



Macrophages Activity and Capacity of Staphylococcus Aureus-induced Male Rattus Norvegicus, Sprague Dawley Strain Subsequent to Extra Virgin Olive Oil and Honey Mixture Administration

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Abstract

Extra virgin olive oil (EVOO) and honey as immunomodulator. Non-specific immune system is the body initial defense for infection, where macrophages are one of its parts. Activated macrophages will move more actively. This study aims to analyze the effect of EVOO and honey mixture administration on the macrophages activity and capacity of Staphylococcus aureus induced male rats (*Rattus norvegicus*) Sprague Dawley strain. This experimental study was conducted during May to June 2019. The total rats examined was 80, divided into 5 groups that were treated orally for 1 to 4 weeks. The groups of rats were K1 (-) Aquades, K2 (+) Stimuno, E1 EVOO, E2 honey, and E3 EVOO and honey mixture. Staphylococcus aureus were induced intraperitoneally into the rats. The macrophages were examined in Bacteriology and Immunology laboratory of FKH IPB University. Macrophages tests were conducted 1 hour subsequent to bacterial induction. The analysis of difference was done using one way anova and the analysis of group difference was carried out using Duncan test.

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The result shows that there are macrophage capacity ($P= 0.000$) differences on the first week but not on the macrophage activity ($P=0.079$), with highest mean value of macrophage activity (62.5 ± 3.32) and capacity (2927.3 ± 42.3) belong to E3 group. There are significant differences of both macrophage capacity and activity on the 2nd, 3rd, and 4th week ($P < 0.005$).

Keywords: Extra Virgin Olive Oil; Honey; macrophages; Phagocytosis.

1. Introduction

One of people's effort to improve body immune system is by consuming extra virgin olive oil (EVOO) and honey. People have easy access to EVOO and honey since they are available in many pharmacies, supermarket, and herb stores. Several studies have proved that EVOO and honey have function as imunomodulator.

EVOO is a functional food ingredient that possesses diseases prevention and treatment activities [1]. It is due to its content that bears high monounsaturated fatty acid, low mono saturated fatty acid, low saturated fatty acid, balanced ratio of essential fatty acid, antioxidant substances, vitamin and mineral. The consumption of monounsaturated fatty acid contained in EVOO significantly reduces adhesion molecule expression among mononuclear cells that decrease inflammation. In 100 g of EVOO there are 13.8-20 % saturated fat, 55-83 % monounsaturated fat, 9.7-16.4 % omega-6, 0.76-1 % omega-3 and 97-99% triglycerides [1,2,3,4]. EVOO contained 1-3% minor components including hydrocarbon, tocopherol, phenolic compound, sterol, chlorophyll, carotenoid, monoglycerides and diglycerides, free fatty acid, ester, and many other components [3,5].

EVOO contributed to improve body immune system most effectively compared to vegetable oil (corn oil and soybean oil) in da Silva's study [6]. Salmonella typhi-induced rat had bacteria colony reduced in their feces after being administered EVOO. EVOO has anti-bacteria and inflammation reduction ability [7].

