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In Vitro Immunomodulatory Activity of Virgin Coconut Oil (VCO) with and without Bromelain Enzyme from Pineapple Waste (*Ananas comosus (L) Merr*)

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Abstract

Immunomodulators are an essential part of the prevention process for treating various diseases related to the body's immune system. This study aimed to determine the immunomodulatory activity of virgin coconut oil (VCO) with and without the enzyme bromelain in pineapple waste extract on the proliferation of mice lymphocyte cells through an in vitro test. VCO was made using two methods: enzymatic using bromelain enzyme (VCOb) from pineapple waste with 10, 25, and 50%, and mixing method without bromelain enzyme (VCOm). The two types of VCO produced were calculated for the yield, moisture content, free fatty acids (FFA), and physicochemical properties. For immunomodulatory activity, the test solution was taken from VCOb and VCOm with a variation concentration of 6.25, 12.5, 25, 50, and 100 µg/mL. Isolation of lymphocyte cells was obtained from the spleen organ of Swiss Webster strain mice which was performed aseptically with a ketamine-xylazine anesthetic. The lymphocyte cell proliferation test was performed using the MTT Assay, and the Optical Density was measured using a microplate reader at 550 nm. The results showed that 50% VCOb produced the highest yield and 22.22% higher than VCOm. However, the results showed that increasing bromelain enzyme concentration would increase the moisture content and free fatty acid content, but still below 0.2%. The VCOb and VCOm had immunomodulatory activity against mice lymphocyte cell proliferation. However, the immunomodulatory activity of VCO with bromelain enzyme from pineapple waste extract (VCOb) was higher than without bromelain enzyme (VCOm). The highest immunomodulatory activity was obtained at 100 µg/mL of VCOb with a percentage increase of 158.26% compared to negative controls, followed by VCOm of 100 µg/mL with a percentage increase of 137.66% compared to negative controls. The optimum dose of VCOm and VCOb for increasing the proliferation of mice lymphocyte cells has not been found.

1. Introduction

Potential vaccines to prevent COVID-19 have been starting distributed to the people of Indonesia. However, the presence of COVID-19 still threatens people's lives, so immunomodulators remain the most crucial part of the prevention and even treatment process. Immunomodulators help the body optimize the function of the immune system, which is the primary system that plays a role in the body's defense against viruses. Immunomodulators are substances that can modulate (change or affect) the immune system in an average direction. Immunomodulators play a role in strengthening the body's immune system (Immune stimulator) or suppressing an excessive immune system

(immunosuppressant). Several in vitro, animal, and human studies support the potential of virgin coconut oil (VCO), lauric acid, and its derivatives as effective and safe agents against viruses such as COVID-19 [1, 2, 3, 4]. VCO is believed to be able to reduce the symptoms of the novel coronavirus [5]. Substances in VCO, such as lauric acid and its derivatives, flavonols, and flavonoids, exhibit antiviral and immunomodulatory activity added to antiviral drugs, prevent host cell infection and viral replication or reduce the inflammatory effect of COVID- 19 [5, 6]. According to the sufficient scientific evidence for the antiviral and immunostimulant activity of coconut oil, lauric acid, and its derivatives, as well as the general safety, it is essential to utilize VCO as an alternative immunostimulant for the prevention of COVID-19, given the abundance of raw materials available.

The abundant availability of VCO raw materials (coconut) can be seen in Indonesia's total area of coconut plantations, which reaches 3.88 million hectares (31.4%) and is the largest coconut plantation area globally. Producing VCO from coconuts can use the enzymatic method combined with fermentation techniques [7]. In the enzymatic method, proteolytic enzymes accelerate the hydrolysis reaction of proteins, thus saving processing time. One of the proteolytic enzymes is the bromelain enzyme. Unwittingly, the waste around us, especially the processing waste of pineapple jam, which is a mainstay product in the Bengkulu specific food industry, contains the enzyme bromelain. Bromelain enzyme can be obtained in the stem, fruit, crown, flower, core, and pineapple peel [8].

The novelty of this research is immunomodulatory testing with VCO obtained from the addition of the bromelain enzyme from pineapple waste. Thus far, no research has been carried out on the immunomodulatory activity of VCO with bromelain enzyme against lymphocyte cell proliferation in vitro. Therefore, this research must be conducted to add scientific evidence and be used as an immunomodulator for complementary therapy in infectious diseases that can reduce immune responses (such as COVID-19) and as a valuable product for increasing immunity. This research is expected to contribute to science by providing information about sources of immunomodulators from natural materials around Indonesian residences. This is beneficial in treating patients infected with the coronavirus (COVID- 19) by increasing immunity (immunostimulant).

