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The potential of bitter melon extract on cellular non-specific immunity of koi fish (*Cyprinus carpio*) after *Aeromonas salmonicida* infection

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Abstract. *Andayani S, Fadjar M, Suprastyani H, Rahmawati A, Nurhalisa, Nadiro VN. 2023. The potential of bitter melon extract on cellular non-specific immunity of koi fish (*Cyprinus carpio*) after* Aeromonas salmonicida *infection. Biodiversitas 24: 5215-5222.* Indonesia has rich and potential fisheries resources both in freshwater (inland), coastal, and ocean. Potential fishery resources in freshwater include the diversity of fish species (germplasm) and fishery land. Utilization of freshwater fisheries potential can be realized by conducting fish farming, especially for economically valuable fish commodities, such as koi fish (*Cyprinus carpio*). Improving koi fish farming can be done by using bitter melon extract as an immunity enhancer. This study was conducted with the aim to determine the effect of bitter melon extract on cellular non-specific immune response. This study used a completely randomized design (CRD) method, with 4 treatments and 3 replicates, consisting of treatment K = not given bitter melon extract and not infected, A = dose of bitter melon extract 100 ppm, B = bitter melon extract 150 ppm, C = bitter melon extract 200 ppm. Before being given the treatment, the test fish were acclimatized with the aim of ensuring that the fish were healthy, not stressed and did not experience death. In addition, the dose of extract treatment and soaking time have been through the LD_{50} test as well as the density of bacterial infection and the length of time of infection have been through the LC_{50} test so that it has been confirmed that it does not cause stress and death in fish, with results SR 100%. A total of 10 fish were immersed into each treatment container (15 liters of water mixed with the extract) for 60 hours. Infection of *Aeromonas salmonicida* bacteria by immersion was carried out 5 days after immersion in bitter melon extract with a bacterial density of 10^7 cells.mL^{-1} for 1 hour. The results showed that lymphocytes increased after being given bitter melon extract and infected with *A. salmonicida* bacteria with a value of 63-83%. Monocytes increased from 3% to 8%, and Neutrophils increased from 4% to 9%. Phagocytosis activity increased from 18.4% to 28.9%, indicating that bitter melon extract can enhance non-specific immunity.

Keywords: Alternative prevention, bitter melon extract, increasing koi fish production, non-specific immunity

INTRODUCTION

Koi fish (*Cyprinus carpio*) provide considerable business opportunities. Koi fish production patterns that are carried out intensively have the opportunity to provide many business options, namely hatcheries, nurseries and enlargement businesses. The production value of aquaculture fisheries for koi fish in 2018 in the East Java region amounted to 242.626,373 thousand IDR (KKP 2021). There is great potential for koi fish in Indonesia in several areas in Jakarta, West Java, Sukabumi, Blitar, Cianjur, and Makassar. The large value of koi fish transactions in Indonesia has encouraged KKP to develop the potential of national ornamental fish. Therefore, the quality of local koi fish must be improved in order to compete with imported koi fish both on a domestic and international scale (Kusrini et al. 2015). However, intensive koi fish production makes koi fish farming vulnerable to diseases, one of which is caused by the *Aeromonas salmonicida* bacteria. This bacteria is dangerous because it can infect the inside and outside of the fish body, or the entire body can be infected (Gan et al. 2015). The rate of bacterial attack on fish hatcheries can occur within a few hours. Bacterial attacks by *A. salmonicida* destroy the internal organs of fish fry and skin wounds (Parvez and Mudarris 2014; Alavinezhad et al. 2021). The ability of *A. salmonicida* to infect fish is related to the bacteria's ability to produce toxins (Soni et al. 2021). *A. salmonicida* belongs to a group of pathogenic bacteria with high virulence (Semwal et al. 2023).

Prevention that is often done by koi fish farmers is by giving antibiotics made from chemicals. The provision of synthetic chemical antibiotics is currently limited and some have been banned because residues have been detected in fish meat. Therefore, other alternatives are needed to treat fish infected with the disease (Setyawan et al. 2019; Heny et al. 2022). An alternative prevention that can be done is by providing natural ingredients. Bitter melon is one of the natural ingredients that can be used in treatment or prevention.

