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Immunomodulator activity of nanosuspension of cocoa (*Theobroma cacao* L.) leaf extract in Balb/c mice

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ABSTRACT: This study intends to examine the immunomodulatory effects of cocoa (*Theobroma cacao* L.) leaves in vivo in mice using two types of comparison between the crude extract form and the nanosuspension form. Cocoa leaf extract is made from young leaves (YL) and old leaves (OL), where the extraction of active compounds from cocoa leaves was carried out by maceration using 96% ethanol. YL and OL in crude extract and nanosuspensions were tested for their immunomodulatory effects *in vivo* in BALB/c mice through tests against nonspecific and specific immune responses. Tests for nonspecific immune responses include carbon clearance tests, organ index determination, and testing against specific immune responses, including measurement of antibody titers as humoral immune responses and cytokine levels as a cellular immune response. Results showed that the three doses of OL and YL extract (*i.e.*, 50,100,200 mg/kg BW), both in crude form extract and nanosuspensions, had immunosuppressive effects characterized by phagocytic index <1. YL and OL have the same immunosuppressive effect. Extract in nanosuspensions show a more substantial immunosuppressive effect than crude extract. In testing against specific immune responses, all three doses of extract in both crude form extract and nanosuspension inhibited antibody, IFN-γ, and IL-2 formation. Based on those results, it can be concluded that all three doses of YL/OL in both crude and nanosuspension forms are immunosuppressive.

KEYWORDS: Cocoa leaf extract; young leaves; old leaves; nanosuspension; immunomodulator activity.

1. INTRODUCTION

The body has a system that protects it from foreign objects in the environment called the immune system. The name immunity describes the body's resistance against outside organisms [bacteria, viruses, parasites] or abnormal cells that are potentially harmful [1][2].

Disorders in the immune system can also lead to the detection of some health problems [3]. Many factors, such as unhealthy diet and lifestyle, can weaken the immune system apart from infection with microorganisms. This, of course, requires agents that can increase the body's immunity. Conversely, Agents inhibiting the immune system are necessary for disorders like autoimmune diseases [4]. Generally, various attempts are made to treat the two conditions above, one of which is immunomodulators.

Substances or compounds known as immunomodulators can alter or affect the immune system [5]. Because of these actions, immunomodulators are commonly classified as immunosuppressants and immunostimulants. Immunosuppressants are chemicals or agents that inhibit the immunological response, whereas immunostimulants can increase or excite the immune system [6].

Many immunomodulators have been created recently, one of which uses natural substances. Many plant sources can be used as sources of immunomodulators [7]. Apart from the beans, the cocoa plant is still rarely used. According to the Indonesian Directorate General of Plantations, in 2020, the area of cocoa plantations reached 1.6 million hectares, with production of 739,483 tons per year. The biggest cocoa producers are on the islands of Sulawesi and Sumatra.

Cocoa contains various phenolic compounds that can modulate the immune system [8,9], with its polyphenol content, cocoa has an impact on human health [10,11]. In several studies, it has been stated that cocoa leaves contain higher polyphenols than green tea leaves as a comparison [12]. It also has good

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antioxidant activity [13–16], and cocoa beans also have good antioxidant and anti-inflammatory activity [17,18]. In other research, it was also found that cocoa pod skin has the potential to be a drug candidate for the management of wound healing, great antioxidant activity, minimizing hyperalgesia, and other in vivo assays [19–21]. Therefore, research must be conducted to see whether cocoa leaves can modulate the immune system like the seeds or rinds.

Nanosuspension is part of nanotechnology that is used to overcome problems such as low bioavailability caused by low solubility, permeability, and stability of drugs [22–24]. In this research, cocoa leaf extract has low solubility. Therefore, a nanosuspension form was made. Nanosuspension was also carried out to see whether there were differences in immunomodulatory activity in mice in vivo.

Based on the conclusion that cocoa leaves are a plant that has quite a large area of land in Indonesia, while the use is mostly only for the seeds, it is very open if use is carried out on other parts of the plant. There is also quite a lot of data regarding previous research which can support that the cocoa plant has potential as an immunomodulator, while this has not yet been done on the leaves. So this research will focus on the part of the leaf that is tested as an immunomodulator, and expand it to parts of young leaves and old leaves, and make it in the form of a nanosuspension to overcome the problems that exist in cocoa leaf extract which is still difficult to dissolve in water.

