challenged group that was sensitized and challenged with ovalbumin and then treated with *Ts* aqueous extract nasally and orally, **OVA TP group**; pre-treated group that was given *Ts* orally, sensitized and challenged with ovalbumin and treated with *Ts* aqueous extract nasally, **H group**; negative histamine-challenged group that was challenged with histamine and treated with PBS nasally, **HT group**; positive histamine-challenged group that was challenged with histamine and treated with *Ts* aqueous extract nasally and orally.

Blood Sampling

A blood sample was taken from each animal at the end of the experiment (day 35) to measure white blood cells (WBC) count and differential WBCs. Sampling was done for all animals at the same time and using the same laboratory materials and procedures. Counts and differentials were compared in order to find any possible differences between them in relation to the different interventions that groups were exposed to.

Experiment Steps

Thymus serpyllum Collection and Identification

Thymus serpyllum plant was collected by us from mountains of Nablus area in October 2013. Another sample was bought. The plant samples were the plant in its mature phase with the long stems and leaves. Both specimens with aerial parts were included in the study. The fresh and healthy parts were separated instantly and packed in a polyethylene bag. The samples then were transported to the laboratory and kept at room temperature until processing.

Exhaustive extraction of Thymus serpyllum

Only aqueous extract was obtained and has been used in the experiment; the popular form of plant used by people. This method is the applicable one in An-Najah National University laboratories.

Aerial parts were dried in the shade for 2 weeks, at room temperature, cut to small pieces and powdered in a mechanical grinder. The powder was exhaustively extracted, this was achieved by re-extracting the ruminants of the powder after the first extract as will be seen in the following steps.

We used 25 g of the powdered plant, which was suspended in 125 ml of 50% ethanol in distilled water with continuous shaking 200 round per minute (rpm) at 25ºC for 72 hours in the shaking incubator. After that, the mixture was filtered by Whitman's No. 1 filter paper using Buchner funnel. The plant materials that accumulated on the filter paper were re-extracted again by adding 125 ml of 50% ethanol in triple distilled water, with continuous shaking for 72 hours in the Shaking Incubator at 25ºC as before.

The aqueous phase was collected and its volume was measured and kept in volumetric flask at room temperature till the next step. After 72 hours of shaking in the Shaking Incubator for the second aqueous extract (as mentioned previously), the mixture underwent second filtration using Whitman's No.1 filter paper. The second aqueous phase was collected after filtration, its volume was measured and added to first aqueous extract. This volume was put in the rotary evaporator for 1 hour at 40ºC, then was put in a pre-weighed freeze dryer bottle for 24 hours, dried completely, weighed and then stored at 8ºC.

Syrian Hamster Models Housing

Syrian hamster was a model for many immunological researches. To test the possibility of inducing asthma in Syrian hamsters by OVA, five animals (tester group) were ordered at first and three of them were sensitized and challenged with OVA while the others were control to assess the significance. Tester group showed significant clinical signs of asthma and it was possible to consider this animal an appropriate model for our research. Animals were ordered taking into consideration that they were not exposed to drugs, toxins or air pollutants and free of any respiratory or allergic diseases. They were housed in plastic cages with wood-chip bedding at temperature of (23±2)ºC, humidity controlled rooms and 12-hour dark-light cycles with access to tap water and normal diet. Diet was withheld 8 hours before the starting time of experiment (day 0) with free access to water.

Ovalbumin Preparation

A 2mg/ml solution of OVA was prepared by dissolving 80 mg OVA (Egg white from chicken (sigma- SLBF2579V)) in 40 ml PBS in a 50-ml Falcon tube. This mixture was vortexed for 5 minutes at 2,000 rpm to mix. A 10 ml of this solution was diluted in 40 ml PBS resulting in 0.4 mg/ml solution. This 0.4 mg/ml solution was distributed as 1 ml aliquots into micro tubes and stored frozen at -20ºC and been used later in allergen challenge phase (phase 2). The remaining 30 ml of the 2 mg/ml solution was used to prepare suspensions of 0.5 mg/dl OVA and 20 mg/dl aluminum hydroxide (alum) (sigma- MKBK2564V)) that was used in allergen sensitization phase (phase 1). The suspension was prepared by adding 80 mg alum to 1 ml of 2 mg/dl OVA and 3 ml of PBS and then it was put on shaker for 4 hours and used freshly.[9]

Phases of Intervention

Phase 1: sensitization phase: on days 1, 2 and 5 hamsters in groups OVA, OVA T, and OVA TP were intraperitoneally (i.p) injected with 100 µl of 0.5 mg/dl OVA and 20 mg/dl alum suspension mentioned previously.