The immune stimulation value of bitter melon extract is based on the content contained in it that can increase the body's resistance, including polyphenols, alkaloids and saponins. Alkaloid content is a hypoglycemic element and this element is concentrated in the fruit (Ahmad et al. 2015). The immune stimulation value of bitter melon

extract is explained by Nurjannah et al. (2013), that alkaloids function to detoxify or reduce toxic levels in the body. Saponins are useful as antibacterial and antiviral, increasing immunity and body vitality. Then added by Boshtam et al. (2016) and Maryani et al. (2018), the mechanism of immune stimulation carried out by saponins is by forming hydrogen bonds with cell membranes so that complex compounds are formed that can damage the permeability of bacterial cell membranes which ultimately results in the death of bacterial cells and increase immunity.

In addition to alkaloids, bitter melon contains polyphenolic compounds and phenol derivatives that have antioxidant activity. Phenolic antioxidants have been used, but only to a limited extent to prevent damage due to oxidation reactions in food, cosmetics and medicines. Polyphenols function as capturers and binders of free radicals or antioxidants, and can also increase endurance (Brandão et al. 2013; Sadowska et al. 2020).

Other non-specific immune responses that have been observed by Andayani (2022) and El-Boshy et al. (2014) include leucocytes, differential leucocytes (lymphocytes, monocytes, and neutrophils), fish leucocytes (white blood cells) consisting of neutrophils, basophils, eosinophils (polymorphonuclear), lymphocytes and monocytes (morphonuclear) and immature white blood cells (leukocytoblasts). Leucocytes have an important role in the immune system of fish (Bello et al. 2014; Widyawati et al. 2020) and in the cellular and humoral defense of the organism against foreign substances. Leucocytes can perform amoeboid movement through the process of diapedesis, in which leucocytes leave capillaries by breaking through between endothelial cells and penetrating connective tissue (El-Boshy et al. 2014; Cahyono et al. 2020). Macrophage and phagocytic activity that results from direct interaction with invading agents, such as microorganisms, can also be activated by the production of lymphocytes stimulated by antigens. Once macrophage cells are activated, they will show their metabolite activity and increased function to phagocytose, kill, and process germs. Based on research that has been done on bitter melon active ingredients to increase cellular and molecular non-specific immunity (Andayani et al. 2020; Andayani 2022), this article examines the effect of bitter melon fruit extract to increase cellular non-specific immunity.

MATERIALS AND METHODS

Test fish

The test fish observed were koi spawned from one broodstock from the Blitar Aquaculture Center. The fish were maintained at a density of 10 fish/15 liters, with an average initial total length of ±8 cm and age 2.5 months. Acclimatization was carried out for 2 weeks, and water circulations of 10-20% of the total volume were carried out weekly. The acclimatization process includes the ethical process of treating test animals. In order for the fish to live comfortably, one of the ethical procedures for test fish to avoid stress, acclimatization was carried out for 2 weeks and water circulations of 10-20% of the total volume were carried out weekly before being treated with bitter melon extract and infected with *A. salmonicida* bacteria.

Extraction method of bitter melon fruit

Extraction of bitter melon fruit was done by maceration method. Bitter melon fruit that has been separated from the skin, then mashed by blending, then dissolved with methanol solvent pa (Pro Analis) which is a polar solvent in a ratio of 1 : 1. Extraction of bitter melon fruit was done 3 times with the same ratio of 500 mL of bitter melon fruit : 500 mL methanol. The results of bitter melon extraction obtained different extract weights from 3 times the extraction process, and the yield value will be obtained.

