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RESEARCH ARTICLE

Histological and Immunological Study of Daily Supplement of Aqueous extract of *Thymus vulgaris* Leaf in Mice Challenged with *Vibrio alginolyticus*

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Abstract

The aim of current study was evaluation the potential effects of daily supplement of aqueous extract of *thymus vulgaris* leaf on immunity system and some of total serum proteins performances in mice was challenged with *V. alginolyticus*, a total of 20 mice was divided into four group, group I:(Normal saline), group II: injected subcutenously *V. alginolyticus*, group III administrated orally for 14 day with 400µgkg of aqueous extract *Thymus vulgaris* leaf. Finally group IV injected subcutenously with *V.alginolyticus* and administrated orally for 14 day with 400 µg/kg of aqueous extract *Thymus vulgaris* leaf. The results of phagocytic activity reported there were significant differences (P≤0.05) between group IV (3.90±0.24) and (2.77±0.24); group II (2.65±0.24), while there was no highly significant difference (P≤0.05) between groups II, group III. In other hand lymphocytes proliferation results were revealed group III, group IV (0.380±0.020, 0.400±0.08) were recorded highly significant differences with Group II (2.65±0.24).In addition, the total serum proteins were showed the highest value was recorded in group III and group IV at day 10 and day 14 with highly significant differences (P≤0.05), Furthermore For delayed type hypersensitivity (DHT), the results were revealed group IV had the highest value after 48, 72 hr, (3. 6±0.33) and recorded a highly significant difference (P≤0.05) with group III, group II after 48, 72 hr. Finally the histopathological examinations of small intestines were showed changes in group II like losing of mucous membranes, damaging in villa, moreover in group IV there were presence of inflammatory cells in damaged area also there were villi partially damaged. This study was revealed depending on the results, the daily supplement of aqueous extract thymus *vulgaris* leaf (400 µg /kg) had positive effects on the immune response, total serum protein, and showed positive effects in histological examinations through protection the small intestine from *V. alginolyticus* invasion.

Keywords: *Aqueous extract of Thymus vulgaris leaf, Histological changes, Immunological response, V.alginolyticus*.

Introduction

Thymus vulgaris plant (thyme) is one of aromatic plants belonged to the Lamiaceae family, the medicine importance of thyme arised from thymol component and carvacrol component, which are the most medically important compounds [1,2] and those evidences came after some studies were revealed the daily feeding of thyme improved many parameters performance of [3,4] Furthermore, thyme essential oil has antioxidant and antimicrobial potentials well like antibacterial [5, 6] anticoccidial [7], and antifungal [8] dues to the volatile components (oil) from thyme that played role in inhibition of microbial growth [9].The major components of thyme playing as antioxidant and antimicrobial are thymol and carvacrol, the phenolic compounds [10] in other side these compounds showed beneficial in poultry health and production and against the pathogenic bacterial growth, [11, 13].Like *V. alginolyticus*, this organism is widely distributed in animals such as seawater and seafood and is probably the most common vibrio found fish [14] also an agent of wound and ear infections, also

some clinical features included mild cellulitis in human, The mechanism of *V. alginolyticus* pathogenic included adhesion and releases some of extracellular substances and products, also neurotoxins, those products were able to degrade the mucus, also had cytotoxic activity for cells in the intestine, the clinical symptoms are included watery diarrhea, vomiting, fever [15].

The objective of study was evaluation the potential effect of daily supplements of aqueous extract of *thymus vulgaris* leaf on immunity system and some of total serum proteins performances ,also this study was aimed to evaluation protection potential of the extract against *V. alginolyticus* in vivo by histological examinations of small intestinal.

Material and Methods

All experiments were done in the laboratories of the in Dijlah University/ collage, and Uruk university/ college, the research was done on male albino mice (Blab-c) with average weight was 22-25 grams.

Isolation and identification vibrio *alginolyticus*

Obtained from Al Numan hospital. Isolated from a patient suffered from otitis and confirmed with Vitek 2.

Preparion of Aqueous Extract of *Thymus vulgaris* Leaf

The aqueous extract preparation was according to [16] by putting (20) g of Thyme powder leaf in (250 mL) of distilled water at boiling point, under reflux for 1 hr. The extract was filtered and evaporated at 50°C to compete dryness: (400 µg / kg) of water extract of *thymus vulgaris* leaf was prepared.

