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The Effect of Ethanol Extract of Dates (*Phoenix dactylifera)* on Blood Level of IFN-γ , IL-12 , and Bacterial Colonies of Mice Liver Infected with *Salmonella typhimurium*

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Abstract : Typhoid fever, is a serious and fatal disease in developing countries. Date fruit (*Phoenix dactilifera*) and its constituents have an important role in the disease prevention through anti-oxidant, anti-inflammatory, and anti-bacterial activities. this study aims to investigate the potential effect of *Phoenix dactylifera* fruits' extract in inhibiting the inflammation cytokines IFN-γ, IL-12 and decreasing the bacterial colonies in the liver of mice infected with *Salmonella typhimurium.* This study used 20 mice that were divided into 5 groups, including negative control (without infection), positive control (infected with *S. typhimurium*), P1 (100 mg/kg BW), P2 (200mg/kg BW), and P3 (400 mg/kg BW). ELISA was used to measure IL-12 and IFN-γ levels, while culture was used to measure the bacterial colonies in the liver. The results indicate that IFN-γ level was significantly decreasing at dose 400 mg/kg BW compared with C1 ($p<0.05$). In contrary, it increased at dose 100mg/kg BW. In addition, IL-12 level was significantly decreasing at dose 400 mg/kg BW compared with C1 ($p<0.05$), but decreased at dose 100mg/kg BW. The bacterial colonies in C1 were significantly different compared to other groups (C0, P1,P2,P3) ($p>0.05$). There were no bacteria found in all treatment groups. In conclusion, the ethanol extract of *Phoenix dactilifera* could improve immune response by decreasing the IL-12 level and decreasing IFN-γ level, as well as inhibiting the systemic disease by killing the bacterial colonies in the liver.
Keywords : Typhoid fever, *Salmonella typhimutrium*, IL-12, IFN-γ, bacterial colony.

Introduction

Salmonella typhi is the causative agent of typhoid fever, a serious, often fatal, disease very common in developing countries[1] and it is virulent in most animals, including mice. However, in mice, because infection with *S. Typhimurium* can increase enteric fever, with symptoms similar to those observed in humans after infection with *Salmonella typhi*, *Salmonella typhimurium* infection in mice is therefore widely accepted as an experimental model for typhoid fever in humans[2].

Salmonella typhimurium is a strain of bacteria that can live in humans. *Salmonella thypimurium* causes a bacterial infection of the intestinal tract and occasionally IL 12 in liver[3]. After oral ingestion, then the bacteria enter the Peyer's patches, which are lymphoid structures lining the intestine from microfold cells (M cell). After penetrating Peyer's patches microfold cells, bacteria access the underlying structure of the lymphoid tissue which is an area rich in phagocytic cells and serves as the initial site of intracellular infection[4]. From the Peyer's patches, *S. typhimurium* moves into the mesenteric lymph nodes and then spreads via the afferent lymph to the circulatory system and eventually gains access to the blood and systemic tissues via transit through efferent

lymphatic vessels[5]. the bacteria crossing the intestinal mucosa infect macrophages and spreads via the blood to the liver where it multiplies intracellular, but manages to survive inside the target cells varies according to the type of cell and depends on the temporal expression of particular genes by Salmonella. Target cells include gut epithelial cells, macrophages, neutrophils, monocytes, dendritic cells, granulocytes, B cells, and T cells[6]. Followed by a phase of several days, during which intracellular multiplication of bacteria occurs and bacterial titers in spleen and liver increase[2].

Studies have shown that Peyer's patches, mesenteric lymph nodes, and spleen cells from mice infected orally with *Salmonella typhimurium* spontaneously express an elevated level of IFN-γ mRNA and produce IFN-γ through in vitro stimulation Recent studies have described the role of interleukin (IL)-12 family of cytokines in the pathogenesis and it potential treatment for immune-mediated inflammatory diseases (IMIDs) [7]. Interleukin 12 is an important immunoregulatory cytokine produced mainly by antigen-presenting cells. The expression of IL-12 during infection could trigger an innate response and specify the type of adaptive immune responses. IL-12 induces interferon-γ (IFN-γ) production[8]. The antimicrobial agent has long been used to treat typhoid fever, but nowadays, recent studies have revealed that the bacteria are resistant to many antimicrobial agents. Therefore, it is necessary to find an antimicrobial agent from the plants as alternative medical sources.