Experimental design

The treatment dose was first tested for LD_{50} and the bacterial density was first tested for LC_{50} and then the experiment was conducted. This aims to find treatment doses and bacterial densities that do not cause >50% mortality, so that the treatment doses and bacterial densities used do not cause severe stress to cause death in fish. The results of the LD_{50} and LC_{50} tests did not cause stress and death, with 100% survival. The treatment plan using a completely randomized design with 3 replications is shown in the following information : (i) A: Treatment of 100 ppm dose of bitter melon fruit extract (in the water) + density *A. salmonicida* 10^7 cells.mL^{-1}. (ii) B: Treatment of 150 ppm dose of bitter melon fruit extract (in the water) + density *A. salmonicida* 10^7 cells.mL^{-1}. (iii) C: Treatment of 200 ppm dose of bitter melon fruit extract (in the water) + density *A. salmonicida* 10^7 cells.mL^{-1}. (iv) K^-: Negative control, which is koi fish without being infected with *A. salmonicida* and without being given bitter melon extract.

Giving bitter melon extract and bacterial infection *Aeromonas salmonicida*

Bitter melon extract was tested by immersion. Immersion was carried out by dividing the immersion container into 3 different treatments, namely treatment A (100 ppm), B (150 ppm) and C (200 ppm). The control fish were kept in a controlled environment or water without bitter melon extract. Each container contains 15 liters of water that is given the extract according to the treatment dose. Then, 10 fish each were immersed into each treatment container for 60 hours. The size of the treatment and control containers was 15 liters filled with 10 fish each. Bacterial infection of *A. salmonicida* was carried out 5 days after immersion in bitter melon extract. Bacterial infection was done by immersing in water containing bacteria with a density of 10^7 cells.mL^{-1} for 1 hour. After being soaked with bitter melon extract and infected with bacteria, the fish were transferred to a normal water container without bitter melon extract and this treatment is one of the ethics in the trial so that the fish are not stressed. Based on the research that has been done, the dose of bitter melon extract soaking treatment and soaking time does not cause fish to experience stress.

Blood collection and parameters observed

Blood collection of koi fish was carried out twice after the administration of the extract, which was observed 5 days after the administration of bitter melon extract by immersion and 1 day after being infected with *A. salmonicida* bacteria. Blood was dripped on a glass slide and erased with an eraser. The dried blood reviews were fixed with 70% methanol for 5 minutes and dried, then stained with 7% giemsa for 20 minutes and dried. After that, it was washed using running tap water and allowed to dry in the open air. Referring to the microscope magnification of 400×, the results of the blood review were observed to see lymphocytes, monocytes and neutrophils. Leucocyte counting is carried out until 100 leucocyte cells are obtained, then the percentage of leucocyte types can be calculated using the following formula (Isyaroh 2020):

Total leucocyte type {%} = leucocyte type % /100 x 100%

Isolation of fish macrophages is done by dissecting and taking the upper kidney on a sterile petri mixed with RPMI solution, injected into the kidney until it expands and chopped until the macrophage suspension comes out, then a drop is taken into the haemocytometer then the number of macrophages is calculated as follows (Irianto et al. 2004):

$$\text{Macrophage count (cells.ml}^{-1}) = \frac{N \times fp}{V}$$

Where:
N : macrophage cell count
fp : dilution factor

Calculation of macrophage phagocytosis begins with a macrophage suspension put into an eppendorf tube then centrifuged at 1000 rpm for 5 minutes so that a pellet precipitate is formed. Macrophage pellets were dripped on a glass object and leveled. Incubated for 60 minutes at room temperature (26°C). The glass object was then washed with RPMI + NCS (RPMI+) to remove non-adherent cells, then added 1.0 ml of yeast suspension 109 cells/ml. Then, the glass object was washed 3 times with RPMI+, fixed with 96% (v/v) methanol and left for 3 - 5 minutes at room temperature. Waited to dry and dripped with giemsa solution, left for 20 - 30 minutes and then washed with running water. Dried, then observed under a microscope. The calculation is as follows (Irianto et al. 2004):

PA = (Σ macrophages that ingest yeast/100 macrophages) × 100%

RESULTS AND DISCUSSION

Fourier Transform Independent Spectroscopy (FTIR) of bitter melon fruit extract

The infrared spectra of the study determined the chemical structure of the polyphenol compounds contained in the bitter melon extract (Figure 1).