Phase 2: challenge phase: on day 33, animals were challenged nasally as drops with 0.4 mg/ml OVA solution (groups OVA, OVA T, and OVA TP) and 0.05 g/ml histamine (groups H and HT). Histamine was freshly prepared by dissolving 0.01 g histamine (Histamine 95% (sigma- K7125-G)) in 200 µl PBS and mixing them on vortex at 2,000 rpm for 5 minutes.

Phase 3: treatment phase: one hour after nasal challenge (phase 2) with previously mentioned substances, animals in groups C, OVA, and H were treated nasally by PBS, and animals in groups CT, OVA T, OVA TP, and HT were

treated nasally with 100 mg/ml aqueous *Ts* extract solution. *Ts* solution was freshly prepared by dissolving 1 g in 10 ml PBS and mixing them on vortex at 2,000 rpm for 2 minutes.

Thymus serpyllum extract of 1 g/ L water was given orally for groups CT and OVA TP on day 0 as prophylaxis and for groups OVA T and HT on day 33 one hour after nasal challenge as a part of the treatment till the time of blood samples collection on day 35.

Blood Sampling

It is important to collect blood samples from experimental animals with least stress possible in order not to affect the outcomes. To achieve that, Samples were collected under terminal anesthesia.[10] Animal was put in a cage with chloroform ($CHCL_3$)-damped cotton. After 5 minutes of inhaling $CHCL_3$, it became anesthetized as it showed loss of passive movements and no response to pain stimulation. In a supine position and using blades, a midline thoraco-abdominal incision was done, chest was opened and a blood sample of (1.5-2) cc was taken from heart slowly using butterfly needles and syringes. Samples were collected in labeled blood collection tubes; EDTA (ethylenediaminetetraacetic acid) tubes, to measure the required parameters (Figure 1).

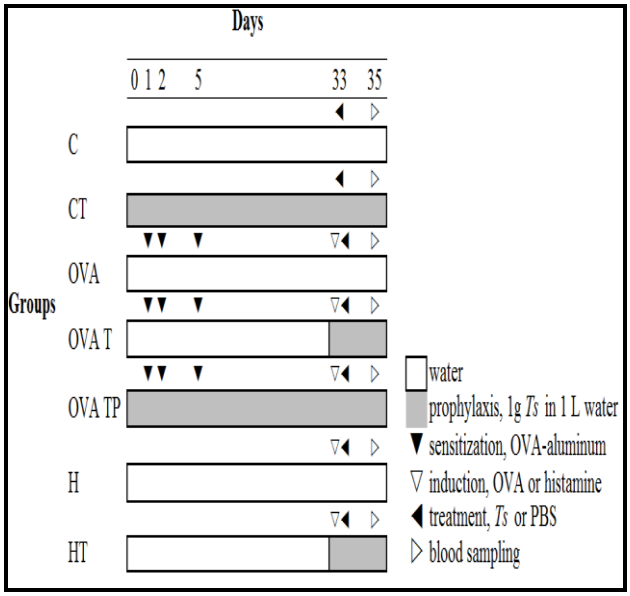

Figure 1: Time Course of Experiment

Clinical Symptoms Assessment

Hypersensitivity reaction was assessed by nasal symptoms frequencies that were evaluated by counting the number of sneezes and nasal rubbings that occurred during the 60 minutes after challenge phase and the 60 minutes after treatment phase.[8]

Measurement of Blood Parameters

***WBC Count*:** manual method of counting WBCs was used. Using 1:20 dilution method[11], 10 µl blood was diluted in 190 µl Turk's solution (3% acetic acid with Gentian violet stain)[12], diluted sample was put in Neubauer counting chamber and WBCs were counted under microscope.[13]

***Differential Count*:** peripheral blood film was prepared from each sample. A drop of blood was put on a clean slide, dragged using another slide slightly backward and then smoothly forward to spread the drop of blood evenly. When dry, it was put in methanol for 2 minutes for fixation then in Giemsa stain for 30 minutes and finally washed with distilled water. Under microscope, 100 WBCs were counted and percentage of each cell type (neutrophils, lymphocytes, monocytes, and eosinophils) was calculated.

