

A Powerful ELISA Technique to Test The Potential of Extra Virgin Olive Oil in Reducing TNF- α Level and Edema Volume in Male *Rattus norvegicus* Exposed to Carrageenan

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Abstract

Extra virgin Olive oil is extracted from fruit that can be used as anti-inflammatory agent. This research aimed to test the potential of extra virgin olive oil in reducing edema volume and TNF- α plasma in carrageenan-induced rats. This research was purely experimental research with the post test control group design. A total of twenty eight Wistar rats were divided randomly into four treatment groups. Group I was a control negative group, while the group II, III, and IV were orally administered with extra virgin Olive oil at the dose of 0.9 ; 1.8 ; 2.7 mL/day, respectively. Paw edema was measured one hour before the rats was induced to carrageenan and every hour until four hours after it was induced to carrageenan. TNF- α plasma was measured at four hours. Analysis of the data was done by calculating the presentation of edema inhibition in every group, then the data was statistically analyzed by Anova, Repeated Anova, LSD and Kruskal Wallis test with 95% confidence interval. The result showed that extra virgin olive oil has an anti-inflammatory effect. The highest decrease in edema volume percentage was in group III (14.21%). There was a significant difference in the edema volume of all treatment groups at each time of the experiment with TNF- α value ($p < 0.05$). In conclusion, the administration of extra virgin olive oil can lower the volume of carrageenan-induced edema in rats depend on the dose. Also, the administration of extra virgin olive oil can be dose-dependent in reducing the levels of TNF- α in carrageenan-induced edema in rats.

Keywords

Anti inflammation, extra virgin olive oil, edema volume, TNF- α , carrageenan

INTRODUCTION

Inflammation is a physiological response to various stimuli such as infection, irritation or tissue injury. Inflammation is also known as a type of non-specific immune response,

and it involves many mediators (1,2). Inflammation is a beneficial response but it can be detrimental to the host because it contributes to numerous pathogenesis of diseases including allergic, autoimmune,

infectious, heart disease, arthritis, osteoporosis, diabetes, myopathy and cancer (3,4).

During the inflammatory process, pro-inflammatory and cytokines are secreted. The vascular reactions cause fluids, blood elements, leukocytes, mediators and pro-inflammatory cytokines to accumulate at the site of injury to remove harmful agents and to repair damaged tissue. The cytokines are immune system proteins regulating interactions between cells and stimulating immune reactivity, either in a specific or non-specific immunity (2,5). Inflammatory cytokine is a small peptide secreted primarily by macrophages and lymphocytes that will activated the tissue in response to trauma stimuli, such as endotoxin, immune complexes, physical and chemical trauma (3,6). The pro-inflammatory mediators respond to various stimuli, including bacterial lipopolysaccharide (LPS), cytokines, and UV radiation that will further induces the activation of Nuclear factor-kappa B (NF- κ B) and activator protein-1 (AP-1). The NF- κ B activates a number of molecules involved in the inflammatory response (proinflammatory cytokines), including iNOS, COX-2, TNF- α , IL-1 β , and IL-6 (7,8). Meanwhile, tumor Necrosis Factor- α (TNF- α) is a cytokine that has a different reaction in different cells. It involved in all process of inflammations and it can be used as an indicator of oxidative

stress, apoptosis or necrosis that occur in the cells. This cytokine induces acute phase reaction and activates the vascular endothelium, leukocytes, platelets and fibroblasts, so the cascade of inflammatory process is initiated by vascular, cellular and humoral immune system (6,9). The vascular changes due to pro inflammatory cytokines induction that will cause the movement of fluid to interstitial tissue called edema, one of the cardinal signs of inflammation (3).

The prevalence of inflammation is associated with place, race and disease. Non-steroidal anti-Inflammatory drugs (NSAIDs) is one of the most commonly prescribed for the treatment of inflammation (10). Thirty million tablets of non-steroidal anti-inflammatory drugs are sold in the United States annually. This number reflects the dependency on anti-inflammatory drugs (4). Additionally, the incidence of inflammatory disease such as osteoarthritis and gout disease has increased. More than fifty percent of NSAIDs are administrated to patients over 60 years old, so it leads to the increase in the side effect of NSAIDs.