Cellular immune parameters

Leucocytes

The function of leucocytes in the body system is very important. One of these functions is detoxifying proteins before they can cause damage to the body (Nurhalisa et al. 2022). Eosinophils are members of leucocytes that are weak phagocytes, while neutrophils and monocytes are strong phagocytes (Cahyono et al. 2020; Widyawati et al. 2020). The results of leucocyte analyses are shown in Figure 2.

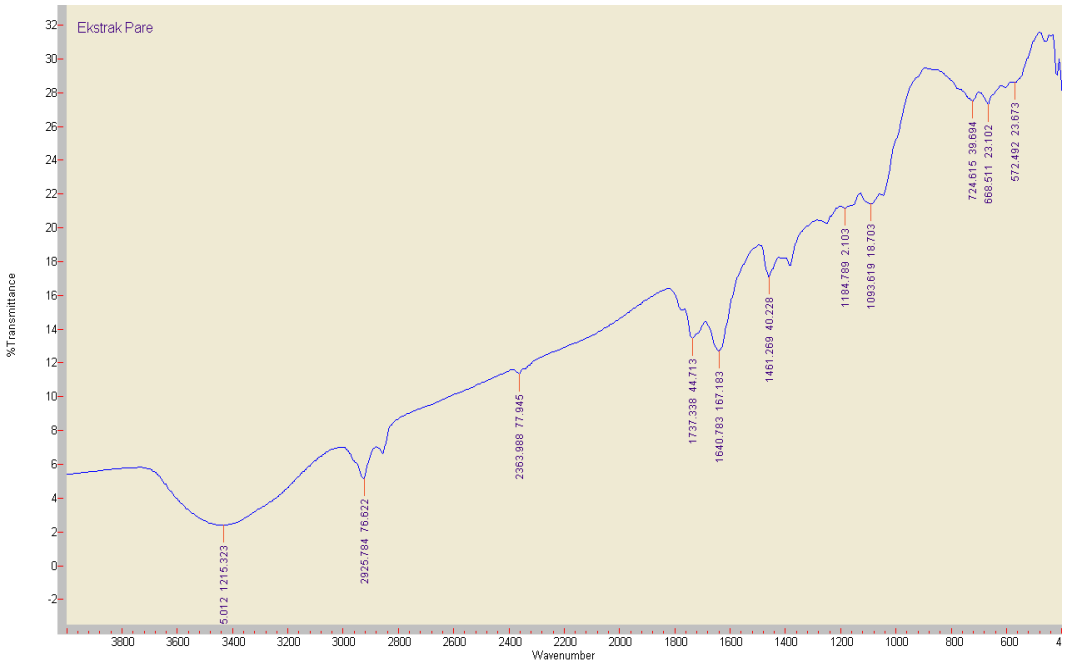

Figure 1. FTIR spectra results of bitter melon fruit extract

Table 1. FTIR spectra data of bitter melon fruit extract

No.	Spectra length	Library on spectra (Kristianingrum 2016; Fangidae et al. 2020)	Ribbon shape	Compound type	Function group
1	3450	3000-3500	Widen	Alcohols	O-H
2	2925	2800-3030	Sharp	Alkanes/Aromatics	C-H
3	2363	2222-2500	Widen	Alkylnitriles	C≡N
4	1737	1630-1680	Sharp	Alkenes	C=C
5	1640	1630-1680	Sharp	Alkenes	C=C
6	1461	1340-1470	Widen	Alkanes	C-O

Differential leucocyte

Lymphocytes

Lymphocytes are the most common type of leukocyte present in some fish, typically making up 85% of the total leukocyte population. Lymphocytes are oval-shaped and have a round purple nucleus and dark blue cytoplasm (Kumar 2016). Based on the observation, it is known that the number of lymphocytes has increased from before infection after being given bitter melon extract until the infection period. To find out the effect of bitter melon extract on the number of koi fish lymphocytes shown in Figure 3 below. Domination and composition structure change to contain lymphocytes.