Statistical Analysis

Data was analyzed using standard statistical methods provided by SPSS software (version 16.0.Ink).

Data was reported as mean and standard deviation. Significance of the differences among groups was determined using analysis of variance (ANOVA) followed by Scheffe *post-hoc* test for comparison between groups. *P* value < 0.05 was accepted as a statistically significant difference.

We did a robust test of equality of mean; Welch test, which is done when there is a violation in ANOVA to reveal if we could proceed with ANOVA results or not. *P* value < 0.05 was accepted as a statistically significant difference and means that we could proceed with ANOVA results.

RESULTS AND DISCUSSION

Aqueous Extract

Thymus serpyllum L. is a common Palestinian flora; 25 grams of the plant powder were subjected to exhaustive ethanolic extraction. The total weight of pure dried aqueous extract was 4.28 g (17.12 % of the total powder weight).

Effect of *Ts* Clinically

There was a significant difference between groups in relation to sneezing (F= 307.604, $p< 0.001$); (Table 1, Figure 2), and nasal rubbing (F= 80.860, $p< 0.001$) frequencies; (Table 2, Figure 3). Welch test showed significant difference between groups in relation to sneezing and nasal rubbing frequencies ($p< 0.001$).

Table 1: Sneezing Frequency Mean and SD of Each Group

Group	N*	Mean	Std. Deviation**	F	*p* value
C	5	2.00	0.707	307.604	< 0.001
CT	5	2.00	0.707		
OVA	5	34.40	3.647		
OVA T	5	7.60	1.140		
OVA TP	5	2.40	0.894		
H	5	43.00	1.871		
HT	5	35.60	4.393		

*Number, **Standard Deviation

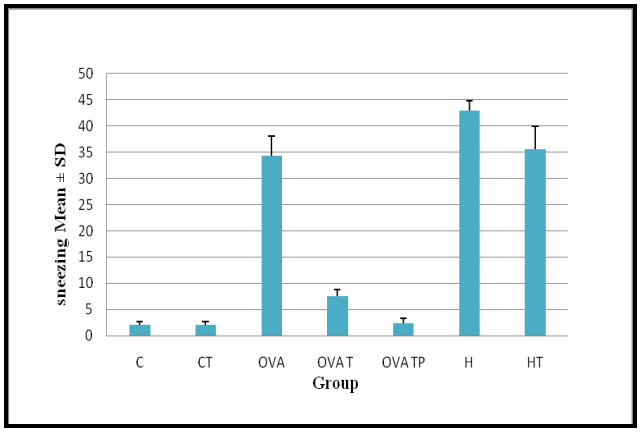

Figure 2: Sneezing Frequency Mean ± SD of Each Group

Table 2: Nasal Rubbing Frequency Mean and SD of Each Group

Group	N	Mean	Std. Deviation**	F	*p* value
C	5	2.40	1.140	80.860	< 0.001
CT	5	3.00	1.225		
OVA	5	14.80	3.114		
OVA T	5	6.40	1.673		
OVA TP	5	2.40	0.548		
H	5	21.80	2.049		
HT	5	11.60	2.074		