There are several ways to prevent or slow down the progress of inflammation, either by using drugs or medicinal plants. Up to now, it is estimated that the Indonesian people still use a variety of plants for an alternative therapy. The use of plants as drugs are expected to have relatively low side effects compared to anti-inflammatory drugs. The

long term use of NSAIDs can cause erosion and bleeding in the lower gastrointestinal tract. It was reported that NSAIDs cause injury and affect the integrity of the mucous membrane of the gastrointestinal tract (10). One of the plants widely used as a drug is olive's fruit. Olive can be processed into olive oil, virgin olive oil and extra virgin olive oil. Extra virgin olive oil has different characteristic from other types of olive oil because of its refining process and its composition (11). Olive oil is used as a dietary component by the Mediterranean community to reduce the risk of illness and death. The Mediterranean people highly value the high oleic acid in olive oil as well as its minor components; the phenolic compounds (12). Phenolic compounds in extra virgin olive oil have been shown to have anti-inflammatory and anti-oxidant properties, as well as anti-microbial activity. Various phenolic compounds in extra virgin olive oil play an anti-inflammatory role in decarboxy methyl ligstroside aglycone (oleocanthal), hydroxytyrosol, flavonoid, and oleuropein (13). The phenol compound of extra virgin olive oil has been shown to decrease the concentration of Interleukin-6 (IL-6), a pro-inflammatory cytokine secreted by response to trauma. Other studies have shown that phenolic compounds in olive oil can inhibit the cyclooxygenase-2 (COX-2) activity. Impellizzer et al. also reported that oleuropein could reduce $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and

NO in carrageenan-induced pleura in rats (8,14). Based on the afore mentioned reasons, the researcher wanted to analyzed the effects of extra virgin olive oil on carrageenan-induced paw edema volume and levels of $\text{TNF-}\alpha$ in rats.

MATERIALS AND METHODS

This research method is an experimental with post-test only design only randomized control group design. The population in this study were 28 male Wistar rats (*Rattus norvegicus*) aged 2 - 3 months, weighing about 200 grams. Animals were acclimatized for 7 days and were divided into 4 groups. Group I was the control group. Group II, III and IV were orally administrated with a single dose of extra virgin olive oil at 0.9 mL/day, 1.8 mL/day, and 2.7 mL/day, respectively. All groups were given 2% of carrageenan injection (0.1 mL).

One hour before the injection of carrageenan, all laboratory animals were subjected to paw volume evaluation. The Extra virgin olive oil was produced in Italy with the brand Bertolli. It was administrated 30 minutes after carrageenan injection, and was followed by edema paw volume measurement at h1 (after 1 hour), h2 (after 2 hour), h3 (after 3 hour) and h4 (after 4 hour) after injection. In h4, all groups were sacrificed under ether anesthesia. Blood samples were taken through the heart after surgery. The blood samples were centrifuged

at 1500 rpm for 30 minutes to obtain serum for TNF- α levels analysis. This research has been approved by the Research Ethics committee of the Faculty of Veterinary Medicine, Airlangga Animal Care and Use Committee (ACUC) with the Ethical Clearance Number 576-KE.

All data was analyzed using Saphiro wilk normality test ($p > 0.05$) and homogeneity of variance Levene's ($p > 0.05$). The differences between unpaired groups were analyzed using ANOVA, REPEATED ANOVA and LSD test for rat paw edema volume, and one-way ANOVA ($p < 0.05$) and LSD ($p < 0.05$) test for the TNF- α . All statistical tests were performed with SPSS program.

RESULTS

The increased volume of edema (Table 1) showed that the control group experienced an increase in edema volume during the first,

second and third hours after induction of carrageenan. Afterwards, there was a decrease in edema volume at the h4. In Group II and III, edema volume increased up to 1 hour after injection of carrageenan, and it began to decrease in the second hour after the injection of carrageenan (Figure 1). The groups were sacrificed by anesthetizing using ether, and the blood samples were taken through the heart after surgery. Blood samples were centrifuged at 1500 rpm for 30 min to obtain serum for TNF- α levels analysis.

In Group IV, there was an increase in edema volume occurred shortly after the injection of carrageenan (t0) and there was a decrease in edema volume at 1 hour after injection of carrageenan (t1). Table 1 shows the percentage of a decline in edema volume in each group. The percentage of reduction in edema volume in the group IV was 14.21%.

