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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Received: 12th October, 19; Revised: 17th November, 19, Accepted: 15th December, 19; Available Online: 25th December, 2019

INTRODUCTION

Thyme (Thymus vulgaris L., Lamiaceae), has is a chemical species, a flowering plant belonged to family Lamiaceae, 15 to 30 cm and about to 40 cm width. 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. The thyme oil acts as an antimicrobial and antifungal agent, as well as with phenolic compounds, it is found to be effective in improving the immune functions, 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, in other hands Proteus mirabilis is one of a gram-negative bacterium belongs to Enterobacteriaceae family is well-known for swarming phenomenon, rod also motile, urease-positive, causes UTI in most common, cell wall has LPS which is the main component responsible for toxicity and, pathogenesis of gram negative bacteria in many immunological studies that revealed the, LPS is play significant functions in immune system such as by enhancing the inflammation environments during lymphocyte activation and as an immunodulator, 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.

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 mirabilis. Study groups were included four groups; Group 1 :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 14 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 immunofluorescent 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 ≤ 0.05), in addition, the humoral immune response results were consisted gel electrophoresis and indirect immunofluorescent 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


Source of support: Nil
Conflict of interest: None

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Isolation and identification *Proteus mirabilis*

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

Susceptibility 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 Ampicillin (AMP), Cefotaxime (CTX), Erythromycin (E), Azithromycin (AZM), Imipenem(IMP), Doxycycline(DO), Ticarcelln(TIC), Ciprofloxacin(CIP), Gentamycin (CN), Amikacin (AK).

Extraction and partial purification of Lipopolysaccharide (LPS) from *Proteus mirabilis*

*Proteus mirabilis* LPS Extraction by Hot EDTA method\(^\text{10}\) and purified by Daisylame membrane (100 – 1000 kDa).\(^\text{11}\)

Preparation of Ethanolic Extract of *Thymus vulgaris* leaf

The ethanolic extract preparation was according to\(^\text{12}\) 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: 25 µg/mL of LPS antigen.
*Group III: 55 µg/mL of LPS antigen.
*Group IV: 85 µg/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:** Ethanolic extract of *Thymus vulgaris* leaf (500 µg/kg).
- **Group III:** Ethanolic extract of *Thymus vulgaris* leaf + (500 µg/kg) of Ethanolic extract of *thymus vulgaris* leaf.
- **Group IV:** Ethanolic extract of *thymus vulgaris* leaf + (500 µg/kg) of Ethanolic extract of *thymus vulgaris* leaf.

Laboratory Assessments

**Innate immune response assay**

- Phagocytic activity by nitroblue tetrazolium (NBT) index

The procedure was done depending on a method presented by.\(^\text{14}\)

Adaptive immune response assays

- MTT Lymphocyte proliferation Test assay

The procedure of MTT was on extraction the lymphocytes from the collected blood.\(^\text{14}\)
- Delayed Hypersensitivity 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.\(^\text{15}\)
- Serum Agarose Gel Electrophoresis

The procedure was done by a commercially available kit.
- Indirect Fluorescent Antibody Test (IFAT)

The procedure was presented by world health organization (WHO)\(^\text{16}\) 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\(^\text{17}\) and the identification was achieved by Ap20 index.

The susceptibility test results were showed one isolate was resist to CIP, CN, Amikacin and Ampicilin 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\(^\text{18}\) 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 µg/mL) was significantly difference (p ≤ 0.05) with others groups as in Table 1.

The results of evaluation of phagocytosis activity of this current study was reported was no significant differences (p ≤ 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 ≤ 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 ≤ 0.05),

<p>| Table 1: IgG concentration rate (mean) of LPS antigen: |</p>
<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage</th>
<th>IgG Mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.5 mL</td>
<td>97 ± 1.8a</td>
</tr>
<tr>
<td>Group II</td>
<td>25 µg/mL</td>
<td>222.2 ± 3.8c</td>
</tr>
<tr>
<td>Group III</td>
<td>55 µg/mL</td>
<td>542.4 ± 6.18a</td>
</tr>
<tr>
<td>Group IV</td>
<td>85 µg/mL</td>
<td>250.3 ± 6.3b</td>
</tr>
</tbody>
</table>

*The different letters denoted that a significant difference between the groups < p ≤ 0.05*

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

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control</th>
<th>LPS antigen</th>
<th>500 µg/kg Thymus vulagris extract</th>
<th>500 µg/kg Thymus vulagris extract + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT</td>
<td>1.72 ± 0.14a</td>
<td>3.15 ± 0.39a</td>
<td>2.52 ± 0.32b</td>
<td>3.52 ± 0.32b</td>
</tr>
<tr>
<td>MTT</td>
<td>0.259 ± 0.02a</td>
<td>0.268 ± 0.02a</td>
<td>0.290 ± 0.08b</td>
<td>0.331 ± 0.14d</td>
</tr>
</tbody>
</table>

*The different letters denoted that a significant difference between the groups < p ≤ 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 ≤ 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 IV 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 vulgaris* on the immune response but the researchers were used different concentrations of extract. Furthermore