Many studies have revealed that date fruit (*Phoenix dactilifera*) comprised of antioxidant, antimutagenic, anti-inflammatory, gastroprotective, hepatoprotective, nephroprotective, anticancer and immunostimulant activities[9]. Date and its constituents have an important role in disease prevention through anti-oxidant, anti-inflammatory, and anti-bacterial activities[10]. The therapeutic effects of *Phoenix dactylifera* are attributed to its polyphenolic content[11]. Due to the anti-bacterial activity of date extract, this study was conducted to investigate the potential effect of *Phoenix dactylifera* extract in decreasing the inflammatory process of cytokines IFN-γ and IL 12 and decreasing the bacterial colonies in the liver of mice infected with *Salmonella typhimurium.*

Material and Methods

Twenty mice were divided into 5 groups (5 mice for each group): negative control (C-) without *Salmonella typhimurium* infection and *Phoenix dactilifera* treatment; positive control (C+) was infected with *Salmonella typhimurium* 10^7cfu/300 µL [12]; P1 was infected with *Salmonella typhimurium* 10^7cfu/300 µL and administered with 100 mg/kg BW of *Phoenix dactilifera* extract; P2 was infected with *Salmonella typhimurium* 10^7cfu/100 µL and administered with 200 mg/kg BW of *Phoenix dactilifera* extract; and P3 was infected with *Salmonella typhimurium* 10^7 cfu/100 µL and administered with 400 mg/kg BW of *Phoenix dactilifera* extract.

Infection Preparation

Data collection was following [13]. Samples were adapted for 1 week in the laboratory by following standard feed. The C- group was not administered with date extract and was not infected with *Salmonella typhimurium.* The C+ group as a positive control was inoculated orally with 300 µL of *Salmonella typhimurium* for each mice with an interval of 2 days. The group of P1, P2, P3 were inoculated orally with 300 µL of *Salmonella typhimurium* for each mice with a 2-day interval. The group of P1, P2, P3 were administered orally with *Pheonix dactilifera* extract with various doses of 100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW for 7 days by using gavage needle. On day 15, all group of mice was sacrificed and the livers were collected.

Extraction

The edible parts of date fruit (*Phoenix dactilifera*) were manually separated, washed, cut into pieces and dried in an oven at 40°-60°C, measured at 100 g, and extracted using ethanol 70 % as much as 900 ml. The solution was shaken for 30 minutes and stored overnight at Pharmacology Laboratory, University of Brawijaya. The upper layer which is the mixture of ethanol and active compound was collected. Thus step was repeated three times. The solution was put in evaporation glass (1L) and placed in the Rotavapor. The water bath was fully filled with water at 90 °C. The solution was centrifuged until the active compound separated from the solution. The residue was collected 900 ml after 2 hours centrifugation. The extract was collected, put into a plastic bottle and stored at freezer until it used in the experiment[14].

IFN-γ and IL12 Examination by ELISA

The serum samples were collected and 50 µL of samples were added to each well in duplicate. The plate was covered and incubated at room temperature (RT) (20-25°C) for 2 hours. Without washing, 50 µL of Biotinylated Antibody Reagent was added to each well and incubated at RT for 1 hour then washed three times. Each well was added 100 µL of Streptavidin-HRP Solution, covered, incubated at RT for 30 minutes, and washed three times. Each well was added 100 µL TMB Substrate then put in the dark at RT for 30 minutes. 100 µL of Stop Solution was added to each well to stop the reaction. The absorbance was measured on a plate reader at 450 nm or 450 minus 550 nm. The results were calculated using graph paper or curve-fitting statistical software.

Calculate Bacteria Colonies In liver

The liver was collected using aseptic technique and then scaled. The liver was obtained and crushed with a mortar and added 4.5 ml of sterile physiological saline. The test tube 6 was filled with NaCl 4.5 ml. Then 0.5 ml of mix solution was inserted into the first tube and homogenized using vortex. The first tube was inserted into the second tube and repeated until the tube dilution VI has reached 1:10 for each dilution. The solution in the last tube was discarded. A sample of each tube was inoculated 0.1 ml on MacConkey agar (BSA) then incubated at 37°C for 24 hours. A number of colonies on each plate dilution containing 30-300 CFU were calculated.