Carbohydrate was proposed as the main component of honey, particularly glucose and fructose [8]. Egypt, Yemen, and Arabic honey contained 64,21-72,36% of reducing sugar, 3,31-3,5% of sucrose. 21,58-26,54% of glucose, and 38,76-50,78% of fructose in El Sohaimy's study [9]. Enzymes and amino acids are available in honey, with proline being the main amino acid in honey. Another amino acids exist in honey include alanine, phenylalanine, tyrosine, glutamate acid, isoleucine, and leucine. Minerals contained in honey are potassium, calcium, copper, iron, magnesium, manganese, phosphor, sodium, zinc, and selenium. Vitamins that are available in honey are ascorbic acid (C), thiamin (B), riboflavin (B2), niacin (B3), pantothenate acid (B5), and pyridoxine (B6). Marcucci's study revealed 38.18% fructose, 31.28% glucose, 7.31% maltose, and 1.31% sucrose content in American honey sample [10]. Honey contained protein (0.5%) in the form of enzymes (diastase or amylase, invertase or sucrase or α -glucosidase, CAT, and glucose oxidase), and amino acids (>20 amino acid), proline is believed as the most important amino acid [11]. Mejías and Montenegro's study discovered the content of Cu (0.602 ± 0.083 $\mu\text{g/g}$), Mn (0.838 ± 0.06 $\mu\text{g/g}$), Fe (1.32 ± 0.13 $\mu\text{g/g}$), and Zn (0.586 ± 0.04 $\mu\text{g/g}$) in honey from Llaima mountain zone [12]. Wiczorek found that the mineral contents in Poland honey, namely K (7.82 ± 3.05 $\mu\text{g/g}$), Ca (0.687 ± 0.04 $\mu\text{g/g}$), Mg (0.240 ± 0.08 $\mu\text{g/g}$) [13]. Alvarez-Suarez proposed that some studies found that the main flavonoid in Manuka honey were pinobanksin, pinocembrin, and

chrysin, whereas quercetin, kaempferol, galangin, luteolin, isorhamnetin, and 8-methoxykaempferol were flavonoid whose concentration are low [14]. Nwese found in their study that *Apis mellifera* honey possessed total content of phenol (439.16 ± 29.06 mgGAE/kg), flavonoid (61.72 ± 3.89 mgCEQ/kg), proline (386.46 ± 71.11 mg/kg), ascorbic acid (156.29 ± 5.48 mg/kg) [15].

The minimum standard of total content of reducing sugar in honey in Indonesia according to SNI 3545-2013 is 65 %. Nora's study revealed the result of qualitative examination in which there were alkaloid compounds, flavonoid, and terpenoid content in bitter-black and sweet-yellow Baduy honey [16]. Several studies have proved the effect of honey consumption in reducing inflammation and improve body immune system.

High content of oleate acid and double-unsaturated fatty acid as well as vitamin E, carotenoid and antioxidant compounds generate the imunomodulator and anti-inflammation property in EVOO. Meanwhile, honey's imunomodulator and anti-inflammation property are attributed to vitamin and mineral, antioxidant compound, carotenoid, and enzyme content. Celep and Yeşilada proposed that there is a possibility of synergic interaction between the antioxidant compounds exist in honey with other compounds that increase health and boost immune system modulation [17].

Generally, Immune system is classified into nonspecific immune system and specific immune system. Nonspecific immune system is the initial body defense against microbes. Nonspecific immune system are manifested as physical defense, biochemistry defense, and humoral defense. Macrophage is included as mononuclear cell in nonspecific immune system that works within tissue. Macrophages are found more inside tissues and cavities such as peritoneum. Macrophages work after being activated by the stimulation from bacterial entry. Based on the previous explanation, this research on EVOO and honey mixture effect on phagocytic action of macrophage subsequent to *Staphylococcus aureus* induction was conducted.

This study aims to (1) demonstrate effect of EVOO, honey and their mixture on macrophage activity of *Staphylococcus aureus*-induced male *Rattus norvegicus*, Sprague Dawley strain and (2) manifest the effect of EVOO, honey and their mixture on macrophage capacity of *Staphylococcus aureus*-induced male *Rattus norvegicus*, Sprague Dawley strain

2. Materials And Methods

2.1. Design, Time and Place

This study is an experimental study using factorial research design The observed variables were macrophages activity and macrophages capacity based on treatments and their time. Factorial design is a research design that has two or more factors, in which each replication has treatment combination, namely interaction effect. The acclimation period, preparation, and treatment to the trial animal were performed in IPB Bogor Veterinary Hospital. Macrophages tests were conducted in Faculty of Veterinary Medicine (FKH) IPB Bogor Bacteriology and Immunology Laboratory. The experiment was carried out from May to June 2019.