Experimental Design

The groups of study were included four groups each group included (5 animals), group I was control group (injected subcutenously with normal saline (0.5 ml), group II was: injected subcutenously with 0.5 ml of suspension of *V.alginolyticus* (1.5 × 108 CFU/ml), group III was administrated orally for 14 day with 400 µg / kg of aqueous extract *Thymus vulgaris* leaf. Finally group IV: was injected subcutenously with 0.5 ml of suspension of *V, alginolyticus* (1.5 × 108 CFU/ml) and administrated orally for 14 day with 400 µg / kg of aqueous extract *Thymus vulgaris* leaf.

Laboratory Assessments

Blood Collection

The mice were sacrificed after the 10 days , and after 14 day , for Phagocytic activity Test assay and Lymphocyte proliferation Test assay, while the serum was collected for serum Agarose Gel Electrophoresis, the gut of group II, IV was collected for histological aspect

Phagocytic activity Test Assay

The procedure was done depending on a method presented by [17], peripheral Blood Leucocytes 1^{χ} 10^{6} cell/ well (100 *ul* Blood, with 175 *ul* MEM media) added to each well of microtiter plate, 25 *ul* of NBT was added and incubated two hours at 28c°. Then supernatant was removed carefully.

Cells were fixed with methanol 100% (v/ v) for 5 min in each well .and washed twice with 125 ml with Methanol 70%.Then drying at air over night. Finally adding 125 ml Potassium hydroxide (2N) and 150 ml DMSO add to each well to dissolve NBT. Then Read by Elisa at 650-wave length.

Lymphocyte Proliferation Test Assay

The procedure of [18] was followed to prepare lymphocytes from mice blood then the cell count was adjusted to 2 x 10^{6} lymphocytes/ml of lymphocyte culture medium. 100 µl of 1% DMSO was added, then 5mg/ml MTT (10% of total volume per well) was added, 80 µl of (46 ug/ml), *V. alginolyticus* was added to each wells, the micro plate was wrapped with a cling film, and incubated in at 37ºC for 72 hours in an incubator supplemented with 5% CO_2.and finally Read by Elisa at 490-wave length.

Delayed Hypersensitivity Test

All mice in the groups was injected with 50 ul of *V. alginolyticus* in the right foot and measured at time zero and at 24, 48 and 72 hours [19].

Serum Agarose Gel Electrophoresis

The procedure was done by following of a commercially available kit steps to determine the total serum proteins.

Histopathological Examination

Pieces of intestines from treated mice in group II , group IV were collected at day 14 and fixed with 10% formalin., the specimens were washed many times with distal water, then dehydrated in a graded series of ethanol(50, 70, 90, 95, 100 and 100% two hrs. for each).

After dehydration, the specimens were processed for paraffin embedding(Blocking) and sections with 4 µm were cut by using Rotary Microtome and conveyed to water bath 50C, fixed on a slide for staining and then placed in Xyline to dissolve paraffin wax, then grades of ethanol (100, 90, and 70) two mins. For each. Finally, the specimens were stained with hematoxylin for two mins., then with water for five mins. And graded of ethanol (70, 90) after that stained with eosin then placed in ethanol (70, 90, 100) one min. for each concentration and Xyline, after that they should be dried, [20].

Results

The bacterial isolates were obtained from Al-Numan hospital. And has been identified according to the morphological characterization, [21] and the identification was achieved by Vitek2. The results of evaluation of phagocytic activity in this study reported there were significant differences (P≤0.05) between group IV (3.90±0.24) and group III (2.77±0.24), group II (2.65±0.24), while there was no significant difference (P≤0.05) between group II and group III as showed in Figure 1.

In other hand lymphocyte proliferation assay was revealed group III, group IV (0.380±0.020, 0.400±0.08**)** respectively were recorded highly significant differences (P≤0.05) with group II (2.65±0.24), in same time there was a significant differences between group III and group IV, as in Figure (2).

