RESEARCH ARTICLE

Evaluation of the Immunomodulatory Effects of Thymus vulgaris Ethanolic Leaf Extract Combination with Partially Purified Lipopolysaccharide from *Proteus mirabilis* in Mice

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ABSTRACT

The current study was carried out to study a high injection dose of the ethanolic extract *thymus vulgaris* leaf (500 ug /Kg) against the immune response combination with partially purified extracted lipopolysaccharide (LPS) from *Proteus mirablis*. Study groups were included four groups; Group I :treated with normal saline. Group II : treated with LPS antigen, Group III: injected subcutaneously ((500 ug /Kg) from ethanolic extract *thymus vulgaris*, group IV : injected subcutaneously (500 ug/Kg) from ethanolic extract *thymus vulgaris* leaf and LPS antigen, the immunological assays were measured through the phagocytic activity as (non specific immunity) after day 8 by using the phagocytic activity index. After day I4 the lymphocyte proliferations was estimated by MTT index. For delayed-type hypersensitivity (DTH) reaction,the result was measured at 24, 48 and 72 hours after LPS antigens injection.While for Humoral immune response, after day 21 and day 28 the antibody production was estimated by indirect immunoflourescent and by Gel electrophoreses.The results were showed no significant difference in the NBT index between Groups but noticed Group III had a value lower than Group II, While the MTT results were revealed, Group IV had the highest value. In the other side of the study the DTH results showed Group IV had the highest value after 48 hr with significant differences ($p \leq 0.05$), in addition, the humoral immune response results were consisted gel electrophoresis and indirect immunoflourescent results showed after day 21 and day 28 Group IV had the highest value.

The result was showed the ethanolic extract thymus in a high concentration combination with LPS from *P. mirabilis* had effects on the immune response particularly Humoral immune response and Cellular immune response but still act as anti inflammatory role as revealed in many previous studies.

Keywords: Ethanolic extract of *Thymus vulgaris* leaf, Immunomodulatory Effects, Immune response, Lipopolysaccharide of *Proteus mirabilis.*International Journal of Pharmaceutical Quality Assurance (2019); DOI: 10.25258/ijpqa.10.4.11

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INTRODUCTION

Thyme (*Thymus vulgaris* L., Lamiaceae), has is a chemical species,[1] a flowering plant belonged to family Lamiaceae, 15 to 30 cm and about to 40 cm width.[1,2] The medically importance of the plant was attributed to many main components which are phenolic components such as Thymol (5-methyl-1-2-isopropyl phenol) and carvacrol (5-isopropyl-2-methyl phenol),,which play as antioxidant scavengers[3,4] reported the antibacterial ctivity, anticoccidial activity, and antifungal potential, as well and also rich in flavonoids and may improve the immune functions,[5] it has a role to enhance the digestibility, also to keep the balance of the gut microbial ecosystem and help stimulation of the secretion many of digestive enzymes and thus improving growth rate specially in boilers chicken,[4,6] in other hands *Proteus mirabilis* is one of a gram-negative bacterium belongs to Enterobacteriaceae family is well-known in swarming phenomenon, rod also motile, urease-positive,[7] causes UTI in most common, cell wall has LPS which is the main component responsibles for toxicity and, pathogenesity of gram negative bacteria 3 8 in many immunological studies that revealed the, LPS is play significant functions in immue system such as by enhancing the inflammation environments during lymphocyte activation and as an immunodulator,[9] the aim of the study is to investigate the role of thymus extract leaves on the immune response combination with LPS derived from Proteus mirabilis cell wall, by using a high concentration,

MATERIAL AND METHODS

All experiments were done in the laboratories of the in Dijlah University Collage, the research was done on male albino mice (Blab-c) with average weight was 22–25 grams.