The materials used were extra virgin olive oil (EVOO) "Guillen", "Madurasa" honey, Stimuno, "Aquades" mineral water, Ketamine Xylazine, "Giemsa" stain, *Staphylococcus aureus* bacteria, methanol, and immersion

oil. The tools used were 1 ml syringe, scalpel and surgical scissors for trial animal, pipette, eppendorf tubes vector image, object glass, microscope cover glass, colony counter, 250 ml glass bottle, 150 ml pyrex beaker glass, 250 ml measuring glass, ux/uw Fujitsu analytical scale, XS-910 Vicom binocular microscope, glove, and mask.

2.2. Trial Animal

Each group consisted of 16 rats. After 7 days of adaptation, rats were administered test material in accordance with the calculation result of feed conversion formula. The administration of test material was performed until day 28. On day 7, 14, 21, and 28, macrophage tests were carried out. Four rats were taken from each group on the observation day (Table 1).

Table 1: Sample amount of each observation.

Group	Negative Control Group	Positive Control Group	Experimental Group 1	Experimental Group 2	Experimental Group 3
Total Sample	16 rats	16 rats	16 rats	16 rats	16 rats
Intervention	No intervention (<i>Aquades</i> administration, once a day)	<i>Stimuno</i> administration, once a day	EVOO administration, once a day	Honey administration, once a day	EVOO and honey mixture administration, once a day

The rats were kept in cages sized 50x30x20 cm. Each cage contained 2 rats. The temperature was 26±2 °C, light (bright: 12 hours, dark: 12 hours), and the humidity was 50-70%. Sawdust were put in the cage to prevent wetness. Additionally, sawdust can be replaced as needed. Before treatment, the rats were acclimated for 7 days to adapt and adjust to the new environment. During acclimation, rats were given standard feed and water inside the cage. Rats were fed in accordance with standard ration, namely, 10-15 gram per rat, and water was administered ad libitum. The ration used was based on AIN-98. Throughout the process, rats were administered anthelmintic medicine (dose 0.2-0.4 ml/kg body weight).

2.3. Bacteria

The bacteria induced intraperitoneally to the rats were *Staphylococcus aureus*. They are gram-positive cocci, grown in room temperature around 27-35°C. *Staphylococcus aureus* were obtained from Integrated Bacteriology Laboratory FKH IPB. A 1ml-dose of *Staphylococcus aureus* with 10⁸ cfu concentration was injected to rats intraperitoneally.

2.4. Treatment Ingredient Doses

Rats being as negative control in group 1 were administered 1ml *Aquades*. Rats in group 2, as positive control, were administered *Stimuno* syrup with 0.25 ml/rat/day dose [18]. Honey with dose 0.8 g/rat/day was administered to rats in group 3, while rats in group 4 was administered 0.185 g/rat/day dose of EVOO. Rats in the remaining group (group 5) was administered honey and EVOO mixture. Doses were calculated based on human consumption of EVOO and honey that was converted to rat dose. Respective dose of EVOO and honey

were mixed without electronic tools or other substances addition.

2.5. Macrophage Activity and Capacity Test

As many as 4 rats from each group were taken out on day 7, 14, 21, and 28. On appointed time, the rats were injected *Staphylococcus aureus* intraperitoneally. After 1 hour of bacterial induction, rats were anesthetized using 75 mg/kg Ketamine and 5-12 mg/kg Xylazine. The rats were then euthanized by exsanguination. Following that, the rats were dissected to get their peritoneal liquid. The carcasses of rats were cremated in FKH IPB incinerator.

The peritoneal liquid was put on smear preparation glass and fixated with methanol absolute for 5 minutes, stained with Giemsa that has been diluted for 20 times with distilled water, set aside for 20 minutes, rinsed with water, and dried. Preparations were read using microscope with 10-100 magnification by immersion oil. Activity and capacity value were determined using the following formula [19].