Monocytes

Based on the observation, it is known that the number of monocytes has increased from the first day until the infection period. The analysis results on bitter melon fruit extract on the number of monocytes of koi fish are shown in Figure 4. The percentage of monocytes ranged from 3.33% to 8.33% during the observation after bitter melon extract administration/before infection, the value of A (3.67%), B (4.67%) and C (5.67%) higher than the control 3.33% and after *A. salmonicida* infection, treatment A (5.00%), treatment B (7.33%), treatment C (8.33%), control 4.33%.

Neutrophils

The analysis of the neutrophil standard is 6-8% (Kumar 2016). Based on the observation, the number of neutrophils increases from the first day until the infection period. The analysis results show that treatment A (5-7%), treatment B (5-8%), treatment C (7-8%), and control (4-5%). To find out the effect of bitter melon extract on the number of koi fish neutrophils is shown in Figure 5.

Macrophage

Based on the results of the study, there was an increase in the number of macrophages following the treatment given, with the control treatment without the administration of bitter melon extract amounting to (4.12-5.38 10^3 cell.mm^{-1}), treatment A (4.34-5.63 10^3 cell.mm^{-1}), treatment B (4.47-5.73 10^3 cell.mm^{-1}) and treatment C (4.64-5.96 10^3 cell.mm^{-1}) as in Figure 6.

Phagocytic activity

The observation on phagocytic activity shows that the number of Treatment A (18.4-23.4%), Treatment B (20.4-27.06%), Treatment C (21.7-28.9%), and control (15.24-16.95%). Phagocytic activity increased in treatments A, B, and C after being given bitter melon extract (Figure 7). Treatment C showed the highest activity, reaching 29.33%. Phagocyte activity increases with the administration of bitter melon extract compared to the control, plus infected with bacteria with the mechanism of phagocytes killing bacteria, phagocyte activity increases (Wibawan et al. 2022).

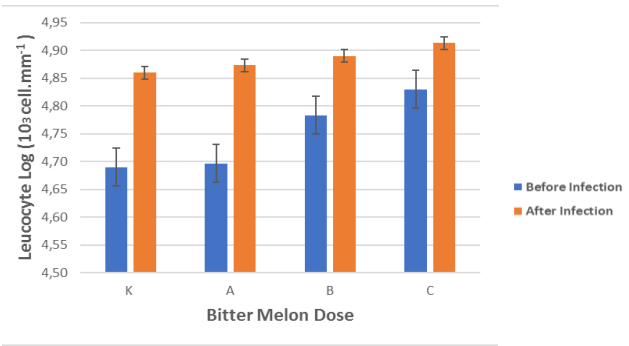

Figure 2. Leucocyte count (10^3 cells.mm^{-1}) before infection after bitter melon extract administration and after infection in koi fish

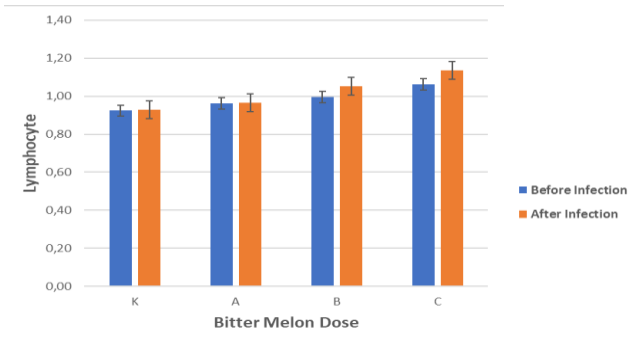

Figure 3. Lymphocyte count (%) before infection after bitter melon extract administration and after infection in koi fish

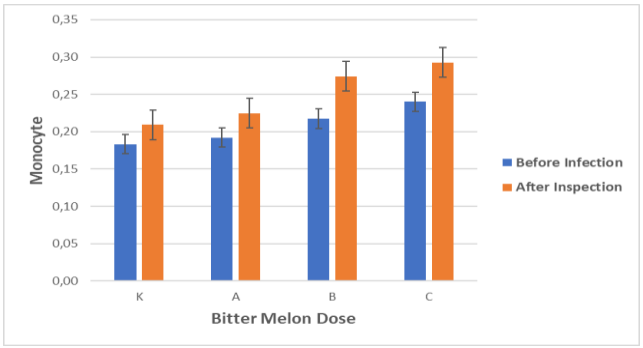

Figure 4. Monocytes (%) before infection after bitter melon extract administration and after infection in koi fish