2. Methodology

2.1. Equipment

The equipment used were analytical balance type FSR-A (Fujitsu, Japan), oven UN 55 53L (Memmert, Germany), sterile surgical dissecting set (Gold Cross, Indonesia), refrigerated centrifuge 5810R (Eppendorf, United States), laminar airflow (Nuaire, United States), and the SPECTROstar Nano microplate reader (BMG Labtech, Germany).

2.2. Materials

The materials used were thick coconut milk from mature coconut (Indonesia), ripe pineapple waste (Indonesia), analytical grade ethanol (Merck, Darmstadt, Germany), 70% ethanol (Merck, Darmstadt, Germany), Phenolphthalein indicator (Merck, Darmstadt, Germany), KOH (Merck, Darmstadt, Germany), HCl (Merck, Darmstadt, Germany), two months old male mice with 25–30 grams in weight from the Swiss Webster line (Indonesia), Roswell Park Memorial Institute medium (Gibco, USA), MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (SangonBio, Indonesia), hepatitis B vaccine (EngerixTM-B, Belgium), Sodium Dodecyl Sulfate (Nzytech, Lisboa, Portugal), 10% ketamine (Netherlands), xylazine (Netherlands), and tris ammonium chloride buffer (Merck, Darmstadt, Germany).

2.3. VCO production with and without bromelain enzyme

In this study, VCO was produced through two methods. The method without bromelain (mixing) was conducted using 1 kg of thick coconut milk left for 2 hours to form two layers: cream and skim. The skim must be removed first to obtain the cream. The cream was stirred using a mixer for 15 minutes at full speed (equivalent to 1500 rpm). The cream was left for 24 hours at 25℃, which aimed to separate the water phase, oil phase, and dregs. In order to obtain VCO, the oil was separated by opening the separatory funnel faucet, and the water phase was removed. The yield of VCO was then calculated.

The other method was using the bromelain enzymatic method. The initial step of extracting the bromelain enzyme was done by peeling the skin and core from the pineapple meat. Then, the skin and core were washed thoroughly, cut into pieces, and mashed using a blender. The results were filtered and left overnight to obtain pineapple waste extract. After that, 2 kg thick coconut milk was left for 2 hours to form two layers: cream and skim. The skim must be removed first to obtain the cream. Pineapple waste extracts in various concentrations (10, 25, and 50%) were added to coconut cream. The pineapple waste extract and coconut cream mixture were stirred until evenly distributed. The mixture was aged for 24 hours to form three layers: dregs in the first layer, oil in the second layer, and water in the lower layer. In order to obtain VCO, the oil was separated by opening the separatory funnel faucet, and the water phase was removed. The yield of VCO was then calculated [9].

2.4. VCO characterization

The resulting VCO was then analyzed for its physicochemical properties, including physical qualities such as smell, taste, and color [10]. The physicochemical properties of VCO were also observed after being stored for one month at room temperature. The moisture content test was performed for all the VCO produced by heating 10 g of the VCO sample in an oven at 105℃ for one hour. The sample was cooled in a desiccator for 30 minutes, then reweighed. Heating and weighing were

repeated until a constant weight of the sample was obtained. The test was repeated three times.

Meanwhile, the levels of Free Fatty Acid (FFA) were determined using the titration method. A 5 g of VCO sample was weighed, put into an Erlenmeyer, and added 50 ml of 95% ethanol. Then three drops of phenolphthalein indicator (PP) were added and titrated with 0.1 N NaOH standard until the color was pink (stayed unchanged for 15 seconds). The test was repeated three times. The water and FFA contents were calculated using equations (1) and (2).

$$\text{Moisture content (\%)} = \frac{\text{initial sample weight} - \text{final sample weight}}{\text{initial sample weight}} \text{x } 100 \quad (1)$$

$$\text{FFA (\%)} = \frac{\text{volume (NaOH) x normality (NaOH) x } 200}{\text{sample weight x } 1000} \text{ x } 100 \quad (2)$$

2.5. Immunomodulatory activity test with MTT assay

The test solution was taken from VCOm with 6.25, 12.5, 25, 50, and 100 µg/mL concentrations. The lymphocyte cells were aseptically isolated from the spleen organs of two-month-old Swiss Webster strain male mice with 25–30 grams in weight. The mice were first anesthetized using ketamine-xylazine to reduce the pain, and then cervical dislocation was performed. Mice were placed in a prone position, and the entire surface of the abdomen and surgical board was moistened with 70% ethanol. Small incisions were made on the skin of abdominal mice to the chest and thighs using scissors assisted with tweezers. The spleen was removed from its peritoneal sheath and placed in a 50 mm diameter petri dish containing 10 mL of RPMI medium. RPMI media was injected into the spleen so that the lymphocytes emerged along with the media.