Statistical Analysis

The data of Gamma interferon and Interleukin-12 level were measured using ELISA method, while the number of bacteria on the liver was calculated. In vivo experiments were applied to four mice per group. Statistical analysis used one-way ANOVA and correlation test at the level of acceptance 95% ($\alpha = 0.05$).

Results

IFN- γ Level in the Serum of mice Infected by *Salmonella typhimurium*

The different average level of IFN-γ as the effect of different dose of *Phoenix dactylifera* treatment is presented in Figure 1.

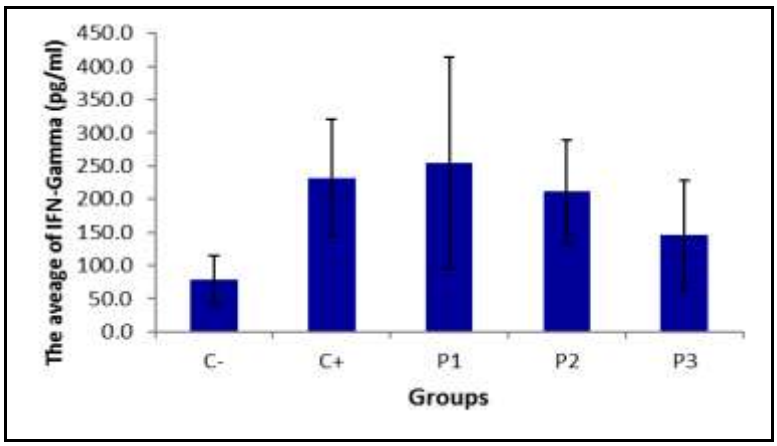

Figure (1): Level of IFN-γ

According to the figure, the level of IFN-γ in each treatment dose varies considerably. The level of IFN-γ in the negative control group is 77.857. On the other hand, the positive control group which was injected with the bacteria shows a higher value, approximately 232 IFN-γ. Surprisingly, the average of IFN-γ in the group administered with 100 mg/kg BW of *Phoenix dactylifera* was higher than the positive control group. The administered group shows an average of 254 IFN-γ, so this group is considered to be ineffective on decreasing the level of IFN-γ. However, the result of IFN-γ level in the group treated with 200 mg/kg BW of *Phoenix dactylifera* was lower than the group treated with 100 mg/kg BW of *Phoenix dactylifera*. Moreover, compared

to the other doses, the treatment of 400 mg/kg BW of *Phoenix dactylifera* could significantly decrease the IFN-γ level to the lowest level, so it considered to be the effective dose on decreasing IFN-γ level in the mice infected with *S. typhimurium.*

IL-12 Level in the Serum of Mice Infected with *Salmonella typhimurium*

The different average level of IL-12 as the effect of different dose of *Phoenix dactylifera* treatment can be seen in Figure 2 as follows.