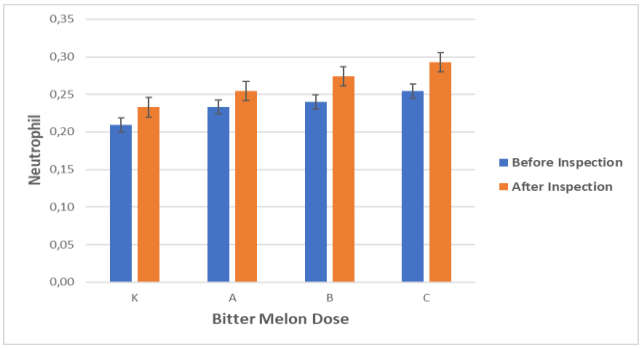

Figure 5. Neutrophils (%) before infection after bitter melon extract administration and after infection in koi fish

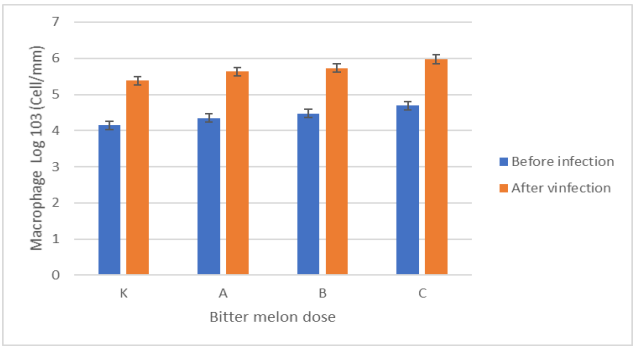

Figure 6. Macrophage before infection after bitter melon extract administration and after infection in koi fish

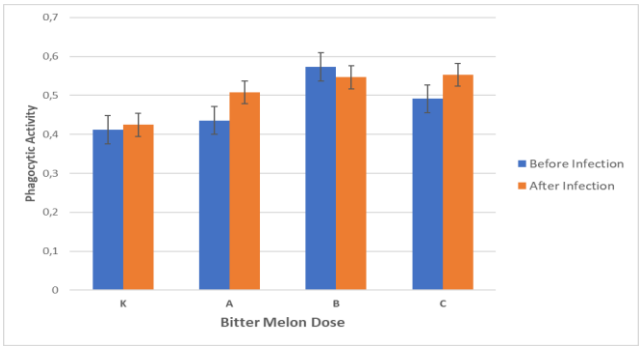

Figure 7. Phagocytic activity (%) before infection after bitter melon extract administration and after infection in koi fish

Discussion

The infrared spectra in this study were used to determine the functional groups in several specific absorption chemical compounds contained in the bitter melon extract (Subayu et al. 2021). Table 1 above shows that bitter melon extract has several functional groups in many specific absorption areas, namely the hydroxy group (OH), which is marked to appear in the absorption area of 3450 cm^{-1}, then there is an absorption area of 2925 $cm^{-1,}$ indicating that bitter melon extract shows the presence of aromatic functional groups (C-H). In addition, the alkyl nitrile functional group (C≡N) is shown in the 2363 cm^{-1} absorption area. Then, an alkene group (C=C) was detected in the 1737 and 1640 cm-1 absorption areas, and the C-C alkane group was identified in the 1461 cm^{-1} absorption area. According to Bautista-Hernández et al. (2021), O-H, C=C, C-H, and C-O groups indicate that a material contains polyphenolic compounds. This is supported by the statement of Wahdaningsih et al. (2022), which states that if a material has several O-H, C-H, C=O, and C-O functional groups, it can be assumed that it contains polyphenolic compounds. Polyphenols have proven to affect non-specific immunity through the white blood of fish macrophages and phagocyte activity (Andayani 2022).