2. RESULTS

2.1 Plant Characterization

The characterization of extract and powder from cocoa leaf are shown in Table 1 and the results of phytochemical screening are shown in Table 2.

Table 1. Quality parameters of powder and cocoa leaf extract

Test parameters	YL powder	OL powder	YLE	OLE
Extract yield (%)	-	-	16.2	14.9
Water content (% b/v)	8	9	15.0	17.5
Water soluble essence content (% b/b)	5.85	5.16	42.50	46.77
Ethanol soluble essence content (% b/b)	7.05	7.64	30.62	33.73
Total Ash Content (%)	6.24	7.25	2.51	1.54

YLE= Young Leaves Extract, OLE= Old Leaves Extract

Table 2. Phytochemical screening of powder from cocoa leaf

Compound Class	YL	OL
Alkaloids	+	+
Saponin	-	-
Flavonoids	+	+
Phenolic	+	+
Tannin	+	+
Steroids	+	+
Terpenoids	-	-
Quinone	+	+

+ = The class of test compounds detected
- = The class of test compounds not detected

2.2 Making Nanosuspensions

The optimization results for making nanosuspensions from cocoa leaf extract can be seen in table 3.

Table 3. Optimization of the effect of sonication duration on particle size

Ingredient	Duration of Homogenization (minute)	Duration of Reduction (minute)	Cycle	Amplitude	Particle size (nm)[a]	Polydisperse index
YLE	10	10	15:15	60	611.9	0.261
	10	15	10:10	65	533.2	0.331
	15	15	15:10	70	283.8	0.385
OLE	10	15	10:10	65	358.2	0.320
	15	15	15:10	70	256.9	0.416

YLE= Young Leaves Extract, OLE= Old Leaves Extract, nm: nanometer

Based on the data, a reduction duration of 15 minutes with an amplitude of 70 can produce a suitable particle size and polydispersion index (good polydispersion index <0.5).

2.3 Non-Specific Immune Response

Carbon clearance testing was done as an initial step to observe the immunomodulatory potential of the test extract with the principle of testing to find the phagocytic rate of the reticuloendothelial system in vivo by inducing carbon ink suspension as an outside substance and then the absorption of carbon particles in the blood would be read at λ675 nm.

The parameters used in testing the immunomodulatory effect are elimination rate (k), corrected phagocytic index (α), and half-life (t1/2). The rate of phagocytosis is determined using the elimination rate. Figure 1 shows the carbon elimination speed curve in the crude extract, nanosuspension, levamisole, methylprednisolone, and control groups.

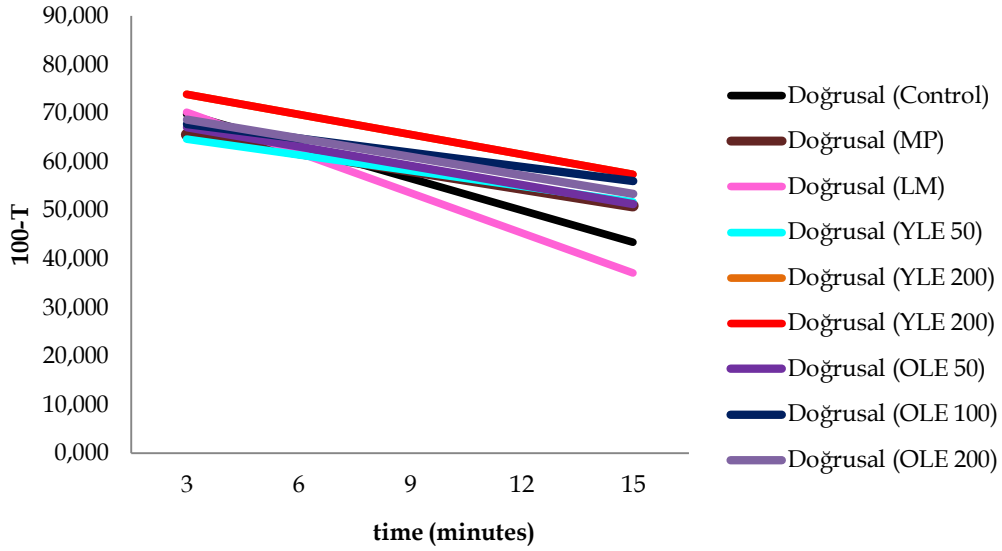

Figure 1. Carbon elimination rate based on linear regression
YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone, LM= Levamisole

Test preparation is considered immunosuppressing if its phagocytic index value is less than 1, moderately immunostimulating between 1 and 1.5, and strongly immunostimulating if it is more significant than 1.5 [25,26].