2.6. Data Analysis

The initial analysis was done by comparing the mean and standard deviation of macrophages activity and capacity of each group on the first week until the fourth week. Anova analysis was performed to see macrophages activity and capacity differences of each group on the first week until the fourth week.

2.7. Ethical Clearance

This study obtained ethical consideration from Animal Ethic Commission of Research and Community Service Institution (LPPM) IPB Bogor number 142-2019 by date 3rd May 2019. This study was declared as qualified for trial animal uses and was concerned of trial animal welfare.

3. Results

This study used EVOO and honey as test material. Some of their contents are described in Table 2 below.

Table 2: The Contents EVOO and Honey Samples.

EVOO	IOC 2016	Total	Honey	SNI 2013	Total
Oleat Acid (%)	55-83	74.7	Fructose (gr/ 100 gr)	-	22,4
Linoleat Acid (%)	3.5-21	7.65	Glucose (gr/ 100 gr), min	-	27,6
Linolenat Acid (%)	Max 1	1.63	Sucrose (gr/ 100 gr), max	5	<0,50
Vitamin E (mg/kg)	-	20.6	Zinc (Zn) mg/kg	0.03-0.40	0,086
Fe (mg/kg)	< 3	1.12	Fe (mg/kg)	-	20,1
Total Flavanoid % (b/b)	-	0.36	Total Flavanoid % (b/b)	-	0.07
Total Carotenoid (mg/kg)	-	25.2	Total Carotenoid (mg/kg)	-	0.64

Table 2 shows that oleat fatty acid in EVOO samples was within the standard threshold and so was linoleat acid, whereas linolenat acid content exceeded the maximum limit of International Olive Council (IOC) standard [20].

The Fe content was in accordance with the standard. IOC did not limit vitamin E, total flavonoid and total carotenoid content. Indonesia National Standard (SNI 3545 2013) determined the glucose, sucrose and Zn content [21]. The honey samples complied the standard

3.1. Macrophage Activity Test

Macrophages activity is the representation of total active macrophage used to destroy or phagocytize microbes. Macrophages perform their function as phagocytes in several stages, namely, recognition, chemotaxis, adhesion, ingestion, digestion, and elimination.

Table 3: The Effect of Extra Virgin Olive Oil (EVOO), Honey, and Their Mixture towards Macrophage Activity of White Male Rats (*Rattus Norvegicus*), *Sprague Dawley* strain.

Group	Day 7	Day 14	Day 21	Day 28
Control (-)	45.92±3.72 ^{ab}	46.17±4.47 ^{ab}	42.25±1.50 ^a	47.78±5.05 ^{abc}
Control (+)	53.75±4.45 ^{bcd}	54.33±5.00 ^{bcde}	56.33±2.23 ^{cdef}	65.08±5.92 ^{fgh}
EVOO	61.17±12.54 ^{defg}	64.0±5.28 ^{efgh}	66.17±6.29 ^{fgh}	67.50±5.02 ^{gh}
Honey	60.53±13.05 ^{defg}	62.0±10.48 ^{defg}	65.92±10.37 ^{fgh}	61.17±3.18 ^{defg}
EVOO + Honey	62.50±3.32 ^{defgh}	63.43±3.12 ^{defgh}	70.10±5.33 ^{gh}	72.12±2.06 ^h
P _{value}	0.079	0.004	0.000	0.000
P _{value}	0.012			

Table 3. shows that the highest mean of macrophage activity on day 7, 21, and 28 occurred in EVOO and honey mixture experimental group, whereas on day 14, the highest mean of macrophage activity occurred in EVOO experimental group (64.0±5.28). The range of macrophage activity mean of EVOO and honey mixture experimental group on day 7 was 62.5±3.32, and kept increasing until day 28 (72.12±2.06). The result indicates that there is significant difference in macrophage activity based on treatment (p=0,000) and based on time of treatment (p=0,012). Stimulus that was given by inducing *S. aureus* to rats activated the macrophages.