Table 1. Mean and Standard Deviation of Rat's Paw Edema Volume (mL)

Group	h-1	h0	h1	h2	h3	h4	Δ Volume (%)
K*	2.63 $\pm$ 0.40	3.34 $\pm$ 0.46	3.42 $\pm$ 0.45	3.48 $\pm$ 0.44	3.6 $\pm$ 0.40	3.29 $\pm$ 0.54	10,25 $\pm$ 5,6
P1*	2.53 $\pm$ 0.38	3.14 $\pm$ 0.29	3.17 $\pm$ 0.36	2.99 $\pm$ 0.33	2.88 $\pm$ 0.44	2.87 $\pm$ 0.37	11,8 $\pm$ 11,6
P2*	2.47 $\pm$ 0.22	3.13 $\pm$ 0.23	3.22 $\pm$ 0.27	3.06 $\pm$ 0.23	2.94 $\pm$ 0.2	2.8 $\pm$ 0.25	13,28 $\pm$ 6,0
P3*	2.59 $\pm$ 0.31	3.23 $\pm$ 0.30	3.16 $\pm$ 0.33	3.16 $\pm$ 0.29	3.08 $\pm$ 0.33	2.85 $\pm$ 0.28	14,21 $\pm$ 4,5

*Anova test : $p < 0.05$

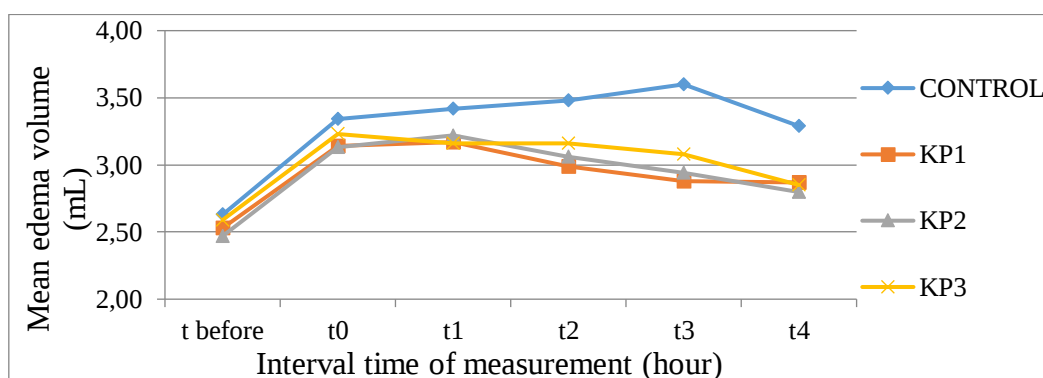


Fig 1. Mean Increase in Edem Volume (mL)

The ANOVA test showed a significant difference in all groups. Thus, there was a significant difference among the time of administration in each group. LSD test of edema volume variables showed that there was much difference in edema volume in the time between h-1 and h3 ($p = 0.001$). In the Group I, the most significant difference in edema volume was found between h-1 and h3 ($p = 0.001$). Meanwhile, in the treatment group III, the most different in edema volume was the time between h-1 and h1 ($p = 0.001$), and time between h-1 and h1 ($p = 0.001$). Furthermore, In the treatment group IV, the most different in edema volume was the time between h-1 and h0 ($p = 0.001$).

A repeated ANOVA test was used to determine whether there were mean differences in the repeated measurements. The results showed an overall significant difference between the time of measurement ($p = 0.001$) and a significant difference in the mean of edema volume between the groups based on the time of measurement ($p = 0.013$).

The LSD test based on the time of measurement that showed a significant difference were: the time group between h-1 and h0 ($p = 0.001$), h-1 and h1 ($p = 0.001$), h-1 and h2 ($p = 0.001$), h-1 and h3 ($p = 0.001$), h-1 and h4 ($p = 0.001$), h0 and h4 ($p = 0.001$), h1 and h3 ($p = 0.019$), h1 and h4 ($p = 0.001$), h2 and h4 ($p = 0.001$) and h2 and h4 ($p = 0.001$). There was a significant difference

between h-1 and h0 ($p = 0.001$). Therefore, it can be concluded that all groups has edema in the rat paw after injection of carrageenan.

The LSD results also showed that there were significant differences in edema volume based on the time of repeated measurement which was found between Group I and II ($p = 0.037$), and Group I and III ($p = 0.042$).

The percentage of reduction in edema volume has a normal distribution yet the variance was not homogeneous, so that the different test used was Kruskal Wallis test. The different test results of the percentage of reduction in edema volume after administration of extra virgin olive oil showed that there was no significant difference ($p = 0.268$) among the groups (Table 1). Thus, there was no difference in the percentage of reduction in edema volume in all of groups.

In the variable of TNF- α level (Figure 2), the highest mean was found in the control group ($2,736.6 \pm 1,535.2$ pg/mL) while the lowest one was found in the group P3 (380.64 ± 90.0 pg/mL) ANOVA test result $P = 0.001$. It indicated that there were significant differences between groups. The result of LSD test showed that there was a significant difference between Group I and Group III ($p = 0.003$), Group I and Group IV ($p = 0.001$), Group II and Group III ($p = 0.033$), Group II and Group IV ($p = 0.001$), as well as Group III and Group IV ($p = 0.012$). The most

significant difference was between Group I and Group IV, along with Group II and IV.