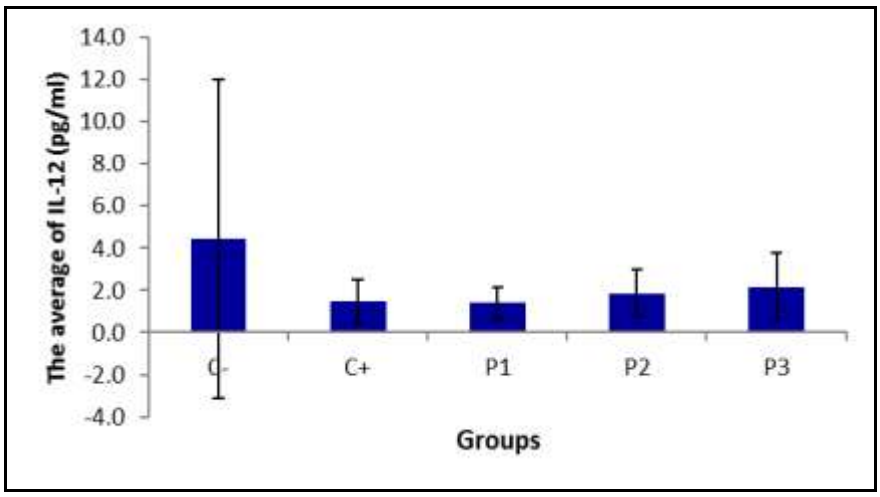

Figure (2): Level of IL-12

According to the figure 2, the level of IL-12 in each treatment decreases along with the decreasing dose of *Phoenix dactylifera*. The level of IL-12 in negative control was at 4.43, while the positive control showed a lower level of IL-12. On the other hand, the group administered with *Phoenix dactylifera* at a dose of 100 mg/kg BW has lower level of IL-12 than the positive control group that only reached 1.392 IL-12. Hence, this group is considered to be ineffective in decreasing the level of IL-12. The treatment of 200 mg/kg BW of *Phoenix dactylifera* demonstrated a higher level of IL-12 compared with the dose of 100 mg/kg BW. The treatment of *Phoenix dactylifera* at a dose of 400 mg/kg BW can significantly decrease the level of IL-12 to the highest level after treatment. Thus, the dose of 400 mg/kg BW is considered to be the most effective dose on decreasing IL-12 level in the mice infected with *S. typhimurium*.

The Number of Bacterial Colony in the Liver of Mice Infected with *Salmonella typhimurium*

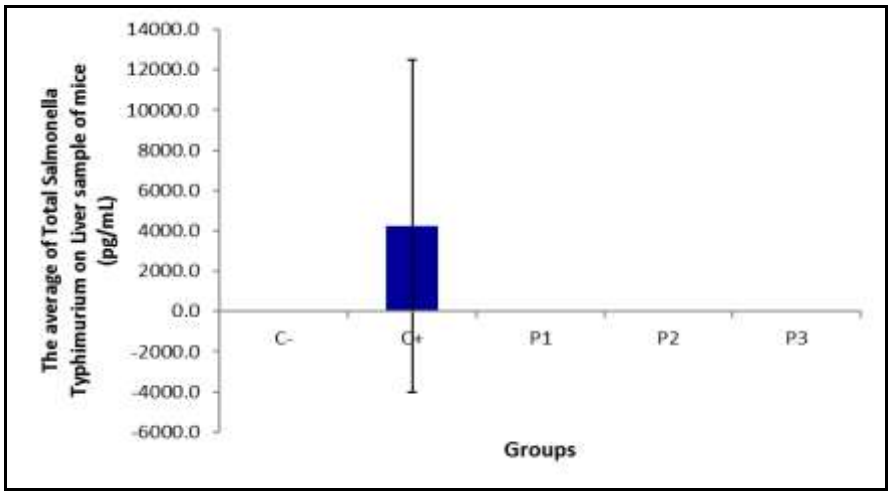

Figure (3): Colony of S. *typhimurium* in each group

Based on the figure above, the analysis shows that the different doses of *Phoenix dactylifera* extract affected the number of *S. typhimurium* colony in the liver of mice. The effect of *Phoenix dactylifera* can be discovered by comparing the colony of *S. typhimurium* bacteria in the positive control group with the other groups. The positive control group presents the highest number of *S. typhimurium* colony. After the treatment of 100 mg/kg BW *Phoenix dactylifera*, the bacterial colony was decreased significantly until zero. The same result also shown by the treatment of 200 mg/kg BW and 400 mg/kg BW *Phoenix dactylifera*. Overall differences in the colony of *S. typhimurium* in each treatment can be depicted in graphic form as follows.

Discussion

Phoenix dactylifera L contains chemical components, including carbohydrates (total sugars 44% - 88%), dietary fibre (6.4% - 11.5%), enzymes, protein (2.3% - 5.6 %), fat (0.2% - 0.4%), minerals, vitamins, phenolic acids and carotenoids. The nutritional and medicinal activities of date fruit are related to its chemical composition[15]. *Phoenix dactylifera* fruit has antioxidant, antimutagenic, anti-inflammatory, gastroprotective, hepatoprotective, nephroprotective, anticancer and immunostimulant activities[9]. Each type of dates has shown medicinal role in the various type of disease prevention. Dates and their constituents take an important role in disease prevention through anti-oxidant, anti-inflammatory, and anti-bacterial activities[10]. *Phoenix dactylifera* and its component play a big impact in the prevention or treatment of bacterial diseases[16].

Effect of *Phoenix dactylifera* extract on the IFN-γ Level

The study shows that the level of IFN-γ found in the group treated with 100 mg/kg BW of *Phoenix dactylifera* did not decrease, instead it become higher than the normal group. On the other hand, the increasing dose of *Phoenix dactylifera* (100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW) was followed by the decreasing of IFN-γ. Therefore, the treatment of 400 mg/kg BW extract resulted in the lowest level of IFN-γ and considered to be the most effective dose on decreasing IFN-γ level in the mice infected with *S. Typhimurium.*

INF-γ (INF type II) has lower specific antiviral activity, ranged from 10 to 100 fold, than INF type I, though it has 100-10000 times more immunomodulatory activities[17]. As a potential inducer of antiviral response, interferon gamma (IFN-γ) as secretary protein is mainly produced by T cells and natural killer cells. Thus has been reported to be involved in the control of HCV replication. IFN-γ triggers several antigen-presenting cell functions and drives the TH1 immune response in infections and IMIDs [18].