Based on the results from Tables 4 and 5, it can be seen that both the YL and OL parts of cocoa have activity as immunosuppressants. The crude extract and nanosuspension forms have immunosuppressant activity compared with the respective normal controls. In NOLE, the 100 mg/kg BW dose was statistically significantly different ($p<0.05$).

Table 4. Phagocytic activity of mice after administration of the test preparation

Groups	Dose (mg/kg BW)[c]	$K_{(el)}$	Groups	$K_{(el)}$
Control		0.029±0.007	**BN**	0.019±0.012
MP	40	0.014±0.005		
Levamisole	2.5	0.041±0.008		
YLE	50	0.016±0.004	**NYLE**	0.003±0.015[b]
	100	0.016±0.008		0.007±0.013
	200	0.019±0.007		0.002±0.013[a]
OLE	50	0.016±0.006	**NOLE**	0.006±0.013[b]
	100	0.013±0.003		0.003±0.016[b]
	200	0.019±0.008		0.010±0.006[b]

YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone, BN = Base of nano (sodium lauril sulfat)
a = ($p<0.01$), b = ($p<0.05$) compared to controls, c= milligrams per kilogram of mice body weight

Table 5. Phagocytic index (IF) of mice after administration of the test preparation

Groups	Dose (mg/kg BW)[a]	IF	Classification of Effects	Group	IF	Classification of Effects
Control		1.00	-	BN	1.00	-
MP	40	0.51	Immunosuppressant			
Levamisole		1.26	Immunostimulant			
YLE	50	0.46	Immunosuppressant	NYLE	0.16	Immunosuppressant
	100	0.52	Immunosuppressant		0.37	Immunosuppressant
	200	0.63	Immunosuppressant		0.11	Immunosuppressant
OLE	50	0.53	Immunosuppressant	NOLE	0.32	Immunosuppressant
	100	0.48	Immunosuppressant		0.15	Immunosuppressant
	200	0.67	Immunosuppressant		0.55	Immunosuppressant

YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone,
BN = Base of nano (sodium lauril sulfat), a= milligrams per kilogram of mice body weight

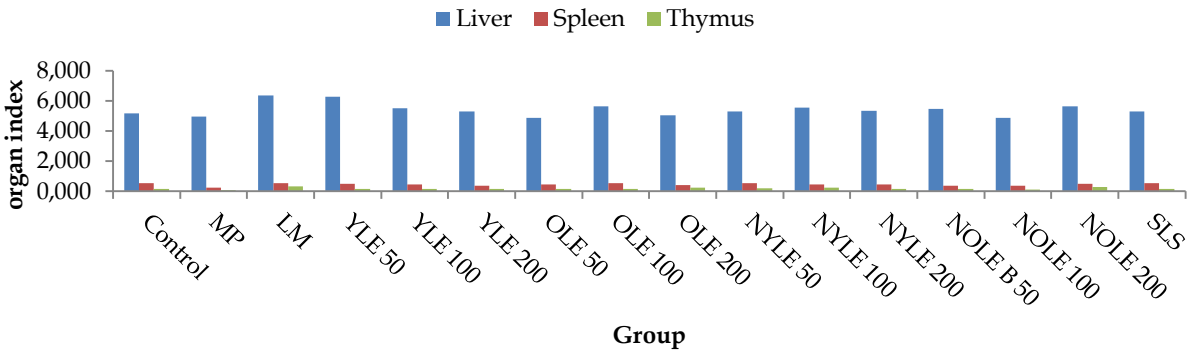

Figure 2. Mice organ index
YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone,

Based on the organ index values in Figure 2, levamisole in the liver and thymus showed a significant increase compared to controls ($p<0.05$). Meanwhile, YLE at a dose of 200 mg/kg in the spleen showed a considerable decrease compared to controls ($p<0.05$).

2.4 Specific Immune Response

Specific immune response was tested to determine the antibody titer value using the hemagglutination method. Determination of antibody titers shows the activity of specific immune responses, especially humoral antibody immune responses to antigens in the form of sheep red blood cells. The antibody titer values of the samples can be seen in Table 6 below.