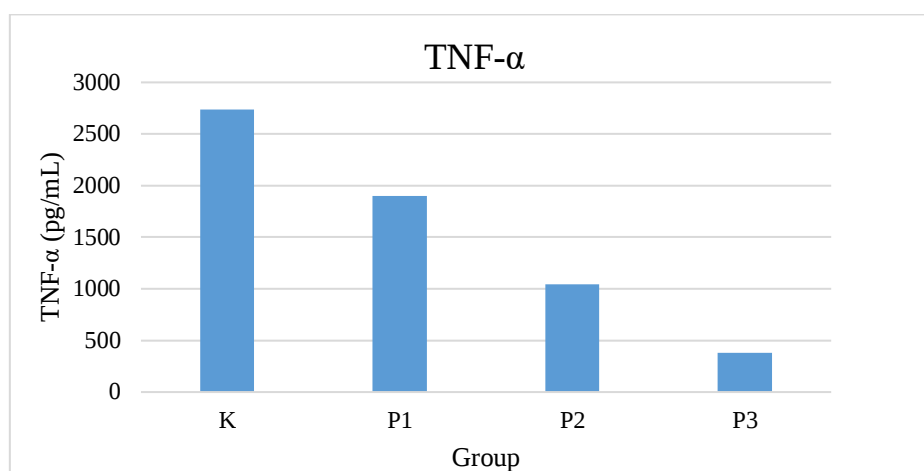


Fig 2. Difference in The Mean of TNF- α

DISCUSSION

Anti-Inflammation Test of Olive Oil to reduce Edema Volume

Based on the Table 1, we can analyse the change of rat paw volume in each treatment group. In h0 (approximately 15 minutes after injection of 2% of carrageenan), there was an inflammation triggered by 2% of carrageenan as shown by the increasing volume of rat paw in all of groups. The control group showed a difference in the increase of edema volume of 0.71 mL compared to the previous leg volume at the time h-1. Group II, Group III and Group IV showed a difference in the increase of edema volume of 0.61 mL, 0.66 mL, and 0.63 mL, respectively. This research is in accordance with the research conducted by Hidayati et al. (5), showing that there was an increase in edema volume at 15 minutes after the injection of carrageenan (5).

Table 1 also showed that the largest dose of 2.7 mL of extra virgin olive oil administered to the group IV has the most rapid anti-inflammatory effect compared to that of the other treatment groups and has the smallest percentage in the increase of edema volume. The highest decrease in edema volume was the treatment in the group IV (14.21%).

Based on result in Table 1, there was a significant difference in edema volume between the measurement time in all of groups. This research is supported by Fezai et al (14), which stating that there was a significant effect on the volume of rat paw edema injected with extra virgin olive oil due to the olive oil phenolic compounds that can lower the prostaglandin level by inhibiting the Cyclooxygenase.

The inhibition of edema volume by extra virgin olive oil was due to the inhibition of

COX-2 which play an important role in the conversion of arachidonic acid to prostaglandin formation, so that the inflammation will not occur (15). During the inflammation, various inflammatory mediators including 5-hydroxytryptamine (5HT), chemotactic factors, bradykinin, leukotrien and prostaglandin are released. Prostaglandins and prostacyclin cause erythema and vasodilatation as well as increase the local blood flow in vitro. Histamine and bradykinin also play a role in the increase of vascular permeability yet the vasodilation effect is not as much as that in the prostaglandins. There is an inflammation in the initial phase due to the release of histamine, serotonin and other similar substances. Then, in the next phase, there is an activation of prostaglandins, protease, lysosomes and other quinine substances (16). This research is also supported by Hidayati (5) study showing that flavonoids can decrease the volume of inflammatory edema-induced rat. Another study conducted by Favacho et al. (17) also said that oleic acid in *Euterpe oleracea* Martmay's fruit has decrease the volume of rat paw edema injected with carrageenan (17).

The Repeated ANOVA test result shows overall significant differences between the measurement time ($p = 0.001$). There were significant differences in mean between repeated measurement edema volume period ($p = 0.013$) among the groups. The LSD test

results showed that there was a significant difference ($p = 0.001$) between the time group h-1 the time group h0 (shortly after the injection of carrageenan), indicating that there was a significant increase in rat edema volume (edema) after carrageenan injection.