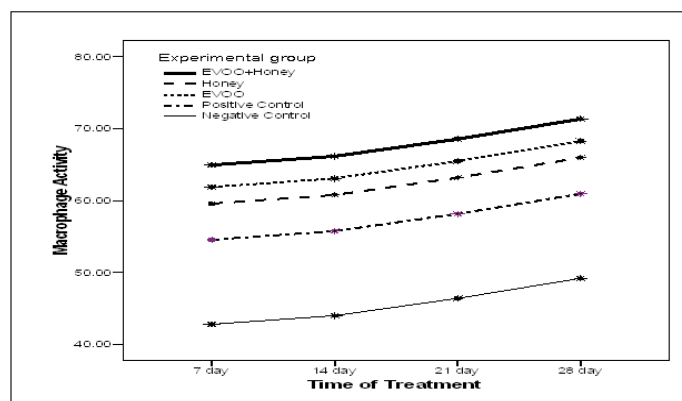


Figure 1: Macrophage Activity.

Figure 1 demonstrates that macrophage activity of EVOO group and positive control (C+) group were also increasing until day 28 of treatment. Meanwhile, macrophage activity means of negative control and honey group did not show increasing trend based on time of the treatment. Macrophage activity of EVOO+honey

mixture group was almost the same on day 14. Macrophage activity of rats in honey group decreased after day 21.

3.2. Macrophage Capacity Test

Macrophages capacity was calculated based on the number of bacteria that has been phagocytized divided by the number of active macrophages. Macrophages capacity describes macrophages' ability to phagocytize *S. aureus* bacteria on digestion stage.

Table 4: The Effect of Extra Virgin Olive Oil (EVOO), Honey, and Their Mixture towards Macrophage Capacity of White Male Rats (*Rattus Norvegicus*), *Sprague Dawley* strain.

Group	Day 7	Day 14	Day 21	Day 28
Control (-)	2241.8 ± 43.8 ^a	2280.3 ± 44.8 ^a	2297.8 ± 106.1 ^a	2172.7 ± 30.1 ^a
Control (+)	2193.5 ± 56.4 ^a	2902.0 ± 508.4 ^{bcd}	3239.3 ± 25.5 ^{ef}	3256.5 ± 26.5 ^f
EVOO	2692.3 ± 41.0 ^b	2834.8 ± 127.4 ^{bcd}	3483.7 ± 38.7 ^g	3552.0 ± 24.0 ^g
Honey	2802.5 ± 42.3 ^{bc}	3010.8 ± 24.7 ^{cd}	3462.8 ± 58.7 ^g	3023.3 ± 43.4 ^{cd}
EVOO+Honey	2927.3 ± 42.3 ^{cd}	3011.3 ± 72.3 ^{cd}	3051.6 ± 265.1 ^{de}	3539.4 ± 59.5 ^g
P _{value}	0.000	0.000	0.003	0.000
P _{value}	0.000			

Table 4 shows that on Day 7, EVOO+honey group has the highest number of macrophage capacity (2927.3±42.3) compare to EVOO group (2692.3±41.3), or to honey group (2802.5±42.3). On day 14, the highest number of macrophages capacity also shown in EVOO+honey group. Nevertheless, on day 21 and day 28, group that has the highest macrophages capacity was EVOO group. It is demonstrated that treatment, as well as time of treatment, have impact on macrophages capacity differences. The interaction between treatment and time of treatment has significant differences on macrophages capacity.