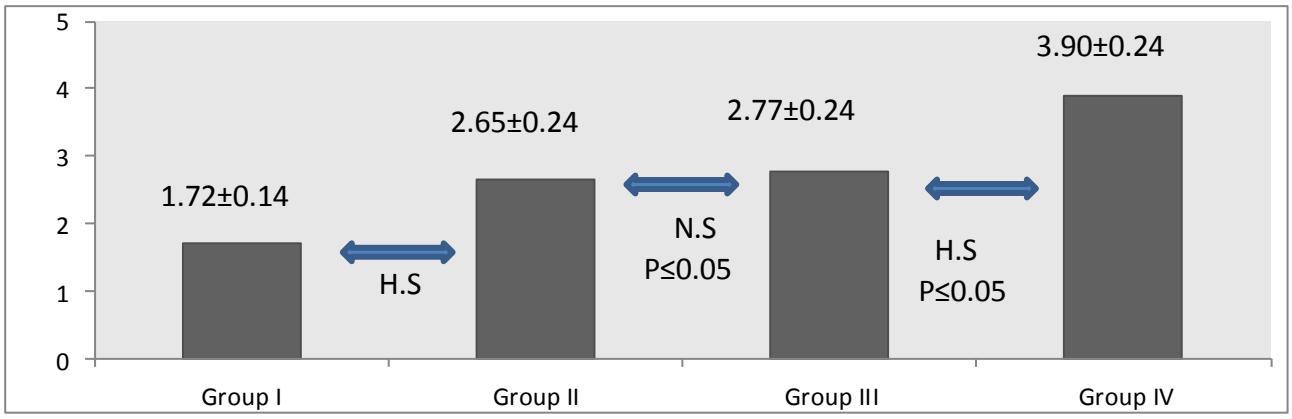

Figure 1: showed the results and significant differences between groups for phagocytic activity assay

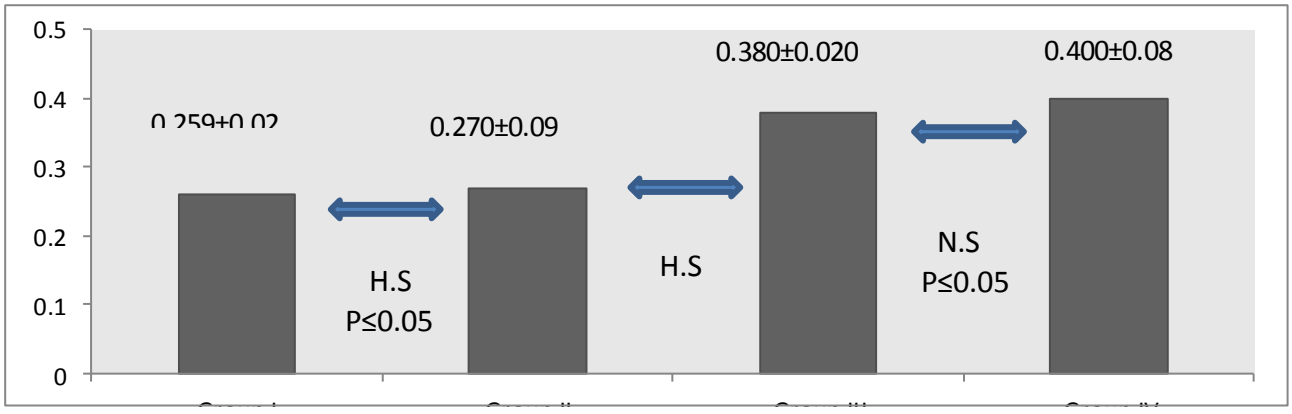

Figure 2: Showed the results and significant differences between groups for lymphocyte proliferation assay

For delayed type hypersensitivity (DHT), the results were revealed group IV had the highest value after 48, 72 hr, (3. 6±0.33) and recorded a highly significant difference (P≤0.05) with group III, group II after 48, 72 hr as showed in Table 1.

Table 1: Results of delayed type hypersensitivity (DTH)

Groups	Time zero	After 24 hr	After 48 hr	After 72 hr
Group1	1.33±0.00[a]	1.33±0.03 [a]	1. 43±0.05 [a]	1.37±0.03 [a]
Group II	1.5±0.00[a]	1.91±0.00[b]	1..7±0.00[c]	1.5±0.00[a]
Group III	1.62±0.20[a]	2.56±0.10[b]	3.0±0.16[c]	2.77±0.04[d]
Group IV	2.20±0.06 [a]	2.57±0.00 [b]	3. 6±0.33 [b]	2.86±0.07 [c]

In addition, the result of the serum proteins (globulin proteins.) assay were showed the highest value was recorded in group III and group IV 10 day in Gamma globulin was (12.34± 0.13, 17.77± 0.13) respectively. In Alpha 1 globulin (7.12± 0.53 ± 7.53±0.62), in Alpha 2 globulin (17.76±0.15, 17.77±0.67), while in beta globulin (22.21± 0.17, 24.8±1.57) with significant differences (P≤0.05) with group II. Moreover the highest values were recorded again in group III and group IV at 14 day, in Gamma globulin was (14.13± 0.13, 19.98±0.27) respectively, in Alpha 1 globulin (8.5± 0.22, 11.31±1.48) respectively, in Alpha 2 globulin (19.23± 0.13, 20.57±0.69), while in beta Globulin (23.56± 0.31, 26.97±1.55) with significant differences (P≤0.05) with group II. Furthermore the results were recorded there were significant differences between day 10 and day 14, in all studied groups as showed in Figure (3, 4, 5, 6) respectively.