*Number, **Standard Deviation

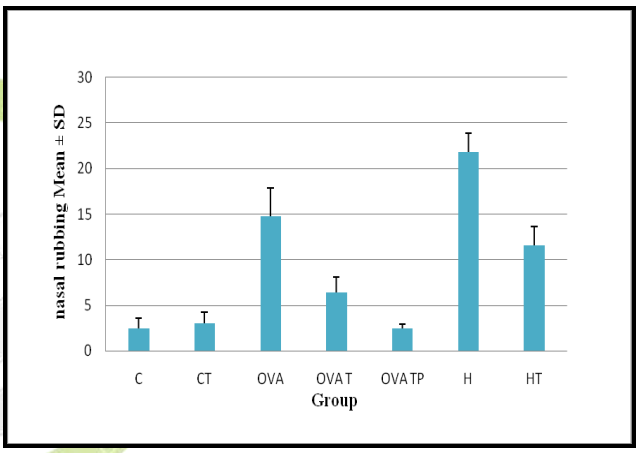

Figure: 3 Nasal Rubbing Frequency Mean ± SD of Each Group

Normal Healthy Groups

Possible side effects of *Ts* were tested on CT group. This group showed immediate sneezing and nasal rubbing lasting for few minutes after *Ts* administration. However, Compared with C group, there was insignificant difference between them in relation to sneezing and nasal rubbing frequencies ($p = 1.0$). Moreover, compared with OVA group, OVA group showed a significantly ($p < 0.001$) higher frequency of sneezing and nasal rubbing than CT group; the mean difference (I-J)= 32.400 for sneezing and 11.800 for nasal rubbing, (I, J go for frequency mean of OVA and CT groups symptoms, respectively). Nasal symptoms are most likely due to local irritation caused by administered *Ts* rather than airway irritation and

hyper-responsiveness, which means that *Ts* does not have any significant side effects.

OVA-Induced Asthma Groups

To investigate the effect of *Ts* on asthma, Syrian hamsters were challenged with OVA to develop models with hypersensitivity reaction in their lungs airways. Nasal allergic symptoms, such as sneezing and nasal rubbing, appeared immediately after starting intranasal OVA challenge on day 33.

Comparison between C group and OVA group showed a significant difference in relation to symptoms frequencies ($p< 0.001$), which means that models were successfully inducted with asthma. (It is worth to mention that those results are the same ones obtained from tester group).

Comparing group OVA T with OVA and C groups in relation to nasal symptoms showed an effective improvement in asthma clinical picture that nearly normalized healthy animals. There was a significant difference in relation to symptoms frequencies ($p < 0.001$) between OVA and OVAT groups; the mean difference (I-J) (I and J go for frequency mean of OVA and OVA T groups symptoms, respectively) was (26.800) for sneezing and (8.400) for nasal rubbing, and insignificant difference in relation to sneezing ($p = 0.059$) as well as in relation to nasal rubbing ($p = 0.110$) frequencies between OVA T and C groups.

Pre-Treated Group

As a prophylactic, *Ts* showed promising results in preventing OVA from causing significant nasal symptoms when it was administration before OVA sensitization/ challenge phases.

Compared with OVA group, *Ts* administration in pre-treated group before OVA sensitization/ challenge had significantly prevented OVA from causing the clinical picture of asthma; $p < 0.001$ in relation to symptoms frequencies. The prophylactic effect was enforced by comparing OVA TP group with C group; it was found that there is no significant difference between them in relation to sneezing ($p = 1.0$) and nasal rubbing ($p = 1.0$).

Histamine-Induced Asthma Groups

Histamine is one of the most important mediators released from mast cells and its involvement in human hypersensitivity reactions is well established.[14]

Nasal symptoms observed in H group were significantly more frequent than C group symptoms ($p < 0.001$ in relation to sneezing and nasal rubbing frequencies) and OVA group symptoms ($p < 0.001$, I-J= -8.600 in relation to sneezing frequency and -7.000 in relation to nasal rubbing frequency, I and J go for frequency mean of OVA and H groups symptoms, respectively).

After *Ts* treatment, nasal symptoms in HT group were less than those in H group in a significant difference ($p < 0.001$). However, comparing the effect of *Ts* on histamine-induced symptoms frequencies with C group showed a significant difference ($p < 0.001$), which means that the effect on histamine was not as on OVA to decrease symptoms frequencies to near control ones.

This may be explained by the fact that histamine acts acutely in causing vasodilatation and inflammation and as samples were taken 48 hours after histamine exposure, it may be the self-limiting process that caused a significant improvement in symptoms frequencies when compared with H group not the effect of *Ts*.

Our results proposed that *Ts* acted on other pathways than histamine in OVA T group so symptoms were near normal frequencies while its effect on histamine pathway was not significant to normalize the symptoms.

Effects of different interventions on the clinical nasal symptoms are summarized in Table 3.