The cell suspension was put in a 10 mL centrifuge tube and centrifuged at 1200 rpm 4℃ for 5 minutes. The pellet obtained was suspended in 5 mL of tris ammonium chloride buffer to lyse erythrocytes. Cells were mixed until homogeneous and allowed to stand at room temperature for 15 minutes or until the color changed to slightly yellowish. Cells were added to RPMI ad 10 mL, centrifuged at 1200 rpm 4℃ for 5 minutes, and discarded the supernatant. The pellets obtained were washed two times with RPMI. Cells were counted with a hemocytometer. The lymphocyte cells were ready to be cultured in an incubator at 37℃ and tested for their activity.

The lymphocyte cell proliferation test using the MTT assay was conducted using 100 µL of lymphocyte cells (density 1.5 x10^6 /mL) distributed to each well of a 96-well microplate. Then, 10 µL of hepatitis B vaccine was added to each well and incubated for 24 hours in an incubator at 37℃. Subsequently, 100 µL of VCO was added to each concentration (6.25, 12.5, 25, 50, and 100 g/mL). Each concentration was replicated three times. The control and treatment groups were incubated for 48 hours, and a solution of 10 µL MTT 5 mg/mL was added. Then both groups were incubated again for 4 hours at 37℃. Live cells will react with MTT to form a purple color. The reaction with MTT was stopped by adding 100 µL of 10% SDS solution (a stopper reagent) in 0.01 N hydrochloric acid into each well and allowed to stand for 24 hours. Then the absorbance was measured using a microplate reader at 550 nm. The test was repeated three times [11].

2.6. Data analysis method

The results of the immunomodulatory activity test on mice lymphocyte cell proliferation were analyzed using analysis of variance (one-way ANOVA) and followed by the student's T-test.

3. Results and Discussion

3.1. The yield of VCO

The different methods of producing VCO resulted in different VCO yields. The highest yield was obtained by the enzymatic method through the addition of pineapple waste extract containing 50% bromelain enzyme (VCOb 50%), followed by the addition of 25% pineapple waste extract (VCOb 25%), then mixing method (VCOm), and the addition of 10% pineapple waste extract (VCOb 10%) (Table 1).

Table 1. The percentage of VCO yield produced by mixing and adding 10, 25, and 50% pineapple waste extract

No.	VCO Types	Concentration	Yield of VCO (%)
			Mean ± SD
1	VCOm	0%	14.92 ± 0.025
		10%	8.28 ± 0.060
2	VCOb	25%	34.85 ± 0.020
		50%	37.12 ± 0.020

Table 1 shows that the pineapple waste extract can increase the yield of the produced VCO. In producing VCO using the addition of the pineapple waste extract method, it can be seen that the bromelain enzyme plays a role in increasing the amount of VCO yield. Protease enzymes in pineapple waste extract can act as a biocatalyst in accelerating the hydrolysis of peptide bonds in the coconut milk emulsion, which causes the breaking of peptide bonds and protein damage, resulting in oil being released from the coconut milk emulsion system [9]. The bromelain enzyme is acidic with a pH of 5.8 [12]. Protease enzymes and acid-assisted carried out protein breakdown process in coconut cream. Thus, the protein in coconut cream will coagulate and separate from the oil [13].

3.2. VCO characterization

3.2.1. Physicochemical properties of VCO

The mixing method produced clear white VCOm with a distinctive coconut taste and aroma. At the same time, all the resulting VCOb had a yellow color that became darker with increasing concentration (Figure 1). Even though they were dominated by the distinctive taste and aroma of coconut, the aroma of pineapple was still detectable, and the taste was slightly sour.

The physicochemical properties of VCO were also observed after one month of storage at room temperature (Table 2), and it was found that both VCOm and VCOb did not change in smell, taste, and color. VCO did not show any signs of rancidity, either aroma or taste, due to the

low moisture and free fatty acid content in VCO, indicating that it can be stored for an extended time.