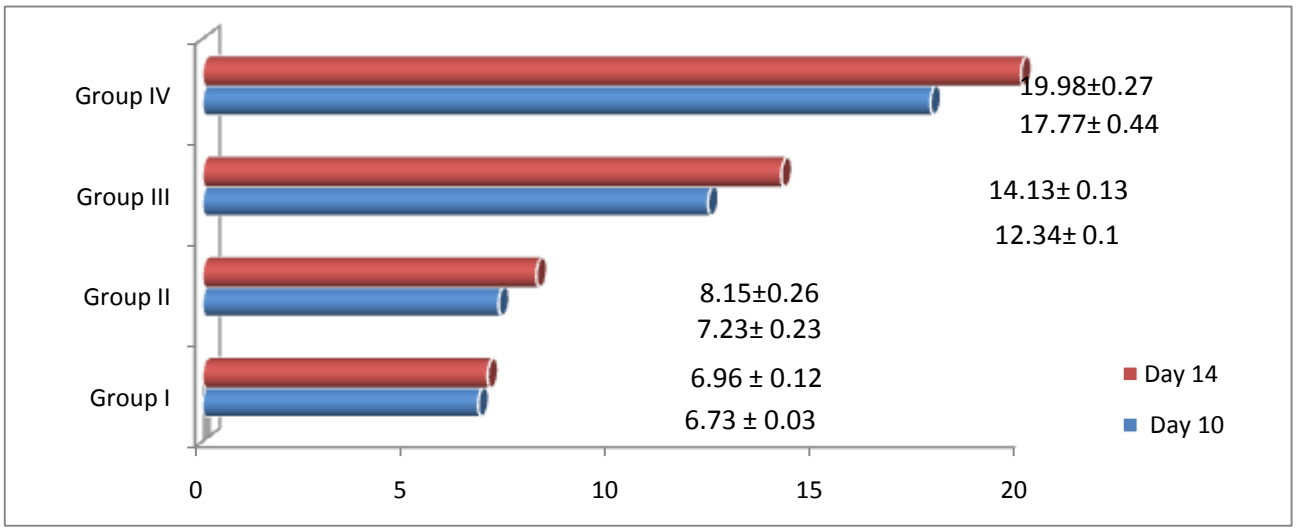

Figure 3: showed Gamma Globulin level results at day 10, and at day 14

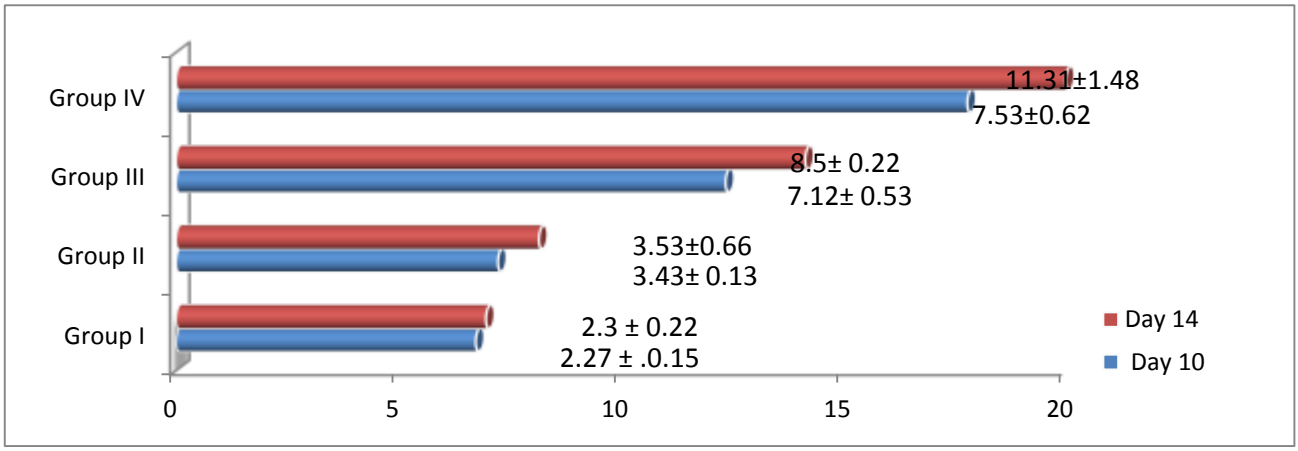

Figure 4: showed of Alpha -1 globulin level results at day 10, and at day 14

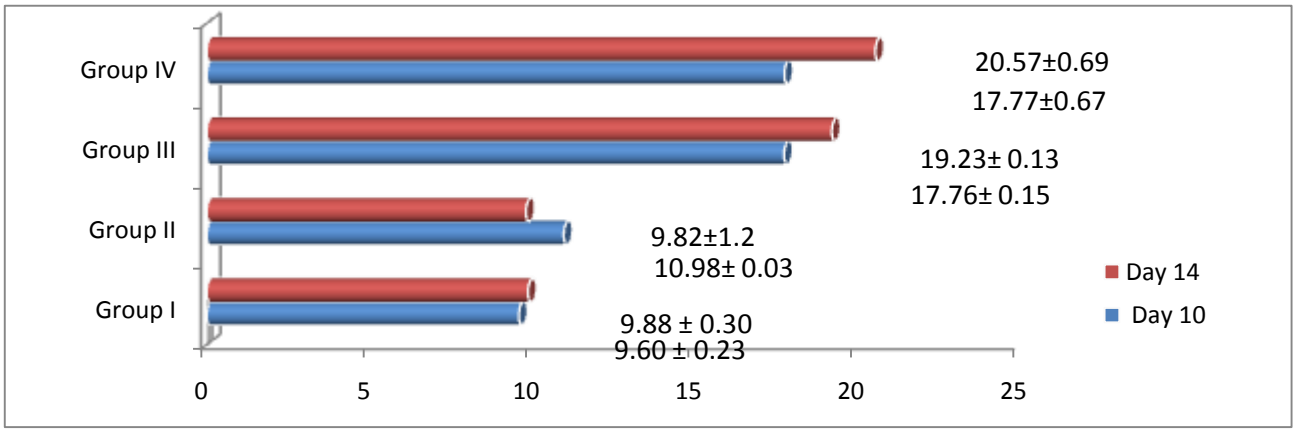

Figure 5: showed Alpha -2 Globulin level results at day 10, and at day 14

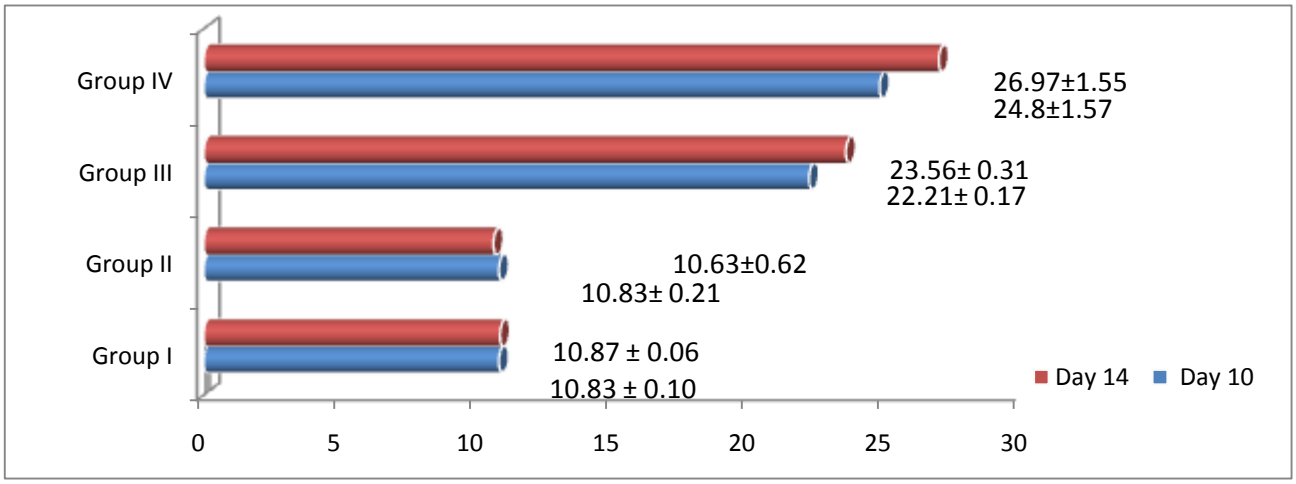

Figure 6: showed Beta Globulin level results at day 10, and at day 14

After noticed the clinical signs in treated mice in group II, which were included weakness and less in activity and finally noticed blood in stools, while group IV there was not any abnormal features in treated mice, this was confirmed by the histopathological examinations of small intestine were showed changes in small intestine of group II like losing of mucous membranes as showed in Figure (7). Moreover villa were damaged, presences of inflammatory cells showed in Figure (8). In addition, the results in this study were revealed infiltration of, macrophage, neutrophiles in damaged area, especially in the lamina propria in group 1V showed in Figure (9), as well as noticed partially damaged villi in small intestine as showed in Figure (10).