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Isolation and identification *Proteus mirablis*

Two *proteus mirablis* isolates were obtained from urinary tract infection (UTI) patiences from Al- Numan hospital and identified by laboratories of Dijlah University Collage

Suscepitibilty test

The antibiotic disks were applied to determine which the isolate that had more resistance to antibiotics, the antibiotic that used in this study are Ampicllin (AMP), Cefotaxime (CTX), Erythromycin (E), Azithromycin (AZM), Imipenem(IMP), Doxycycline(DO), Ticarcillin(TIC), Ciprofloxacin(CIP), Gentamycin (CN), Amikacin (AK)

Extraction and partial purification of Lipopolysaccharide (LPS) from *Protues mirablis*

Protues mirablis LPS Extraction by Hot EDTA method[10] and purified by Dailysis membrane (100 – 1000 kDa).[11]

Preparion of Ethanolic Extract of *Thymus vulgaris* leaf

The ethanolic extract preparation was according to[12] by putting (50) g of Thyme powder leaf in (Soxhelt) and added (350) mL of ethanol (80%), extraction continued for (12) hours at (40)°C by using vacuum rotary evaporator, also at (35)°C, then: (500 µg/kg) of Ethanolic extract of *thymus vulgaris* leaf was prepared.

Determination Lipopolysaccharide (LPS) antigen doses

This experiment included Four groups. three different doses of LPS antigen and Control group was injected subcutaneously in mice group included five mice to determine the level IgG in serum by using Radial Immunodiffusion (RID).

*Group I (Control Group): Normal saline (0.5 mL) .
*Group II: 25ug/mL of LPS antigen.
*Group III: 55ug/mL of. LPS antigen .
*Group IV: 85 ug/mL of LPS antigen.

Groups of Study

This current study was included 4 groups, the mice in groups were treated by subcutaneous injection as following

Group I : Normal saline (0.3mL)**.**

Group II : LPS antigen.

Group III: Ethanolic extract of *Thymus vulgaris* leaf (500 µg/kg).

Group IV: LPS antigen + (500 µg/kg) of ethanolic *Thymus vulgaris* leaf .

Laboratory Assessments

Innate immue response assay

- Phagocytic activity by nitroblue tetrazolium (NBT) index

The procedure was done depending on a method presented by.[14]

Adaptive immune response assays

- MTT Lymphocyte proliferation Test assay

The procedure of MTT was on extraction the lymphocytes from the collected blood.[14]

- Delayed Hypersenstivity Test.

All mice in the groups was injected with 50 ul of LPS in the right foot and measured at time zero and at 24 and 48 hours.[15]

- Serum Agarose Gel Electrophoresis

The procedure s done by a commercially available kit .

- Indirect Fluorescent Antibody Test (IFAT)

The procedure was presented by world health organization (WHO)[16] was followed for determination anti LPS antibodies titers.

Statistical Analysis

The Statistical Analysis values by SPSS by probability value ≤ 0.05.

RESULTS

The bacterial isolates were obtained from Al- Numan hospital. and has been identified according to the morphological characterization, swarming phenomenon in young culture on blood agar according to[17] and the identification was achieved by Ap20 index.

The suspectibility test results were showed one isolate was resist to CIP, CN, Amikacin and Amplicilin while the other isolate were showed sensitivity to those antibiotics, the isolate that showed resistance to antibiotics was selected to extract the LPS from the cell wall as this isolate has more pathogenicity. The isolate was activated with nutrient broth because those media are enrichment[14] to increase the growth for extraction of LPS from the cell wall.

Result of LPS antigen doses were showed the concentration IgG in Group III (55 ug/mL) was significantly difference ($p \leq 0.05$) with others groups as in Table 1.

The results of evaluation of phagocytsis activity of this current study was reported was no significant differences ($p \leq 0.05$) between the groups but Group IV showed the highest value 3.52 ± 0.32.

In other hand the MTT result were revealed Group III, Group IV (0.290 ± 0.08, 0.331 ± 0.14 **)** respectively were showed significant differences ($P \leq 0.05$) with other groups, Group I, III as in as in Table 2.

