Effects of Lipopolysaccharides of \textit{Pseudomonas Aeruginosa} and Aqueous Extract of \textit{Ginkgo Biloba}, Ginkgoaceae, on Cellular Immune Response in Mice Balb/c

\textit{Pseudomonas Aeruginosa} ve Ginkgo Biloba, Ginkgoaceae Sulu Özü Lipopolisakkaritlerin Balb/c Farelerinde Hücresel Bağışıklık Yanıtı Üzerindeki Etkileri

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\textbf{ABSTRACT}

In this study, lipopolysaccharides (LPS) of \textit{Pseudomonas aeruginosa} IANS isolate and aqueous extract of \textit{Ginkgo biloba} leaves were used to investigate the cellular immunity in mice Balb/c \textit{(in vivo)}. Some parameters were used to achieve this study, are percentages of polymorphonuclear neutrophils (PMNs), phagocytosis coefficient of PMNs at different time periods 30, 60, 90 and 120 minutes, Formazan granules formation in them, and migration inhibition factor (MIF). The immunization of mice with the LPS antigen affected delay-type hypersensitivity and increased activity of PMNs in \textit{Candida} sp. and reduced NBT, while inhibited migration of PMNs. The immunization did not affect the macrophages of PMNs. The injected treatment with LPS and \textit{Ginkgo biloba} extract showed the best results significantly \((p<0.05)\) compared to LPS and control treatments, whereas the \textit{Ginkgo biloba} extract alone showed no significant differences \((p<0.05)\).

\textbf{Key Words:} LPS, immunology, herbal extract, \textit{in vivo}, maidenhair tree.

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\textbf{ÖZET}

Bu çalışmada, \textit{Pseudomonas aeruginosa} IANS izolatının lipopolisakkaritleri \textbf{(LPS)} ve \textit{Ginkgo biloba} yapraklarının sulu özü, Balb/c farelerde \textit{(in vivo)} hücresel bağışıklığı araştırılmıştır. Bu çalışmanın gerçekleştirilmesinde bazı parametreler kullanıldı; polymorfonükleer neutrofiller (PMNs) yüzdesi, PMNs'ın fagositoz katsayısı, içlerinde Formazan granül oluşumu ve göç önleme faktörü (MIF). Farelerin LPS antijeni ile immünizasyonu, geçici tip aşın duyarlılığı etkilemiş, \textit{Candida} sp. deki PMN aktivitesini artırmış, NBT'yi azaltmış ve PMN'lerin göçünü inhibe etmiştir. Bağışıklama, PMN'lerin makrofajlarını etkilemedi. LPS ve \textit{Ginkgo biloba} ekstresi ile en iyi edilen tedavi, LPS ve kontrol tedavileri ile karşılaştırıldığında en iyi sonuçları \((p<0.05)\) gösterirken, \textit{Ginkgo biloba} ekstresi tek başına anlamlı farklılıklar göstermedi \((p>0.05)\).

\textbf{Anahtar Sözcükler:} LPS, immünoloji, bitkisel öz, \textit{in vivo}, baldrıkara ağacı.

\textbf{Geliş Tarihi:} 20.01.2018 \hspace{1cm} \textbf{Kabul Tarihi:} 03.06.2018
INTRODUCTION

Many medical and experimental studies declared the importance of cellular immunity induction (1-2). Tang et al. (2017) referred that murine acid-rich LPS induces monocytes to produce a high level of INF and IL-1 which increase the adherence of basophils with endothelium vessels then inhibition their migration to the infection place. The results referred that the purified LPS inhibits migration of phagocytes then inhibition of specific immunity responses (4). Lipopolysaccharides induce allergic interactions which lead to filtered acidophil and Th2 cells into respiratory canals. Th2 secretes IL-4 which causes B lymphocytes to produce IgE and IL-5 and to accumulate acidophil (2). Helper T-cells has differentiation CD4+ antigen on their outer surfaces that differentiated LPSs and lead to induce helper T-cells for proliferation and to release Lymphokines. Lipopolysaccharides induce macrophages and monocytes to secrete TNF-a, IL-6, and IL-1 through their linking with Lipopolysaccharide Binding Protein to form Lipopolysaccharide Binding Protein Complex which links with the receptors CD14 or TLR-4 (5).