Effect of *Ts* on Hematological Parameters; WBC Count and Differential WBC

WBC Count

There was a significant difference between groups in relation to WBC count ($F= 95.733$, $p < 0.001$), Welch test had a p value less than 0.001.

Table 3: *p* value and Mean Difference of Sneezing and Nasal Rubbing

Group		**Mean Difference I-J**		***p* value ***	
(I)**	**(J)****	**Sneezing nasal Rubbing**		**sneezing nasal rubbing**	
C	CT	.000	-.600	1.000	1.000
	OVA	-32.400	-12.400	.000	.000
	OVA T	-5.600	-4.000	.059	.110
	OVA TP	-.400	.000	1.000	1.000
	H	-41.000	-19.400	.000	.000
	HT	-33.600	-9.200	.000	.000
OVA	CT	32.000	11.800	.000	.000
	OVA T	26.800	8.400	.000	.000
	OVA TP	32.000	12.400	.000	.000
	H	-8.600	-7.000	.001	.000
H	HT	7.400	10.200	0.005	.000
* *p* value significant when < 0.05, **Frequency mean					

Table 4: WBC Counts Mean Std. Deviation for Each Group

Group	**N**	**Mean**	**Std. Deviation****	**F**	***p* value**
C	5	3680	258.844	95.733	< 0.001
CT	5	3740	207.364		
OVA	5	22950	3109.662		
OVA T	5	9740	2016.928		
OVA TP	5	6880	947.101		
H	5	14250	650		
HT	5	12050	1316.245		

The mean of WBC counts of each group members was normal in C and CT groups and elevated in other groups as shown in Table 4.

- Compared with C group, WBC count was not significantly elevated in CT group (p = 1.0) which means that *Ts* did not have a significant effect on WBC count.
- Compared with C group, WBC count elevated significantly in OVA group (p < 0.001, the mean difference (I-J) = -19,270.0, I and J go for the mean of WBC count in C and OVA groups, respectively) as well as H group (p < 0.001, the mean difference (I-J) = -10,570.0, I and J go for frequency mean of C and H groups, respectively).

After *Ts* administration, WBC count of OVA T group was significantly reduced compared with OVA group (p < 0.001) but not to the extent that approximating C group WBC count because there was a significant difference (p < 0.001) between OVA T group and C group in relation to WBC count. This explains the improvement at the clinical level that was mentioned previously. With regard to HT, *Ts* effect was not significant in reducing WBC count because there was no significant difference compared with H group (p = 0.553) and this reinforces the fact that *Ts* does not act on histamine pathway.

In OVA TP and with comparison with OVA group, prophylactic effect of *Ts* was promising as the significant difference in relation to WBC count (p < 0.001) showed. In other words, *Ts*

administration in OVA TP group before OVA sensitization/ challenge was effective in preventing the significant increase in WBC count caused by OVA and keeping WBC count near the normal; $p = 0.142$ in relation to WBC count of OVA TP and C groups.

Differential WBCs

There was a significant difference between groups in relation to differential WBCs; lymphocytes (F=19.947, $p < 0.001$), neutrophils (F= 47.841, $p < 0.001$), and eosinophils (F= 12.155, $p < 0.001$). However, in relation to monocytes, there was no significant difference between groups (F= 1.439, $p = 0.235$). (Figure 4). Welch test showed significant results ($p < 0.001$) in relation to lymphocytes, neutrophils, and eosinophils but insignificant one (p = 0.205) in relation to monocytes.

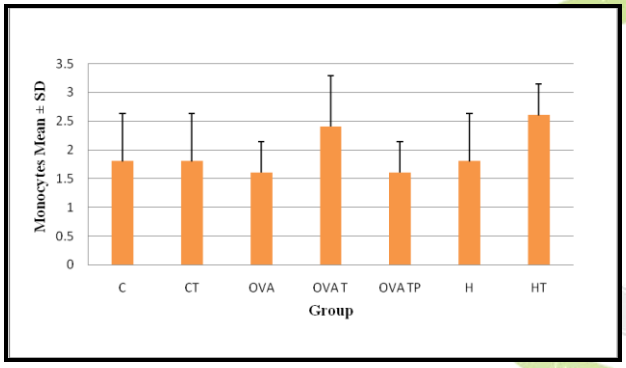

Figure 4: Monocyte Mean ± SD of Each Group

Blood films had showed the lymphocyte being the dominant cell type in all models regardless to the changes occurred in differential WBC as a whole picture. It was found that morphology of WBCs of hamsters differs from that known in human (Figure 5).