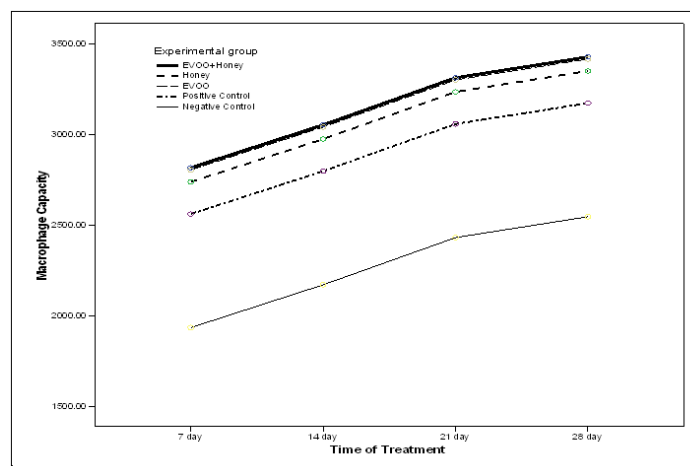


Figure 2 : Macrophage Capacity.

Figure 2 displays that the effect of EVOO and honey mixture on macrophages capacity was the highest in result and had positive linear pattern. From day 7 to day 28, there was found a significant difference of macrophages capacity after intervention. However, the number of macrophage capacity shown in group other than

EVOO+honey mixture, did not have linear pattern.

4. Discussion

Macrophage mechanism can be divided into two, namely M1 and M2 activation. In M1, macrophage works by destroying bacteria, in which IFN- γ cytokine is produced to activate macrophages to kill bacteria or foreign substances. In M2, Th2 is stimulated to generate IL-4 and IL-3 cytokines. Macrophages function as phagocytes, antigen-presenting cell, and several cytokines producer [22]. Macrophages activity is not a single process. It happens due to several causes including germs entry, cytokines production mainly IFN- γ , T lymphocytes that works with support of CD4+, inflammation, trauma, and working antigen antibody complex. Germs destruction can happen extracellularly or intracellularly. Extracellular process produces superoxide and radical halogen as cytotoxic agent to kill germ. Meanwhile, intracellular process is related to the work of histocompatibility complex II (MHC II).

The total macrophages activity influenced by the ingredients, dose, and time of the treatment as well as the type of bacteria induced in trial animal. The macrophages activity test can be performed in vitro or in vivo. Afiyata found that macrophages activity of mice that have been treated by *tempe* (fermented soy) for 12 days (760.6 \square 109.9) was higher than those who weren't given the treatment (9.6 \square 2.839) and those who were given imboost (244.2 \square 70.159). [23]. Reni study discovered macrophages activation subsequent to the administration of horse milk to mice was in the range of 51.23 \square 9.72. [24]. Arifah asserted that mice macrophages activity following the earth peg (*Eurycoma longifolia*) administration was around 85.4-95.4, the intervention doses were significantly different to macrophages activity [25]. Setyawan proposed that the average macrophages activity was 59.67-62.33, after being intervened with kejobeling leaves (*Strobilanthes crispus*) for 8 days [26]. Aldi discovered that after 7-day- administration of leafflower (*Phyllanthus niruri* Linn), the macrophages activity was 63.5-96 [27]. Nugroho showed that the potion of betel fruit, coleus (*Plectranthus scutellarioides*), honey, and yolk can increase phagocytic activity of macrophages cells [18].

Macrophage capacity turns out to be the number 1 ability indicator of macrophages in doing bacterial phagocytosis. Macrophages ability can be increase by the existence of bioactive substance content in the treatment ingredient, the length and doses of the treatment, as well as the type of bacteria induced in the trial animal. Some of the studies results showed different total macrophages capacity. Arifah found that after the administration of earth peg (*Eurycoma longifolia*), the macrophages capacity was 469 \square 2.55 on 10 \square g/ml dose, 439 \square 4.3 on 50 \square g/ml dose, and 364 \square 3.9 on 100 \square g/ml dose [25]. The smallest dose has the highest macrophages capacity. Aldi proclaimed that macrophages capacity after leafflower (*Phyllanthus niruri* Linn) treatment for 7 days was between 81.24-109 [27]. Setyawan stated that the mean of macrophages capacity was 4,43-5,23 following the administration of kejobeling leaves (*Strobilanthes crispus*), high dose doesn't guarantee high macrophages capacity [26].