Ginkgo tree is one of the most popular drugs and herbal supplements in Europe and the rest of the world (6). *Ginkgo biloba* is the sole surviving member of the Ginkgoaceae family. Its common name is maidenhair tree (7). Some specimens are over 30–40 meters high and several hundred years of age, and nowadays it is cultivated in many countries (8). The name “ginkgo” derived from the Chinese word for silver apricot and this name is called to the tree because of the size and coloring of the fruit (9). Leaves of *Ginkgo biloba* have been extensively studied for their potential therapeutic properties. Ginkgo leaves contain two primary active ingredients, flavonoids and terpenoids (10).

Studies on mammalian cells indicate that *Ginkgo biloba* extract can scavenge nitric oxide and may prevent its production, consequently protecting mammalian cells against nitric oxide reactivity (11). Traditionally, *Ginkgo biloba* leaves had been used as a drug for bronchial asthma and as wound-plasters (8). Some studies suggested that *Ginkgo biloba* extract is conserving mitochondrial metabolism and adenosine triphosphate (ATP) production in tissues, thus partially inhibiting morphologic distortion and signs of oxidative damages due to mitochondrial aging (12). Therefore, this work investigates the induction of the response of cellular immunity in vivo by *Ginkgo biloba* extract and Lipopolysaccharides of *Pseudomonas aeruginosa* IANS (local isolate).

MATERIALS and METHODS

Preparing migration medium

This medium was prepared by dissolving 1.5 g agarose in 100 ml D.W and then sterilized using the boiling. Afterwards, the medium is cooled to 45 ºC and added Hank’s balanced Salt (pH 7.2) (prepared from Flow Laboratories by v:v) and inactive sera to obtain the final concentration 10% in the medium. All ingredients were wholly mixed and stored in 4 ºC until the use.

Phosphate buffer saline (pH 7.2)

This buffer is prepared according to method of Hudson and Hay (1980). Only 0.85 g KH$_2$PO$_4$, 0.795 g NaHPO$_4$ and 9 g NaCl are dissolved in 1000 ml D.W and sterilized using Autoclave for 15 min. This buffer is prepared according to method of Hudson and Hay (1980). Only 0.85 g KH$_2$PO$_4$, 0.795 g NaHPO$_4$ and 9 g NaCl are dissolved in 1000 ml D.W and sterilized using Autoclave for 15 min. This buffer was used as a control in this test.

Phosphate buffer solution (pH 7.2)

This buffer was prepared by dissolving 1.5 g agarose in 1000 ml D.W and then sterilized using Autoclave for 15 min. This buffer was used as a control in this test.

Table 1 Effect of immunization of mice using LPS antigen of *Pseudomonas aeruginosa* and *Ginkgo biloba* extract on PMNs

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percentage of PMNs</th>
<th>Percentage of PMNs formed Formazan</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS of <em>P. aeruginosa</em></td>
<td>93.0±0.71a</td>
<td>35.4±1.00b</td>
</tr>
<tr>
<td><em>Ginkgo biloba</em> extract</td>
<td>93.2±0.42a</td>
<td>32.5±1.00c</td>
</tr>
<tr>
<td>LPS &amp; <em>Ginkgo biloba</em></td>
<td>93.2±0.42a</td>
<td>32.5±1.00c</td>
</tr>
</tbody>
</table>

The different letters in the same column refers to the significant differences (p<0.05).

RESULTS and DISCUSSION

Effect of the immunization by using lipopolysaccharides (LPS) of *Pseudomonas aeruginosa* and extract of Ginkgo (*Ginkgo biloba*) on the survival of basophile is reported in table 1. LPS of *P. aeruginosa* and *Ginkgo biloba* extract did not exhibit any significant differences (p<0.05) in mice. The higher percentage is 93.6±0.84% by LPS antigen and *Ginkgo biloba* extract compared with the control (phosphate buffer) 93.2±0.42%. *Ginkgo biloba* extract and LPS isolated from bacteria individually show lower percentages reach to 93.2±0.42% and 93.6±0.84% respectively. The rate of survival of basophile is height percentage that never provides the effect of antigens toward it. This result agrees with results of Hoffbrand et al. (2004).