More further, the results of DHT were showed Group IV had the highest value after 48 hours significant different ($P \leq 0.05$),

Table 1: IgG concentration rate (mean) of LPS antigen:

Groups	*Dosage*	*IgG Mg/dL*
Group I	0.5 mL	97 ± 1.8^{e}
Group II	25 ug/mL	222.2 ± 3.8^{c}
Group III	55 ug/mL	542.4 ± 6.18^{a}
Group IV	85 ug/mL	250.3 ± 6.3^{b}

Table 2: Result of NBT index and MTT assays in studied groups

Assay	*Control*	*LPS antigen*	*500 µg/kg Thymus vulagris extract*	*500 µg/kg Thymus vulagris extract + LPS*
NBT	1.72 ± 0.14^{a}	3.15 ± 0.39^{b}	$2 .52 \pm 0.32^{b}$	3.52 ± 0.32^{b}
MTT	0.259 ± 0.02^{a}	0.268 ± 0.02^{b}	0.290 ± 0.08^{c}	0.331 ± 0.14^{d}

*The different letters denoted that a significant difference between the groups < $p \leq 0.05$

in other side there was no significant difference between Group III and Group IV in time zero and after 24 hours, as in Table 3.

In addition, The humoral immune response was measured by gel electrophoresis and Indirect fluorescent antibody, the results of gel electrophoresis were showed the Group IV, Group III have significant differences ($p \leq 0.05$) in comparison with Group II but the highest value showed in Group IV as in Table 4.

In other side of this study the indirect fluorescent antibody results were revealed after 21 day as Groups I, showed no anti- LPS antibodies, While Group II, III, IV were showed positive reaction but Group IV was recorded a highest at the titer of antibodies which was 1:256, as presented in (Table 4). Furthermore the result after 28 days were revealed Group II showed the titer of Anti LPS was 1:32. But the highest titer of anti LPS antibodies was revealed in Group III at titer I: 128, and Group 1V at titer 1: 156 as in Table 5 and Figure 1 and 2.

DISCUSSION

This study was continued the previous studies about role *of thymus vulagris* on the immune response but the researchers were used different concentrations of extract. Furthermore

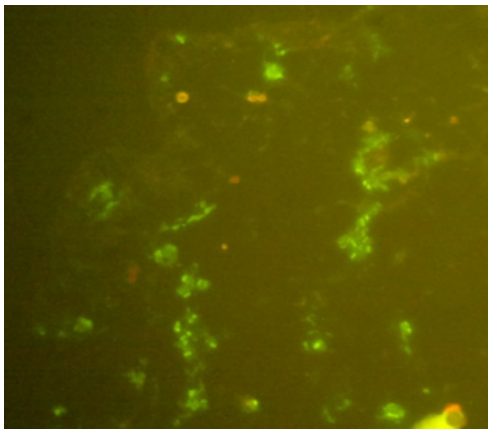

Figure 1: Anti LPS antibodies at titer **I:** 128

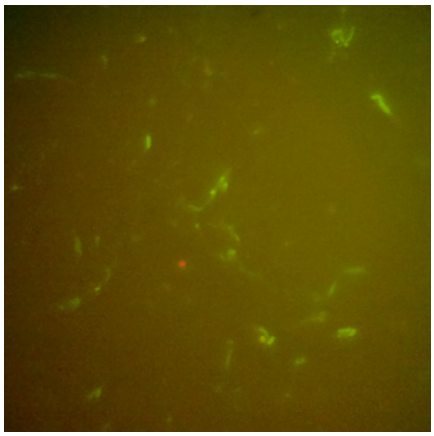

Figure 2: Anti LPS antibodies at titer **I:** 256

Table 3: Delayed type hypersensitivity (DTH) in studied groups

	DTH index mean ± S.E mm		
Group	*Time zero*	*After 24 hours*	*After 48 hours*
Group I	2.0 ± 0.00^{a}	2.07 ± 0.03^{a}	2.1 ± 0.05^{a}
Group II	2.2 ± 0.11^{a}	2.09 ± 0.14^{b}	3.33 ± 0.17^{b}
Group III	2.37 ± 0.27^{a}	$\pm 0.00^{b}3.0$	3.41 ± 0.20^{bc}
Group IV	2.57 ± 0.10^{a}	3.64 ± 0.06^{b}	3.81 ± 0.20^{c}

Table 4: The Results of Gamma globulin serum, *Alpha-1 level,* Alpha-2 level, Alpha-beta level