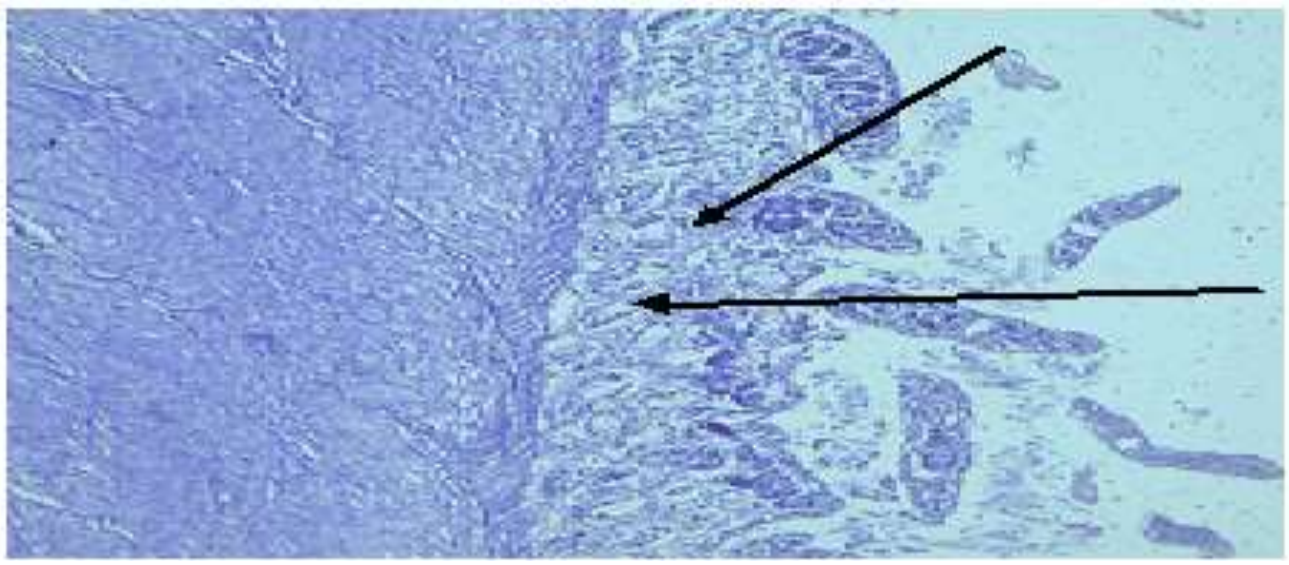

Figure 7: Showed the lost of mucous membrane

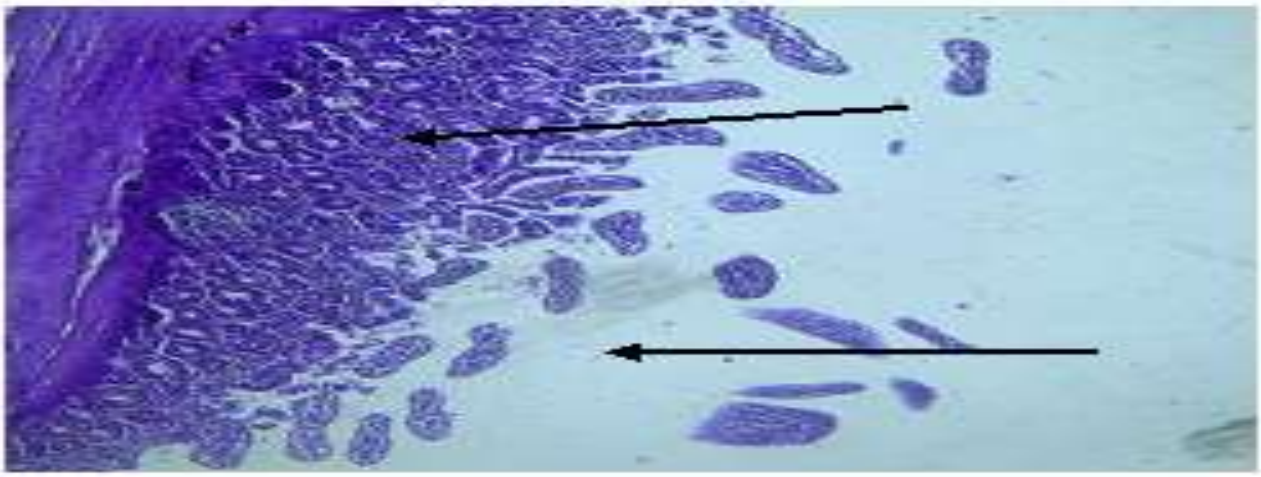

Figure 8: showed damaged of villa of small intestine and infiltration of inflammatory cells