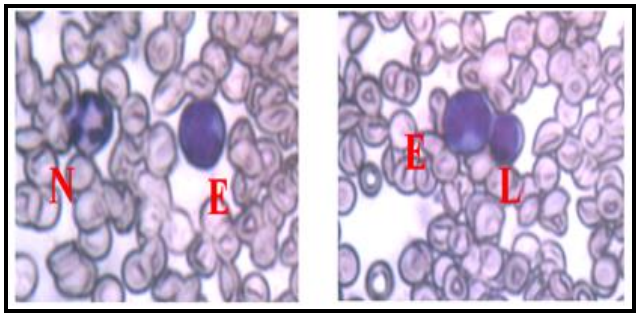

Figure 5: Blood Films of OVA-induced Asthma Models, L: Lymphocyte, E: Eosinophil, N: Neotrophil

- Compared with C group, OVA group showed a significant difference in relation to each cell type of WBCs ($p < 0.001$). OVA increased lymphocytes and eosinophils and decreased neutrophils from the C group levels. Looking for H group and with comparison with C group, there was a significant difference in relation to lymphocyte and neutrophils ($p < 0.001$) but was not a significant one in relation to eosinophils ($p = 0.432$), as shown in (Figure 6). Histamine had elevated lymphocytes and reduced neutrophils from C group levels.

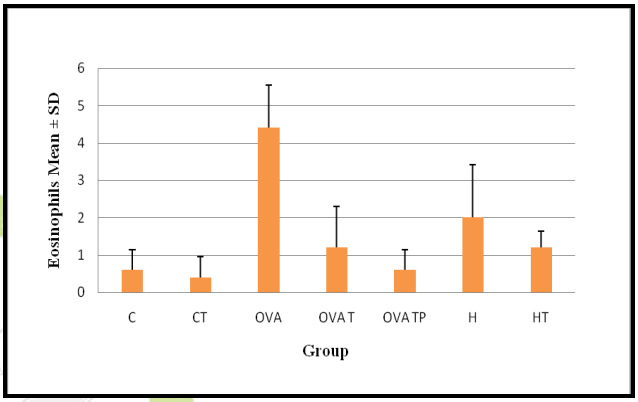

Figure 6: Eosinophil Mean ± SD of Each Group

- After treatment with *Ts*, differential WBC of OVA T group was significantly different in relation to each cell type when compared with OVA group ($p < 0.001$). *Ts* had changed differential WBC in the opposite direction of OVA; it had reduced lymphocyte and eosinophils and elevated neutrophils. Such changes brought the differential WBC picture to be near C group one (no significant difference between C and OVA T groups in relation to lymphocytes, neutrophils, and eosinophils ($p = 0.999$, 1.0, 0.978 respectively). Eosinophils play a major role in exacerbating allergen-induced asthma. *Ts* treatment of OVA induced asthma had significantly normalized the differential WBCs and this explains the significant improvement in nasal rubbing and sneezing as the effect on eosinophils decreased the release of immunoglobulin E (IgE) and subsequently the inflammatory process that underlying allergic asthma.