Table 6. Antibody titers

Groups	Dose (mg/kg BW)[a]	Antibody Titers Primary	Secondary	**Groups**	Antibody Titers Primary	Secondary
Control	-	1:1024	1:256	**BN**	1:512	1:256
MP	40	1:128	1:64			
Levamisole	2,5	1:1024	1:1024			
YLE	50	1:512	1:128	**NYLE**	1:128	1:64
	100	1:256	1:128		1:64	1:64
	200	1:256	1:128		1:64	1:32
OLE	50	1:256	1:256	**NOLE**	1:128	1:64
	100	1:128	1:256		1:128	1:64
	200	1:128	1:128		1:64	1:32

YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone,
BN = Base of nano (sodium lauril sulfat), a= milligrams per kilogram of mice body weight

Measurement of IFN-γ levels using a standard absorbance value curve obtained y = 0.0018x + 0.1682 and R^2 value = 0.9902. So, the levels of IFN-γ as follows are shown in Table 7.

Table 7. IFN-γ Levels

Groups	Dose (mg/kg BW)[c]	Levels (pg/mL)[d]	Groups	Levels (pg/mL)
Control		79.63 ± 13.97	**BN**	24.52 ± 6.27
MP		27.98 ± 14.05		
YLE	50	25.76 ± 9.06[a]	**NYLE**	22.98 ± 6.05
	100	6,98 ± 3.97[a]		17.19 ± 9.75
	200	29.94 ± 5.8[a]		8.80 ± 0.45[b]
OLE	50	40.65 ± 9.76[a]	**NOLE**	22.00 ± 6.22[b]
	100	13.02 ± 7.04[a]		30.28 ± 1.50
	200	12.33 ± 3.24[a]		22.0 ± 6.22

YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone,
BN = Base of nano (sodium lauril sulfat)
a = (p<0,05), b = (p<0,01) compared to controls, c= milligrams per kilogram of mice body weight, d= picograms per milliliter

3. DISCUSSION

Cocoa samples belong to the Sterculiaceae family and are of the *Theobroma cacao* L. type. In this case, powder is from cocoa leaves that have been dried and powdered. At the same time, the extract is a powder of cocoa leaves made by extracting it based on the appropriate method, in this case, maceration. The extract was made from 2 types of leaves [13], YL and OL. Young leaves and old leaves were used in this study to see whether there were differences in providing immunomodulatory activity, while based on previous research showed that differences in young leaves and shoots had quite different levels of polyphenols [13], and differences in leaf parts also showed differences in the metabolism of some metabolite [27]. The results of phytochemical Screening of powder from cacao leaf show that in both YL and OL, there is no difference in phytochemical content, such as alkaloids, phenolic flavonoids, tannins, steroids, and quinones. Other studies have variations in results depending on the extraction method, solvent used, and plant part [28].

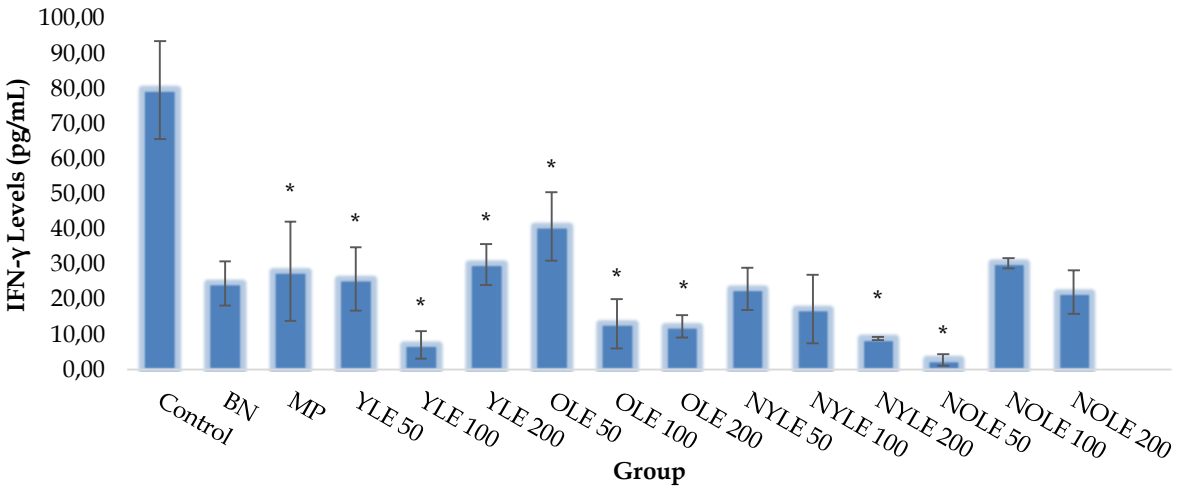

Figure 3. IFN-γ Levels
YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone,
BN = Base of nano (sodium lauril sulfat), * = (p<0,05), (p<0,01) for NOLE 50

Measurement of IL-2 levels using a standard curve obtained y = 0.0059x + 0.1207 and R^2 = 0.9936. So, the IL-levels are as follows in Table 8.