The result of LSD test among all treatment groups based on repetition of measurement showed that there was a significant difference between group I and group II treated with olive oil at the dose of 0.9 mL. Furthermore, a significant difference was also obtained between group I and group III treated with olive oil at the dose of 1.8 mL while those in groups I and group IV treated with olive oil at 2.7 mL showed no significant difference. This result is consistent with the previous research elaborated by Fezai et al., (14) showing that the largest dose of extra virgin oil has no statistically significant difference. It is possible that the relation between the volume of carrageenan injected was slightly different in each animal so that the volume of edema was not similar. The results of different tests indicated the percentage of reduction in edema volume $p = 0.480$ ($p > 0.05$), meaning that there was no significant difference in the effect of extra virgin olive oil on the percentage of reduction of edema volume among the groups. However, the clinical percentage of reduction in edema volume was greater along with the increasing dose. It is likely due to the absorption, distribution, metabolism and

excretion of the active compounds in extra virgin olive oil will vary depending on the body. Therefore, the dose of olive oil in this study can not be used as a reference of effective dose of olive oil as an anti-inflammatory agent.

Anti-Inflammation Test of Olive Oil on TNF- α

The carrageenan injected in rat's paw involves several mediators. In the early phases of inflammation, the first detected mediators are histamine, serotonin and bradykinin. In the next phase, the detected mediator is prostaglandin, a mediator that causes an increase in the vascular permeability. Additionally, local or systemic inflammation due to carrageenan was associated with the increase in pro-inflammatory cytokines such as TNF- α , IL-1 and IL-6 (18). The administration of extra virgin olive oil containing phenolic compounds such as hydroxytyrosol, oleochemical and flavonoids can lower TNF- α levels through the blocking of IKK phosphorylation resulting in inhibition of the degradation of I κ B proteasomal, thus preventing the activation of NF- κ B (19). The level of TNF- α increased four hour after injection, it is based on the research conducted by Ogata et al. (20) showing that elevated levels of the highest plasma TNF- α present at the fourth hour in rats injected by carrageenan intraperitoneally.

Furthermore, the results showed that the highest level of TNF- α was in the control group, and there was a decrease along with an increasing dose. This is in line with the research conducted by Amijaya et al. (21) which suggested that flavonoids may lower the levels of TNF- α in inflammatory-induced mice. The research conducted by Hardyanto et al. (22) also showed that flavonoids can decrease TNF- α in rats induced by urolithiasis.

The result of LSD test showed that there was a significant difference between control group and P2 group administrated with olive oil at the dose of 1.8 mL. Additionally, there was a significant difference between control group and P3 group treated with 2.7 mL olive oil. This statistic difference was not similar from clinically decreased levels of TNF- α . The highest dose of olive oil has the highest anti-inflammatory effect in lowering plasma TNF- α levels. It is possible that the absorption, distribution, metabolism and excretion of active compounds of olive oil in the body vary depending on the microenvironment.

In a study conducted by Nugraheni (11), the percentage of oleic acid in olive oil was 77.478%. Oleic acid contained in olive oil acts as an anti-inflammatory agent by inhibiting COX-2 regulation. At 30-50 g of olive oil is consumed, with a concentration of 180 mg/kg of phenol compounds, it is estimated that 8 mg of phenolic compounds

such as hydroxytyrosol, tyrosol, oleocanthal and flavonoids are absorbed (23).

The molecular mechanism of phenolic compounds as anti-inflammatory agents includes enzyme inhibition of pro-inflammatory, such as cyclooxygenase (COX-2) and lipoxygenase (LOX) and inducible nitric oxide synthase (iNOS). It involves the activation of the peroxisome proliferator activated receptor gamma (PPAR γ) and inhibition of nuclear factor-kappa B (NF-kB) (24).

The result of volume edema showed significant differences between Group I and group II, Group I and group III. Also, TNF- α levels showed significant differences between group I and group III as well as group I and group IV. This is presumably related to the capture point difference of each active compounds of extra virgin olive oil, causing differences in the effectiveness of extra virgin olive oil in various dosage levels of the edema volume and serum TNF- α . Therefore, this study could not show the

effective dose of anti inflammatory response of extra virgin olive oil.

CONCLUSIONS

This study concludes that extra virgin olive oil can reduce the volume edema in rats according to the dose and it can be dose-dependent in reducing the levels of TNF- α in carrageenan-induced edema in rats.

Further studies are needed to determine the effectiveness and toxicity of the extra virgin olive oil dose that has an anti inflammatory activity. In addition, the purification, identification, and quantification of the active substance in the extra virgin oil that has an anti inflammatory activity is also necessary to be analysed. TNF- α tissue examination and immunohistochemistry examination of rat's paw expression are also needed.

CONFLICT OF INTEREST

There are no conflicts of interest.

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