Groups	*Gamma globulin level*	*Alpha-1 level*	*Alpha-2 level*	*Alpha-beta level*
Group I	6.73 ± 0.13^{a}	$2.27 \pm .0.15^{a}$	9.60 ± 0.23^{a}	10.83 ± 0.10^{a}
Group II	$15.39 \pm 0{,}60^{b}$	2.3 ± 0.33^{b}	7.64 ± 0.65^{b}	11.13 ± 0.25^{b}
Group III	16.94 ± 0.38^{c}	5.46 ± 9.2^{c}	10.62 ± 0.47^{c}	15.8 ± 1.18^{c}
Group IV	17.83 ± 0.86^{d}	7.54 ± 0.38^{dc}	$18.5 \pm 0.72b^{d}$	18.5 ± 0.57^{b}

*The different letters denoted that a significant difference between the groups < $p \leq 0.05$

Table 4: Anti- LPS antibodies antibody titer after 21 days

	Anti- LPS antibodies Titer after 21 day									
Groups	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	*1024*
I	0	0	0	0	0	0	0	0	0	0
II	2	4	8	16	32	0	0	0	0	0
III	12	4	8	16	32	64	128	256	0	0
IV	2	4	8	16	32	64	128	0	0	0

Table 5: Anti- LPS antibodies antibody titer after 28 days

	Anti- LPS antibodies Titer after 28 day									
Groups	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	*1024*
I	0	0	0	0	0	0	0	0	0	0
II	0	0	8	0	0	0	0	0	0	0
III	12	4	8	16	32	64	128	256	0	0
IV	2	4	8	16	32	64	128	0	0	0

using the extract against induced immune response by *Proteus mirabilis* LPS antigen to investigate the main role of the plant on the immune response, considering thymus vulagis is an immunodulator, which mediates the effectors mechanism of the immune system through stimulation of immune to given antigens.[18] Therefore the current study was carried out the effect of thymus vulgaris in induced immune response in mice in a high concentration dose.

P. mirabilis causes most of urinary tract including cystitis and pyelonephritis. UTIs are more common in individuals aged 20 to 50 years and most common in women of this age group. Proteus spp caused for 5%. Complicated UTIs specially in old people,[19,20] in this study, the result of the suspitibilty The suspectibility test results were showed three isolates were resist to ciprofloxacin (CIP), gentamycin (CN), amikacin and amplicilin and this agreed with[21] overall all the pathogenic bacteria got ability to resist to antibiotics This horizontal gene transfer (HGT) can allow antibiotic resistance genes to be transferred between different species of bacteria.[22] Resistance can also occur through mutation.[23] and overuse, of antibiotics are overprescribed worldwide.[10] used those isolates that showed more resistance to next experiment to extract LPS from the cell wall because the LPS an important role in pathogensis of gram negative bacteria as endotoxin but many studies used LPS as an immunomodulator which stimulate the immunity cells, macrophage, to produce inflammatory mediates and also help to produce co stimulatory molecules.[9]

There was no morbidity ratio in all groups during experiment because of thyme oil contained components like thymol and carvacrol, have a potent antioxidant and antibacterial properties[24] also thymus extract help in increase serum protein albumin and globulin which are very important in metabolism of animal and growth.[25] In experiment of determining the dose of LPS Group III that treated with (55 ug/mL) was showed better immue response in RID and there was a significant difference comparson with the Group II, Group1V that Attributed to the immune system is showed showed tolerance to those groups.[26]