- Concerning HT group, comparison with C group showed a significant difference in relation to lymphocytes and neutrophils ($p < 0.001$) (Figures 7 and 8) and an insignificant difference in relation to eosinophils ($p = 0.978$); (Figure 6) which is something resembled the relation between H and C groups. This is explained by the insignificant difference between HT and H groups ($p = 1.0$ in relation to lymphocytes and neutrophils, $p = 0.914$ in relation to eosinophils) which means that *Ts* did not have a significant effect in normalizing the differential WBC of H group to be as C group as it did with OVA.
- In CT group, the effect of *Ts* on WBC differential was not significant in relation to lymphocytes, neutrophils, and eosinophils ($p = 1.0$); differential WBC of CT group remained near C group differential WBC. As mentioned previously, there is a possibility that *Ts* may cause side effects influencing the study results. As the observations showed, *Ts* caused insignificant asthma-like nasal symptoms. However, the insignificant effect on WBC count and differential WBC which were near normal supports the fact that those symptoms were caused because of local irritation rather than airway hypersensitivity reaction.
- In OVA TP and with comparing it with OVA group differential WBC, there was a significant difference in relation to each cell type ($p < 0.001$) (Figures 6, 7 and 8) which means that *Ts* was effective in preventing OVA from changing the differential picture that remained near C group one; there was an insignificant difference between C and OVA TP differential WBC ($p = 0.996$ in relation to lymphocytes, $p = 0.997$ in relation to neutrophils, and $P = 1.0$ in relation to eosinophils frequencies).
- As a prophylactic, *Ts* showed promising results in preventing the inflammatory process of OVA-induced asthma. Administration of *Ts* before OVA sensitization/ challenge significantly prevented OVA from causing significant nasal symptoms which is explained by the prophylactic effect of *Ts* in preventing the change in WBC count and differential WBC which remained near normal; there was no significant cellular inflammatory process specifically at eosinophils level.

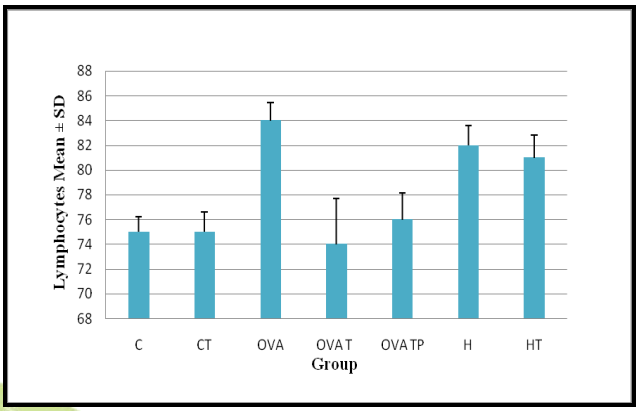

Figure 7: Lymphocyte Mean ± SD of Each Group

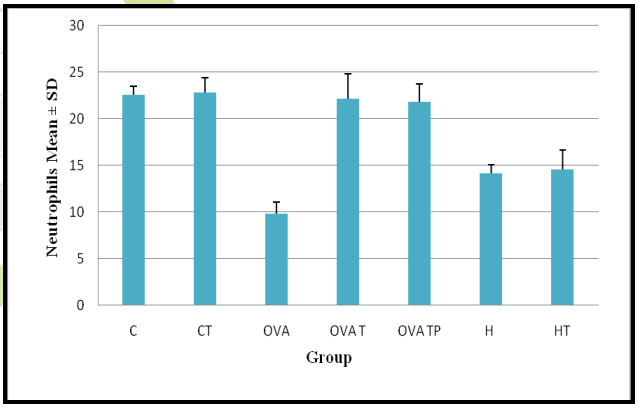

Figure 8: Neutrophil Mean ± SD of Each Group

CONCLUSION

Through this work, an attempt was made to discover possible therapeutic and prophylactic effects of *Ts* on asthma models.

Ts significantly improved asthma clinical picture caused by OVA by decreasing eosinophils which are responsible for the release of IgE and subsequently the inflammatory process underlying allergic asthma. However, histamine-induced asthma models did not show a significant improvement in either the clinical picture or the hematological parameters

suggesting that *Ts* did not act on histamine pathway.

In addition to therapeutic effect, *Ts* showed a prophylactic effect on the clinical picture as well as on the cellular inflammatory process specifically eosinophils level.

ETHICAL CONSIDERATIONS

All animal procedures were conducted under an animal protocol of Institutional Animal Care and Use Committee (IACUC), focusing on the following points:

- Cages allowed for social interactions, adequate ventilation and minimal disturbance to them. Also, they provided safe and secure environment that permits the normal physiologic and behavioral needs of the Syrian hamsters to be expressed.
- Cages were readily accessible to food and water.
- Identification of the Syrian hamsters was by colored stains.
- All Syrian hamsters received food that is palatable and of sufficient quantity and nutritive value to maintain their good health. In addition, water bottles were replaced rather than refilled.

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