In contrary, cytokines of the Th1 (IFN-γ, IL-12, IL-18) are crucial in the protection activities against *Salmonella* in mice and humans, moreover, the different mutant of *Salmonella* strains require alternative cytokines for controlling the infection[19].

Effect of *Phoenix dactylifera* extract on the IL-12 Level

This study represents that the ethanol extract of *Phoenix dactylifera* fruit affected the decreasing level of IL-12 in groups P2 and P3. *Phoenix dactylifera* at a dose of 100 mg/kg BW could not significantly increase the level of IL-12 in serum. This dose considered to be ineffective in decreasing the level of IL-12 due to its lower level of IL-12 than that in the normal group. However, *Phoenix dactylifera* fruit extract at a dose of 200 mg/ kg BW could decrease the IL-12 level in serum of mice infected with *Salmonella typhimurium*. Moreover, the highest level of IL-12 in serum could be reached when the mice are treated with 400 mg/kg BW of *Phoenix dactylifera*. Thus, 400 mg/kg BW of *Phoenix dactylifera* is considered to be the most effective dose on increasing IL-12 level in the mice infected with *S. typhimurium.*

Interleukin-12 is essential for the induction and maintenance of TH1 immune response, with some shared functions, such as arising from the common subunit IL-12 p40 and its interaction with the IL-12Rb1 receptor subunit. Interleukin-12 stimulates the activation of T cells and natural killer cells to synthesize multiple proinflammatory factors, most importantly the TH1-specific cytokine, IFN-γ [18].

Effect of *Phoenix dactylifera* extract on the Number of Bacterial Colony in the Liver

The study indicates that the administration of *Phoenix dactylifera* extract can inhibit the growth of *S. typhimurium* colony in the liver of mice infected with *Salmonella typhimurium*. All treatment doses of ethanol extract of *Phoenix dactylifera* could kill the bacterial colony in the liver. This demonstrates the antibacterial activity of *Phoenix dactylifera* fruit extract.

Antibacterial activity of *Phoenix dactylifera* was examined by using leaf, seed, fruit and bark of the plant. The results demonstrated that all parts of the plant have antibacterial potential. The aqueous extract has less antimicrobial activity than methanol and acetone. On the other hand, fruit and leaf extracts had better antibacterial activity than seed and bark. Antibacterial activity of *Phoenix dactylifera* is predicted to be affected by its constituents, such as alkaloids, flavonoids, and tannins as the antibacterial properties[20].

Besides, Aqueous extract of *Phoenix dactylifera* fruits can significantly reduce the adhesion of *C. albicans, C. tropicalis* and *C. kefyr* in human buccal epithelial cells[21] and inhibit the growth of *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi,* and *Pseudomonas aeruginosa* [22, 23]. A Recent study described that *P. dactilifera* acts as antimicrobial on *Klebsiella pneumonia* and *Escherichia coli* and also has a role in the reduction of methyl side effects while uses as drugs. The extract of *Phoenix dactylifera* has an antibacterial effect against *E. faecalis,* thus proved that this extract can be used to treat intestinal diseases[24].

According to this study, the ethanol extract of *Phoenix dactylifera* is proven to be affected the number of bacterial colonies. The experiments showed that the bacterial colony is only found in the group injected with *Salmonella typhimurium.* However, there is no *Salmonella typhimurium* colony found in the mice treated with *Phoenix dactylifera* extract. The result was similar to the condition of the negative control group. This indicates that *Phoenix dactylifera* extract effective in inhibiting the development of bacterial colony in the liver of mice injected with Salmonella *typhimurium.*

Conclusion

Phoenix dactylifera extract of 400 mg/kgBW is the most effective dose on decreasing IFN-γ level and decreasing IL-12 level in the mice infected with *S. typhimurium*. In addition, *Phoenix dactylifera* extract is effective to decreasing the development of the bacterial colony of mice injected with *Salmonella typhimurium.*

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