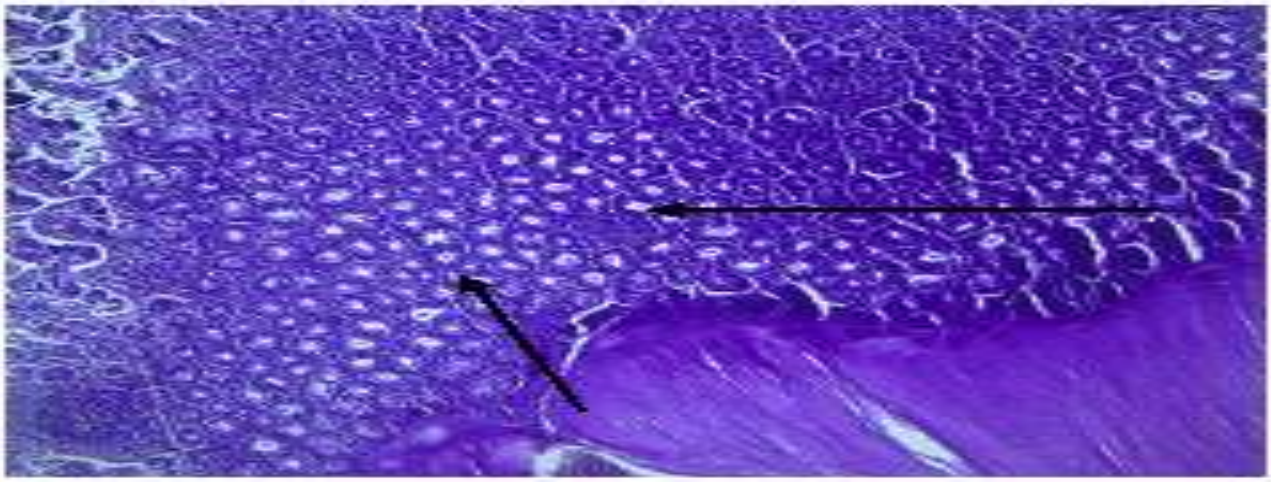

Figure 9: showed inflammatory cells, macrophage, neutrophiles in damaged area

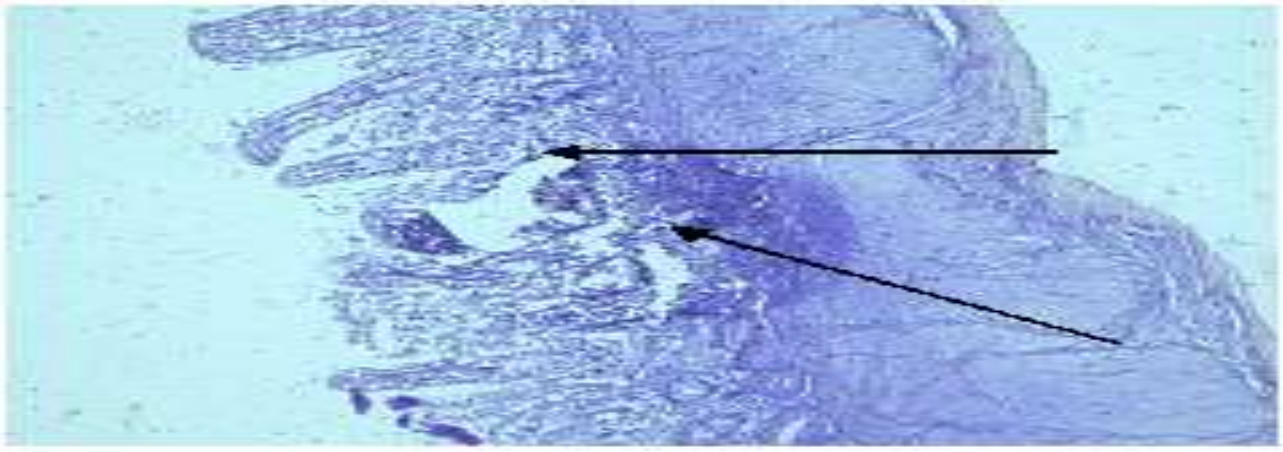

Figure 10: showed the partially damaged villi

Discussion

V. alginolyticus is medically important and causes otitis and able to adheres and wound infection [1].Also it founds in many of animals more common in fish, and responsible for the production neurotoxin, tetrodotoxin [22]. The result of Phagocytic activity were showed there was no significant differences between group II, group III, this result were agreed with [23, 24] who reported that thymus extract reduce the production and reduce the gene expression that important to produce the pro-inflammatory mediators from the cells such as TNF-*a*, IL-1B, and IL-6 but in same time increase produces the antiflammtory such as IL-10 [25, 26].