The immune stimulation value of bitter melon extract is based on the content of bitter melon itself that can increase the body's resistance, such as polyphenols, alkaloids and saponins. The immune stimulation value of bitter melon extract is explained by Nurjannah et al. (2013), that alkaloids function to detoxify or reduce toxic levels in the body. Saponins are useful as antibacterial and antiviral, increasing immunity and body vitality. Added by Boshtam et al. (2016) and Maryani et al. (2018), the mechanism of immune stimulation carried out by saponins is by forming hydrogen bonds with cell membranes so that complex compounds are formed that can damage the permeability of bacterial cell membranes, which ultimately results in the death of bacterial cells and increase immunity. In addition, polyphenols and phenol derivatives contained in bitter melon extract have antioxidant activity. Polyphenols function as capturers and binders of free radicals or antioxidants and can also increase endurance (Brandão et al. 2013; Sadowska et al. 2020).

The observation of fish leucocytes before treatment was 46.400 cells.mL^{-1}, indicating that the total leucocytes were in the normal range, as reported by Suryadi et al. (2021), that the total leucocytes of normal fish ranged from 20.000-150.000 cells.mL^{-1} of blood. Based on research after being given bitter melon extract (before infection), there is an increase in leucocytes compared to the control. To prove that there is an increase in immunity from leucocyte parameters, infected with bacteria, it turns out that after infection showed an increase in leukocyte values with values K (4.59-4.86 10^3 cells.mm^{-1}), treatment A (4.70-4.87 10^3 cells.mm^{-1}), treatment B (4.78-4.89 10^3 cells.mm^{-1}) and treatment C (4.83-4.92 10^3 cells.mm^{-1}). The increase in

leukocyte counts continued as the dose and duration of the infection process increased. Leucocytes are the body's defense system against pathogenic infections. Leucocytes, or white blood cells, are responsible for the body's defense system. Most leucocytes are transferred to areas of infection to provide a rapid and potent defense against any infectious gene (Marshall et al. 2018). Bacteria that have entered the body will continue to adhere to host cells. Once the bacteria have settled at the initial site of infection, they will multiply and spread directly through the tissues into the bloodstream.

Lymphocytes after the administration of bitter melon extract or before infection increased. This proves that bitter melon extract containing polyphenols and alkaloids can stimulate the formation of lymphocytes (Maryani et al. 2018; Andayani et al. 2020). It can increase non-specific immunity through lymphocytes. Increased immune cells can increase fish's defense against adverse conditions. After infection with *A. salmonicida*, lymphocytes showed an increase in each treatment. treatment A (67-68%), treatment B (69-77%), treatment C (75-83%), control (63-65%), can be seen in Figure 3. According to Parvez and Mudarris (2014), *Cyprinus carpio* fish infected with bacterial Hemorrhagic Septicemia (BHS), the number of lymphocytes is 39 % and after treatment with chloramphenicol the number of lymphocytes is 80%.

The percentage of monocytes ranged from 3.33% to 8.33% during the observation after bitter melon extract administration / before infection. The value of A (3.67%), B (4.67%) and C (5.67%) was higher than the control (3.33%) and after *A. salmonicida* infection, treatment A (5.00%), treatment B (7.33%), treatment C (8.33%), control (3-4%). Monocytes are blood cells derived from fish lymphoid tissue and can differentiate into macrophages. This cell functions in the fish body to block the invasion of incoming pathogens (Figure 4) after being given bitter melon. This indicates that bitter melon extract succeeded in increasing the number of fish monocytes because the flavonoid content can disrupt the cytoplasmic membrane, inhibit the synthesis of nucleic acids, and inhibit the energy metabolism of bacteria. According to Parvez and Mudarris (2014), *Cyprinus carpio* fish infected with bacterial Hemorrhagic Septicemia (BHS) had a monocyte count of 14% and after being given chloramphenicol treatment had 45%. So bitter melon extract with polyphenol content can inhibit bacteria so as to increase cellular non-specific immune power through monocytes.

The number of neutrophils increases from the first day until the infection period. The analysis results show that before infection after being given bitter melon treatment A (5.33%), treatment B (5.67%), treatment C (6.33%) and control (4.33%). The content of bitter melon can cause the death of bacteria tested with *A. salmonicida*, so that non-specific immune power increases as shown in the following data, neutrophil values after infection for treatment A (6.33%), B (7.33%), C (8.33%) and control (5.33%), can be seen in Figure 5.