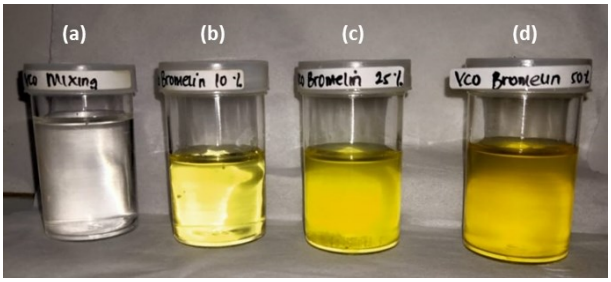

Figure 1. Difference color of (a) VCOm, (b) VCOb 10%, (c) VCOb 25%, and (d) VCOb 50%

Table 2. Physicochemical properties of the produced VCO

No.	VCO	Initial			After one month		
		Odor	Taste	Color	Odor	Taste	Color
1	VCOm	Typical of coconut oil	Typical of coconut oil	Clear	Typical of coconut oil	Typical of coconut oil	Clear
2	VCOb 10%	Slightly pineapple (+)	Slightly sour (+)	Yellow	Slightly pineapple (+)	Slightly sour (+)	Yellow
3	VCOb 25%	Slightly pineapple (++)	Slightly sour (++)	Yellow	Slightly pineapple (++)	Slightly sour (++)	Yellow
4	VCOb 50%	Slightly pineapple (+++)	Slightly sour (+++)	Deep yellow	Slightly pineapple (+++)	Slightly sour (+++)	Deep yellow

3.2.2. The moisture content of VCO

The amount of moisture content in the VCO will affect the quality of the obtained VCO. The less amount of moisture content in the VCO, the better the quality of the VCO. The results of the moisture content testing for all the produced VCO can be seen in Table 3.

Table 3. The moisture content of the produced VCO

Number	VCO Types	Concentration	Moisture content (%)	SNI 7381:2008
			Mean ± SD	
1	VCOm	0%	0.0044 ± 0.0005	0.2% (maximum)
2	VCOb	10%	0.0503 ± 0.0031	
		25%	0.0130 ± 0.0010	
		50%	0.0262 ± 0.0017	

Based on Table 3, VCO obtained by the mixing method and bromelain enzymatic method has a moisture content of less than 0.2%, which is the quality standard of VCO in SNI 7381:2008. Thus, VCO produced by mixing and bromelain enzymatic methods concurred with the standard given by the SNI. This confirms the findings of Ishak *et al.* [14] that the addition of pineapple extract in VCO production can affect the moisture content of the VCO, where the more addition of pineapple extract, the higher the moisture content obtained. The moisture content strongly influences the quality of VCO. The moisture content has a role in the oxidation and hydrolysis processes which can cause the oil to become rancid. The high moisture content will accelerate the hydrolysis process and cause rancidity in VCO [15].

3.2.3. Free Fatty Acid (FFA) level of VCO

The level of FFA is one of the essential parameters in determining the quality of VCO because FFA is closely related to the level of VCO degradation due to the hydrolysis process [16]. Based on SNI 7381:2008, fatty acids in VCO do not exceed the recommended SNI limit (0.2%). All produced VCO fulfilled the FFA quality standard in this study, as shown in Table 4.

Table 4. Free Fatty Acid (FFA) level of produced VCO

No.	Method	Concentration	Level of Free Fatty Acid (%)	SNI 7381:2008
			Mean ± SD	
1	Mixing	0%	0.0573 ± 0.0021	0.2% (maximum)
2	Bromelain enzymatic	10%	0.0650 ± 0.0044	
		25%	0.0663 ± 0.0015	
		50%	0.0713 ± 0.0012	

According to Anwar and Salima [17], the hydrolysis of VCO is caused by the moisture content, enzymes, and the activity of microorganisms. The high water content in VCO will lead to higher free fatty acids produced due to the hydrolysis process. Moreover, high fatty acids can affect the taste of VCO. In this study, VCO showed high free fatty acids due to the addition of pineapple extract, which contributed to the high water content in VCO. Saturated fatty acids contained in VCO will be hydrolyzed into free fatty acids and glycerol due to the presence of water [14].

3.3. Immunomodulatory activity of VCO

The VCOm and VCOb 50% were evaluated for immunomodulatory activity. These VCO samples were selected based on the yield and characterization results. VCOm exhibited the best characterization results compared to the other three VCOs and could also be used as a negative control (VCO without pineapple waste extract containing bromelain enzyme). The VCOb 50% had the best yield compared to the other three VCOs, and the characterization results showed only a slight difference between VCOb 10% and VCOb 25%. Immunomodulator activity is comprehended through the immune response of VCO to lymphocyte cell proliferation of Swiss-Western strain mice.

The immunomodulatory activity of both samples on lymphocyte cell proliferation was performed using the MTT assay. The principle of the MTT assay is based on the conversion of the tetrazolium salt. Yellow-colored MTT is converted to purple-colored formazan in the mitochondria of metabolically active cells. MTT is broken down through a reduction reaction to formazan salt by the enzyme succinate dehydrogenase present in the active mitochondria [11]. Formazan salt formed was measured using a microplate reader at a wavelength of 550 nm. The color intensity of formazan crystals that are read on a microplate reader is equivalent to the number of proliferating lymphocytes; thereby, the measurement of lymphocyte cell proliferation activity can be determined from the optical density (OD) value [18, 19].