Effect of immunization by LPS and *Ginkgo biloba* extract on reducing Nitro blue tetrazolium dye using Nitro blue tetrazolium reduction test (NBT blood test). However, table 1 shows that LPS of *P. aeruginosa* and *Ginkgo biloba* extract exhibit higher significant differences (p<0.05) in the percentage of PMNs formed Formazan at percent 37.5±1.12% in comparison with the control (32.5±1.00%). Followed 35.4±1.00% by LPS treatment alone. The lower percentage is 32.5±1.00% by Ginkgo extract individually. Also, in an apparent form, figure 1 shows PMNs formed Formazan under compound microscope X100. That is agreeing with Clark (1999) and Al-Kubaissi (2002). NBT is used to test phagocytosis of microbes by PMNs through their ability to produce superoxide anion O$_2$ which reduces the soluble yellow stain (NBT) to precipitate dark blue granules (formazan) in the cytoplasm of PMNs (19-21). These granules appear under a light microscope as in figure 1. This test showed the enzymatic activity of PMNs in their vacuoles after induction of NADPH oxidase to oxidize NADPH to NADPH+ (17,22).
Effect of LPS and Ginkgo extract on phagocytosis of the killed *Candida albicans*, as exhibited in Table 2. Generally, LPS and Ginkgo showed best phagocytosis coefficient with significant differences (p<0.05) reach to 73.9, followed 69.6 by LPS in comparison with the control (64.7). Ginkgo extract alone gave lower phagocytosis coefficient 64.7. Figure 2 presented killing *C. albicans* was devoured by MNPs. The differed time periods from 30 to 120 minutes for each treatment were investigated on phagocytosis coefficient. After 30 and 60 minutes, LPS and the plant extract exhibited together higher phagocytosis coefficient 75.3±0.36 and 74.5±0.31 significantly (p<0.05) and then decreased with increasing the time after 90 and 120 min to 73.7±0.33 and 72.1±0.37 respectively. The bacterial LPS exhibited higher phagocytosis coefficient after 30 min (70.7±0.34) then declined to 67.7±0.43 after 120 min. Ginkgo extract showed coefficients 66.1±0.62, 66.0±0.66 and 64.1±0.57 after 30, 60 and 90 min respectively, then dropped significantly (p<0.05) to 62.6±0.63 after 120 min. The control one showed 66.1±0.62 and 66.0±0.66 after 30 and 60 min respectively. Afterwards, phagocytosis coefficient declined to 64.1±0.57 and 62.6±0.63 respectively.

Macrophages are induced by microbes and their byproducts, immune complexes, inflammation cases, and wounds to produce many lysozymes which are leading to raise the organism temperature or to activate lymphocytes for releasing cytokines or producing nitric oxide as an antibiotic according to types of macrophages and microbes (21). Furthermore, Cortes et al. (2002) referred to the reason of increasing phagocytosis coefficient in the treated cells of mice with LPS of *Klebsiella pneumoniae* returns to the ability of these antigens to prompt completion system compounds especially C5a and C3b which contribute in Opsonization and attracting basophils to infection site respectively.

Table 2 Study influence of injection using LPS of *P. aeruginosa* and Ginkgo extract on phagocytosis of *Candida albicans* killed by the heat

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Phagocytosis coefficient of PMNs at different time periods (minutes)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS of <em>P. aeruginosa</em></td>
<td>70.7±0.34a 70.0±0.13a 70.0±0.38a 67.7±0.43b 69.6B</td>
<td></td>
</tr>
<tr>
<td>Ginkgo biloba extract</td>
<td>66.1±0.62a 66.0±0.66a 64.1±0.57b 62.6±0.63c 64.7C</td>
<td></td>
</tr>
<tr>
<td>LPS &amp; Ginkgo biloba</td>
<td>75.3±0.36a 74.5±0.31a 73.7±0.33a 72.1±0.37b 73.9A</td>
<td></td>
</tr>
<tr>
<td>Phosphate buffer (control)</td>
<td>66.1±0.62a 66.0±0.66a 64.1±0.57b 62.6±0.63c 64.7C</td>
<td></td>
</tr>
</tbody>
</table>

The different small letters in the same row refers to significant differences (p<0.05) in the period for each treatment. The different capital letters in the last column indicate significant differences (p<0.05) among averages of treatments.