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

<table>
<thead>
<tr>
<th>Group</th>
<th>DTH index mean ± S.E mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time zero</td>
</tr>
<tr>
<td>Group I</td>
<td>2.0 ± 0.00a</td>
</tr>
<tr>
<td>Group II</td>
<td>2.2 ± 0.11a</td>
</tr>
<tr>
<td>Group III</td>
<td>2.37 ± 0.27 a</td>
</tr>
<tr>
<td>Group IV</td>
<td>2.57 ± 0.10 a</td>
</tr>
</tbody>
</table>

*The different letters denoted that a significant difference between the groups < p ≤ 0.05

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Gamma globulin level</th>
<th>Alpha-1 level</th>
<th>Alpha-2 level</th>
<th>Alpha-beta level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>6.73 ± 0.13a</td>
<td>2.27 ± .015 a</td>
<td>9.60 ± 0.23 a</td>
<td>10.83 ± 0.10 a</td>
</tr>
<tr>
<td>Group II</td>
<td>15.39 ± 0.60b</td>
<td>2.3 ± 0.33 b</td>
<td>7.64 ± 0.65 b</td>
<td>11.13 ± 0.25 b</td>
</tr>
<tr>
<td>Group III</td>
<td>16.94 ± 0.38c</td>
<td>5.46 ± 9.2 c</td>
<td>10.62 ± 0.47 c</td>
<td>15.8 ± 1.18 c</td>
</tr>
<tr>
<td>Group IV</td>
<td>17.83 ± 0.86d</td>
<td>7.54 ± 0.38dc</td>
<td>18.5 ± 0.72d</td>
<td>18.5 ± 0.57 b</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Anti- LPS antibodies Titer after 21 day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Anti- LPS antibodies Titer after 28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
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</tbody>
</table>
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 vulgaris is an immunomodulator, which mediates the effectors mechanism of the immune system through stimulation of immune to given antigens.\(^\text{18}\) Therefore the current study was carried out to evaluate 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,\(^\text{19,20}\) in this study, the result of the susceptibility The susceptibility test results were showed three isolates were resist to ciprofloxacin (CIP), gentamycin (CN), amikacin and ampicillin and this agreed with\(^\text{21}\) 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.\(^\text{22}\) Resistance can also occur through mutation.\(^\text{23}\) and overuse, of antibiotics are overprescribed worldwide.\(^\text{24}\) used those isolates that showed more resistance to next experiment to extract LPS from the cell wall because the LPS an important role in pathogenesis 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.\(^\text{9}\)

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 \(^\text{25}\) also thymus extract help in increase serum protein albumin and globulin which are very important in metabolism of animal and growth.\(^\text{26}\) In experiment of determining the dose of LPS Group III that treated with (55 \(\mu\)g/mL) was showed better immune response in RID and there was a significant difference comparison with the Group II, GroupIV that Attributed to the immune system is showed showed tolerance to those groups.\(^\text{26}\)

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.\(^\text{27}\) who used different thymus oil and showed all those oil has no effect on the phagocytic activity and there are reduction in intralukin (IL)-1b and intralukin-6 (IL-6) level and agreed with Oca˜na et al.\(^\text{28}\) 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-\(\alpha\), IL-1B, and IL-6 but in same time increase produces the antiflammatory such as IL-10.\(^\text{29,30}\) Neutrophiles and macrophage are phahocytic cells and kill the bacteria by phagocytosis which s led by proinflammatory mediators and release inflammation mediates to induce inflammatory response.\(^\text{31}\) Many studies found the thymus have antiflammatory effect due to Thymol and carvacrol also exhibit anti-inflammatory activity, and induce high level of intinflammatory Cytokines IL 10, TGF-\(\beta\) which those effect directly or 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.\(^\text{32}\) 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 \(^\text{33}\) 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.\(^\text{34}\) 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.\(^\text{35}\) in Other hand some compounds such as tannin and thymine are enhanced the activity and actions of innate immune 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+.\(^\text{36}\) The reason behind this is thymus vulgaris extract enhancing activation of T cell is by suppressing the inflammatory factors as AP-1 and NFAT-2 and preventing the production of IL-2 and IFN-\(\gamma\). The inhibitory role of Thymus vulgaris on the IFN-\(\gamma\), 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\(^\text{29,30}\) and those were showed in this DHT result that revealed no significant differences between group II, III, VI. As we discussed the carvacrol and thymol effected on IL-\(\text{Thymus vulgaris}\) 10 and TGF-\(\beta\) expression.\(^\text{29,37}\) The TGF-B is produced by Treg cells. IL-10 is produced and released from T reg, Breg, and Th2 cells.\(^\text{38}\) TGF-\(\beta\) helps the differentiations and survive of T reg as well as maintaining self-tolerance.\(^\text{39}\) 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 vulgaris 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 Group V1 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 continue the study by using higher concentration dose by using extracted phenolic components which has the main role in benefit of thymus vulgaris.

ACKNOWLEDGMENTS
The authors are grateful to Dijlah university college, Iraq support us during the study and for providing the facilities.

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