The results (Figure 2) show that the OD value will increase with increasing VCOm concentration, which

indicates that the number of living cells is also increasing. VCOm for each concentration increased the OD value compared to the negative control (without the addition of VCOm). The percentage increase in OD values for concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL against the control were 26.37%, 66.86%, 99.63%, 107.99%, and 137.66%, respectively. The ANOVA one-way test with α = 0.05 obtained F count > F table, which shows differences in VCOm immunomodulatory activity between treatment groups. The results of the student's T- test for all VCOm samples with concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL had significant differences (T count < T table in 95% confidence level).

The bromelain enzymatic method (addition of 50% pineapple waste extract) showed that every increase in VCOb concentration would be followed by an increase in the OD value and the number of living cells. The VCOb for each concentration increased the OD value compared to the negative control (without the addition of VCOb). The percentage increase in OD values for VCOb concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL against controls were 44.56%, 100.07%, 125.42%, 142.02%, and 158.26%, respectively. The analysis using the ANOVA one-way test method with p = 0.05 obtained F count > F table, which shows differences in VCOb immunomodulatory activity between treatment groups. Furthermore, the results of the student's T-test for all VCOb samples at concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL also had significant differences (T count < T table at 95% confidence level).

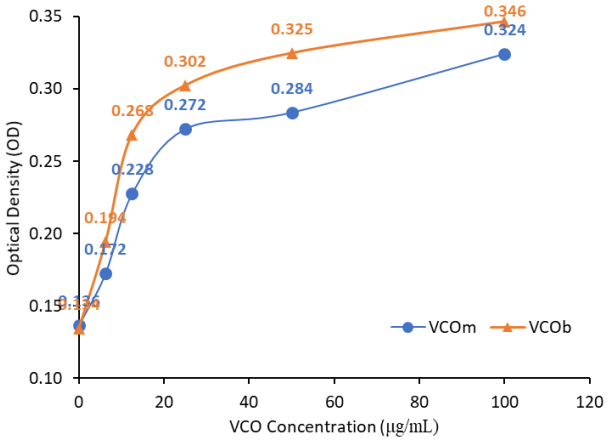

Figure 2. Value of optical density (OD) VCOm and VCOb 50% on mice lymphocyte cell proliferation

Based on Figure 2, VCOb with 100 µg/mL can produce the highest proliferative activity, and the average OD value reaches 0.3465 ± 0.004. This result is nearly the same as the positive control, Phytohemagglutinin (PHA), which can reach an OD value of 0.408 [11]. However, the optimal concentration of VCOm and VCOb was unknown because it was still increasing. However, further research is necessary to determine the effect when the concentration is increased above 100 µg/mL.

This proliferation-increasing activity is probably due to the content of flavonoids, lauric acid, and their derivatives from VCO. Flavonoids can increase the proliferation of lymphocyte cells because they can increase the production of IL-2. IL-2 has a significant role in activating T lymphocyte cells to proliferate. The binding of IL-2 regulates Antigen-stimulated T lymphocyte proliferation to its receptor. In addition, IL-2 also stimulates the proliferation and differentiation of B lymphocytes and Natural Killer (NK) cells [20]. In addition, there are three mechanisms to explain the antiviral activity of lauric acid and monolaurin (which are present in VCO): first, they cause disintegration of the viral envelope; second, they can inhibit the final maturation stage in the viral replication cycle; and the last, they can prevent the binding of viral proteins to the host cell membrane [1]. The presence of bromelain enzymes contained in pineapple waste extract can increase the levels of FFA (lauric acid content) formed in VCO. Therefore, the immunomodulatory activity of VCOb was higher than that of VCOm, which could be seen from the increased proliferation of mouse lymphocytes. However, further research is still needed on using direct bromelain extract to support this study's results.

4. Conclusion

VCO produced by the mixing method and bromelain enzymatic method (addition of pineapple waste extract) can increase the proliferation of mice lymphocyte cells in vitro. Statistical analysis showed that the VCOm and VCOb of 6.25, 12.5, 25, 50, and 100 µg/mL showed significant differences (p < 0.05), thus, all concentration groups possess immunomodulatory activity. The immunomodulatory activity of VCO with bromelain enzyme by adding pineapple waste extract was higher than VCO without bromelain enzyme.

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