LPS and *Ginkgo biloba* were used to investigate zone of migration (Figure 3) and migration inhibition factor (MIF) of PMNs, table 3. LPS and Ginkgo extract jointly showed a lower zone of PMNs migration reached to 7.15±0.12 mm significantly (p<0.05), while LPS antigen individually exhibited migration zone of 11.4±0.10 mm compared with the control (16.4±0.04 mm). Also, LPS and *Ginkgo biloba* extract showed higher migration inhibition factor (MIF) 0.43, followed 0.69 by LPS individually compared with the control which reached to 1.00. Using *Ginkgo biloba* separately did not record any significant differences (p>0.05).

Kulseng et al. (1996) referred to that injecting the purified alginate from *Pseudomonas aeruginosa* in mice is inducing macrophage to produce high levels of IL-1 and TNF which leads to adherence of PMNs on endothelium layer of blood vessels then inhibition of migration to the infection site. This study agrees with Al-Kubaissi (2002) who mentioned that *Entamoeba histolytica* inhibits migration of PMNs.
Table 3 Influence of injection of mice using LPS and Ginkgo extract on the migration of PMNs

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Zone of migration (mm)</th>
<th>Migration inhibition factor (MIF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS of <em>P. aeruginosa</em></td>
<td>11.4 ±0.10 c</td>
<td>0.69</td>
</tr>
<tr>
<td>Ginkgo biloba extract</td>
<td>16.4±0.04 a</td>
<td>1.00</td>
</tr>
<tr>
<td>LPS &amp; Ginkgo biloba</td>
<td>7.15±0.12 d</td>
<td>0.43</td>
</tr>
<tr>
<td>Phosphate buffer (control)</td>
<td>16.4±0.04 a</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Treatment of LPS of *P. aeruginosa* and Ginkgo extract showed the delayed type hypersensitivity (DTH) by an increase of foot thickness of mice 2.51±0.101 mm, 2.44±0.113 mm and 1.55±0.084 mm respectively in comparison with the mice before the treating (1.19±0.034 mm) significantly (*p*<0.05), as shown in figure 4. Furthermore, the delayed type hypersensitivity of Ginkgo biloba extract individually reached to 1.18±0.027 mm before the treating, and that declined to 0.34±0.028 mm after 72 hr in which is similar to the control (0.34), table 4.

In table 4, Ginkgo biloba extract and phosphate buffer did not exhibit any irritation area in the treated mice. LPS of *P. aeruginosa* and Ginkgo biloba extract jointly showed significant (*p*<0.05) area of irritation reached to 0.55±0.016 mm², 0.44±0.016 mm², and 0.36±0.016 mm² after 24 hr, 48 hr and 72 hr respectively. The reactions of the delayed type hypersensitivity are considering an indicative of the cellular immunity without the effect of antibodies (22,25,26). Results of this study show changing in the delayed-type hypersensitivity because of influence antigen type on this reaction as mentioned by Abbas et al., (2000) and Benschop et al., (1999). The responsible cells of the immune response are CD4 T-cells (TDTH) by producing Cytokines which effect on the activity of macrophage (27,28). The increase in thickness of mice’s foot may belong to the ability of the injected antigens to enhance lymphokines which lead to migration of WMCs selectively.

Table 4 The delayed type hypersensitivity test after various periods (mm) and area of irritation after different periods (mm²)

<table>
<thead>
<tr>
<th>The injected antigens</th>
<th>Foot thickness after the treating (mm)</th>
<th>Area of irritation after the treating (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before the treating</td>
<td>After 24 hr</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>1.1±0.024a</td>
<td>1.28±0.023b</td>
</tr>
<tr>
<td>G. b</td>
<td>1.18±0.027a</td>
<td>1.23±0.028b</td>
</tr>
<tr>
<td>LPS and G. b</td>
<td>1.19±0.034a</td>
<td>2.51±0.101a</td>
</tr>
</tbody>
</table>

Conflict of interest
No conflict of interest was declared by the authors.

REFERENCES