The result of Phagocytic activity were showed there was no significant differences between the treated group Group II, III, IV this result were agreed with Vetvicka, V. *et al.*[27] who used different thymus oil and showed all those oil has no effect on the phagoctic activity and there are reduction in intralukin (IL)-1b and intralukin-6 (IL-6) level and agreed with Oca˜na *et al*.[28] who reported that thymus extract reduce the production and reduce the gene expression that important to produce the proinflammatory mediators from the cells such as TNF-*α*, IL-1B, and IL-6 but in same time increase produces the antiflammtory such as IL-10,[29,30] Neutrophiles and macrophage are pahocytic cells and kill the bacteria by phagocytosis which s led by proinflammatory mediators and release inflammatory mediate to induce inflammatory response.[31] Many studies found the thymus have antiflammatory effect dued to Thymol and carvacrol also exhibit anti-inflammatory activitie, and induce high level of intinflammatory Cytokines IL 10, TGF-β which those effect directly on indirectly against macrophage and neutrophile by regulation the polymorphonuclear (PMN) of phagocytosis while the absence of changes in CR1 (C3b) or Fc receptors expression, leads to the Inhibition of phagocytosis .in addition the inhibition of superoxide production from PMA-stimulated PMN, by IL 10 suppressed the respiratory burst,[32] The other reasons is the extract of leaves have many effects in different levels such as effect on proteins such as C-reactive protein (CRP), also effect on molecules adhesion such as vascular cell adhesion molecule- 1, and finally effect on matrix metalloproteinase 9[33] and those were showed in values group III which had value lower than Group II while the group II which the animals injected with only LPS showed a high value than group III, this attributed that the LPS act on the TLR4 receptor system, leading to the production of pro-inflammatory cytokine as well as interferons, thus launching the inflammatory and immune response.

In addition The MTT result was showed difference effects between group IV and Group III and Group II and this agreed with Menati1, J.K. *et al*.,[34] who treated the boiler chicken with different concentration of eqouse thymus vulagris and report there was increasing in level of the T lumphocytes, Natural killer cell and B lymphocyte, in addition the results of this current studies were agreed those facts attributed to thyme (*Thymus vulgaris*) that are full of flavonoids, can extend the activity of vitamin C, act as antioxidants, and may enhance the immune responses As well as contain compounds such as Thymol and carafacrol and vitamins that helps in increase level of immune response and make the titer of Abs high and play as antioxidant.[35] in Other hand some compounds such as tannin and thymine are enhanced the activity and actions of innate immue response as macrophage and neutrophiles and also natural killer cells and associate to development T lymphocyte and balance between the assistance and inhibitory development, in addition to increase production of cytokines the important in humoral immune response, IL4 (26), and increase CD4+, CD8+,[36] The reason behind this is thymus vulgaris extract enhancing activation of T cell is by suppressing the transcription factors as AP-1 and NFAT-2 and preventing the production of IL-2 and IFN-γ. The inhibitory role of of Thymus vulgaris on the IFN-γ, IL-6 and IL-17 expression may be directly could be done via the modulation of signaling pathways and transcription factor that associated with these cytokines in Th1, macrophages, and Th17 cells[29,30] and these resulted confirmed with our DHT result that revealed no significant differences between group II, III, VI. As we discussed the carvacrol and thymol effected on IL- *thymus vulagris* 10 and TGF-β expression.[29,37] The TGF-B is produced by Treg cells. IL-10 is produced and released from T reg, Breg, and Th2 cells.[38] TGF-β helps the differentiations and survive of T regs as well as maintaining self-tolerance.[39] This is the other role of enhancing effects of Thymus vulgaris on TGF-B and IL-10 production may effects on T reg, B reg and The cells. (Thymus important), for humoral immune response result in this study was showed the serum protein was increased in

Group III, Group IV with significant difference comparison with Group II this result agreed with[40] who studied the effect of dairy *thymus vulagris* on boiler chicken and reported there was significantly increasing the serum total protein and globulin levels, Globulins are responsible for humoral immunity., the and agreed with many studies,[41] also thymol played a role in to increase the total IgA and IgM levels.[36] As this result was confirmed result of the current study as showed in Group III and GroupV1 after 21 days, and 28 days the (mention the titer) and this because the thymol enhanced the B cell and also improved/Th2 cell release IL4 and help B cell to produce antibodies.[26]

CONCLUSION

The results were demonstrated the ethanolic thymus extract had positive effects in a high concentration combination with extracted LPS from *P. mirabilis* on the immune response particularly Humoral immune response and cellular immune response but still act as anti inflammatory role as revealed in many previous studies, the researchers will contuine the study by using higher concentration dose by using extracted phenolic components which has the main role in benefit of *thymus vulgaris.*

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