Neutrophiles and macrophage are pahocytic cells and kill the bacteria by phagocytosis which led by pro inflammatory mediators and release inflammatory mediate to induce inflammatory response [27]. Many studies found the thymus have antiflammatory effect

by induce Cytokines IL 10, TGF-ß that effect on the regulation of phagocytosis of macrophage and Also the IL 10 inhibition of respiratory burst [28]. Also some studies showed thymus extract might have influence on molecules adhesion such as vascular cell adhesion molecule-1, and finally effect on matrix metalloproteinase 9 [29].However, the results were revealed the group IV had The highest value with highly significant difference with group III this agreed with [30] who revealed thyme essential oil for a period of 30 days, improved the survival of Atlantic salmon when challenged with *Saprolegnia parasitica as well* as agreed with similar studies that used different extract such as *Coriolus versicolor* [31], who revealed the supplementary diets with suitable doses of *Coriolus versicolor* polysaccharides enhanced the WBC count in crucian carp when challenged with *Aeromonas hydrophila*.

This attributed to to a stimulation of the immune system with thyme essential oil due to phenolic compounds such as eugenol (2-methoxy-4-(2-propenyl) phenol), thymol and carvacrol present in essential oils[30]. Due to the role of WBC in non-specific or innate immunity [32], increasing in WBC count and its functions was quite likely to result in an enhancement of the non-specific defense. Many studies have shown that immunostimulants, such as tuftsin, *C. versicolor* polysaccharides, azadirachtin and ginger elevated the WBC count of fish [33, 35].In addition, Lymphocytes proliferation was highly significantly differences in group IV and group III than group II and this agreed with [36], who revealed the lymphocytes in boiler chicken was increased with different treatment concentration of thymus vulgaris. Those facts tend to thyme (*Thymus vulgaris*) that are full of flavonoids, in Other hand some compounds such as tannin and thymine are enhanced the activation of development lymphocyte, increase production of cytokines the important in humoral immune response, IL4 [23] and TGF-B Treg cells as well as release IL-10 from T reg, Breg, and Th2 cells [37].

TGF-ß helps the differentiations and survives of T regs, B reg and Th2 as well as maintaining self-tolerance [38].And these suggestions was confirmed in DHT result that revealed the highest values were recorded in Group III, Group IV after 72 hr. As we discussed the carvacrol and thymol effected on IL 10 and TGF-ß expression [39, 40].The TGF-B is produced by Treg cells. IL-10 is produced and released from T reg, Breg, and Th2 cells [37].

TGF-ß helps the differentiations and survives of T regs as well as maintaining self-tolerance [38]. Furthermore the results of serum protein were revealed there were increasing in highest values were reported in group IV at 10 days and 14 days and this agreed with the results obtained from [40] who reported the daily *thymus vulagris* diet on boiler chicken increased the serum total protein and globulin levels, Globulins (Gamma Globulins) are responsible for humoral immunity, also the results came with agreements with [41] some blood parameters, antioxidative metabolism of the serum and liver t issues of quails fed with 150, 300 and 450 mg/kg recorded increasing in level of Total serum proteinsand revealed that In result, thyme essential oil positive affected the performance of serum parameters in (450 mg/kg), also effect on the antioxidant metabolisms. The importance of the daily supplement of thymus were showed in results of histopathology examination, where noticed V. alginolyticus caused damages in intestine like an loss of mucous membrane in Figure (7) and damage of villa of small intestine as in Figure (8), due to the ability to *V. alginolyticus* to dhere to the intestine and d hydrolytic activities of extracellular substances and products of *V. alginolyticus* those the products were degraded the mucus. *V. alginolyticus* was showed cytotoxicity on the cells in the intestine, moreover, the extracellular products could degrade tissues (15).

While group IV, the treated animals with extract and challenged with bacteria was showed the ability of thymus extracts to increase requirement of inflammatory cells as discussed above also showed the protection to the microvilli, that responsible for the absorption of nutrient materials. Stimulation the appetite, and induce secretion of endogenous digestive [42].Against the *V.* alginolyticus products as showed in Figure (10).

Conclusions

This study was revealed depending on the results, the daily supplement of aqueous extract thymus *vulgaris* leaf (400 µg / kg) had

positive effects on the immune response, total serum protein, and showed positive effects in histological examinations through protection the small intestine from *V. alginolyticus* invasion.

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Acknowledgments

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