EVOO and honey posses flavonoid content that can increase macrophages activity and capacity. Flavonoid compound in EVOO and honey can also increase IL-2 activity and proliferation of lymphocytes. Lymphocytes proliferation will affect CD4+ cells, so Th1 cells are activated. Afterwards, Th1 cells will affect the Specific

Macrophage Activating Factor, where the IFN- γ molecules activate macrophages. Honey contains polyphenol in the form of phenolic acid and flavonoid. The efficacy of honey is mainly influenced by flavonoid, which can increase enzymes production, inhibit free radicals, and stimulate hormones. Pasupuleti declared that kaempferol, chrysin, galangan, quercetin, caffeic acid, acetin, and pinocembrin in honey were related to mitochondria membrane induction process, apoptosis induction process, mitochondria activating process, and inflammation repair by suppressing TNF- α and activating NF-KB. [28]. Amiot asserted that flavonoid in EVOO benefits were to protect cells structure, increase vitamin C effectivity, and anti inflammation [2]. Mark stated this oleate acid advantages were to give effect in membrane cells and help repairing damaged cells and tissues [29]. Bogdanov expressed that the protein and vitamin content in honey increased body immune system that works by mitigating inflammation and killing bacteria that entered human body [30]. By consuming 1.5 gram/kg body weight of honey, the antioxidant level in the blood can increase [31].

Scotece stated that the oleocanthal content in the polyphenolic compound EVOO affects the work of macrophages [32]. EVOO and Honey contain flavonoids that can increase the activity and capacity of macrophages. The flavonoid compounds contained in EVOO and honey support the work of IL-2 and proliferating lymphocytes. CD4+ is affected by proliferating lymphocytes. CD4+ stimulated, activating Th1 cells. Continued activation of Th1 cells affects the Specific Macrophage Activating Factor, where IFN- γ molecules activate macrophages. Siswanto stated that processes in cell membranes are related to T cell proliferation and the number of cytokines released. Vitamin E can increase the work of cell membranes by stabilizing membrane permeability [33]. Cell membranes also enhance immune function through the cooperation of Helper cells with antigen-presenting-cells (APCs). Oelschlaegel stated that honey has high antimicrobial activity. The antimicrobial activity comes from the phenolic acids, flavonoids, and norisoprenoids present in honey [34]. Other fatty acids such as lauric acid which is also contained in EVOO have significant antimicrobial activity against gram-positive bacteria, as well as some fungi and viruses [35]. Lauric acid is more effective at inhibiting gram-positive bacteria than gram-negative bacteria [36]. The limitations of this study, at the stage of examining the chemical properties of EVOO and honey, only used 1 sample of each brand and a total of 7 samples of EVOO and 5 samples of honey. Another limitation, this study did not conduct a dose-based treatment study. This study only used 1 dose of EVOO and honey, which was then converted to a dose of experimental animals with. Another limitation, this study was not able to control the occurrence of stress in experimental animals when treated with a probe. Another limitation is that the calculation of the length of the villi and the size of the depth of the crypts are strongly influenced by the position of the intestinal organs in the paraffin. The limited price of EVOO products, which are not cheap, so that the use of EVOO in improving health can only be purchased by people with middle and upper economies.

5. Conclusion

EVOO and honey treatment showed the highest macrophages activity compared to other treatment group, on day 7, 21, and 28, but not on day 14. The mixture of EVOO and honey treatment group also exhibited the highest macrophages capacity than other treatment groups on day 7 and 14. Macrophages activity and capacity of EVOO and honey mixture treatment group showed an increase based on time. The highest result of macrophages activity of EVOO and honey treatment group was on day 28, while the highest result of

macrophage capacity test of EVOO treatment group was also on day 28.

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