Neutrophils are blood cells that are critical for fish defense and maintaining homeostasis. These cells are the first leucocytes to be recruited to the site of inflammation and eliminate pathogens through complementary mechanisms. Once activated, neutrophils become powerful killers and use toxic intracellular granules. Neutrophils are rapidly recruited from the blood to inflammation by chemotactic signals derived from infectious agents or molecular patterns associated with damage (Havixbeck and Barreda 2015). This is supported by Mayadas et al. (2014) and Burn et al. (2021); in principle, neutrophil degranulation is similar to the granule-phagosome membrane fusion mechanism. However, neutrophil degranulation occurs at the plasma membrane. Thus, soluble proteins derived from granules are released into the extracellular space. In vitro evidence clearly shows granules fusing with the plasma membrane and the release of cargo proteins to kill bacteria.

Based on the research results, there was an increase in the number of macrophages following the treatment given, with the control treatment without the administration of bitter melon extract amounting to (4.12-5.38 10^3 cell.mm^{-1}), after given bitter melon or before infection treatment A (4.34 cell.mm^{-1}), treatment B (4.47 cell.mm^{-1}), treatment C (4.64 cell.mm^{-1}) and after infection *A. salmonicida*, treatment A (5.63 10^3 cell.mm^{-1}), B (5.73 10^3 cell.mm^{-1}) and C (5.96 10^3 cell.mm^{-1}), can be seen in Figure 6. There is an antigen in the form of bitter melon with polyphenol content that macrophages digest and increase, plus antigens in the form of *A. salmonicida* macrophage function digests and kills bacteria so that non-specific immunity increases through macrophages.

The results of Elwira et al. (2021) study of goldfish *Cyprinus carpio* after being given *Gracilaria verrucoasa* seaweed extract and after being infected with *Aeromonas hydrophila* increased the number of macrophages. The increase in macrophages is due to tissue damage by bacterial activity; it secretes chemicals that can bring in more macrophages. Bacteria themselves, such as endotoxins (lipopolysaccharides) can activate macrophages. In addition, macrophages have properties like other phagocytic cells, which have protective properties carried out by phagocytic cells against the infection of foreign materials/microorganisms (Pattipeiluhu et al. 2022).

Macrophages that respond first to antigens are tissue macrophages. *Aeromonas salmonicida* bacteria, which are gram-negative bacteria, have LPS on the outside; one of them is recognized by macrophages. According to Wibawan et al. (2022), macrophages then release interleukins to stimulate T cells to proliferate and form lymphokines, attracting more macrophages to their place. The macrophages that are brought in and circulate in the bloodstream are monocytes that undergo maturation on their way to the wound area. The amount of phagocytic activity follows the increase in the number of macrophages.

An increased phagocytosis index indicates increased immunity. According to Boshtam et al. (2016), the phagocytosis process is followed by an increase in metabolism in the form of a "Respiratory burst." Phagocytosis events begin with contact between the cell membrane and particles (toxins) that will activate the

flavoenzyme system on the membrane NADP (*Nicotinamide Adenine Dinucleotide Phosphate*) oxidase so that *Reactive Oxygen Intermediates* (ROI) are formed. NADP oxidase will react and form *Superoxide Anion* (O^{-2}). *Superoxide Anions*, with the help of *Superoxide Dismutase* (SOD), could catalyst into *Hydrogen Peroxide* (H_2O_2) and *Hydroxyl Radicals* (OH^-), which are toxic to *A. salmonicida* organisms (Desai and Bhilave 2018).

After giving bitter melon extract, non-specific immune parameters of leucocytes, differential leucocytes, macrophages and phagocytic activity increased compared to the control. After being challenged with *A. salmonicida* bacteria, the value of non-specific immune parameters increased, so it was necessary to add more doses to increase non-specific immunity and bacterial infection until the maximum immune value was obtained and then the immune power decreased, which needed to be given a bitter melon extract booster to increase immunity.

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