Table 8. IL-2 Levels

Groups	Dose (mg/kg BW)[b]	Levels (pg/mL)[c]	Groups	Levels (pg/mL)
Control		14.887 ± 7.9	**BN**	11.853± 3.22
MP	40	9.988 ± 1.55[a]		
YLE	50	8.351 ± 0.77[a]	**NYLE**	7.367 ± 2.63
	100	8.436 ± 1.74[a]		7.316 ± 2.3
	200	7.83 ± 0.52[a]		10.288 ± 0.9
OLE	50	8.288 ± 0.7[a]	**NOLE**	4.559 ± 2.08[a]
	100	10.305 ± 2.22		7.92 ± 2.55
	200	8.655 ± 2.08[a]		6.02 ± 1.81[a]

YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone,
BN = Base of nano (sodium lauril sulfat)
a = ($p<0.05$) compared to controls, b= milligrams per kilogram of mice body weight, c= picograms per milliliter

Characterization of the extract, where it found that the percent water-soluble essence content was greater than the percent ethanol-soluble essence content. Then, the determination of the total ash content was carried out to provide an overview of the internal and external mineral content originating from the initial process until the formation of the powder from the cocoa leaf. Based on the test results, the total ash content in the powder was no more than 5.5% [29].

Based on the nonspecific immunomodulatory test results, the elimination rate of Levamisole was higher than that of the control and extract group, with a Phagocytic index number of 1.26. It can concluded that Levamisole has an immunostimulant effect. Meanwhile, methylprednisolone as an immunosuppressant control has a line slope that is smaller than the control, so methylprednisolone has an effect as an

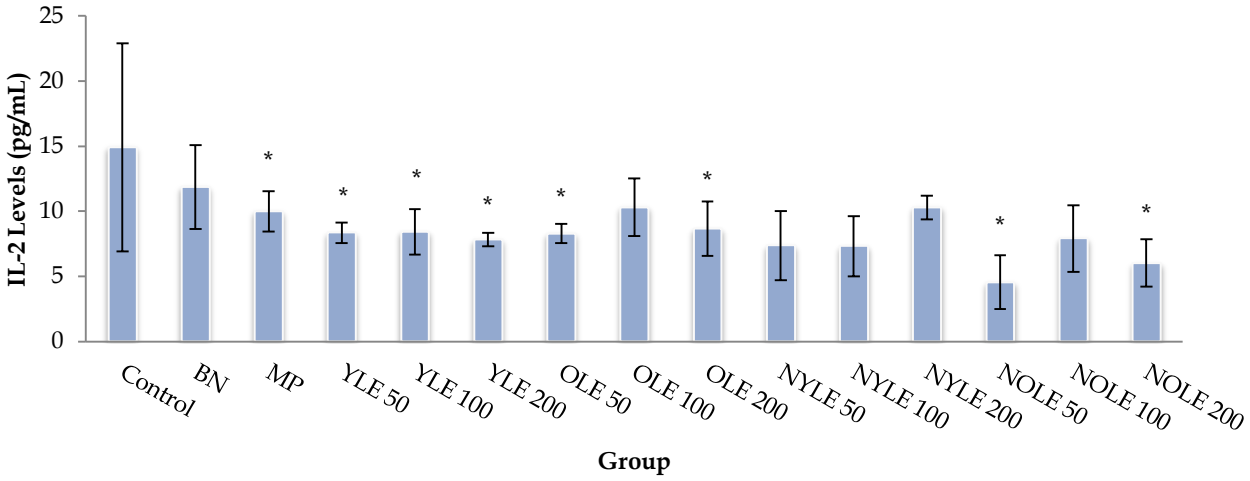

Figure 4. IL-2 Levels
YLE= Young Leaves Extract, OLE= Old Leaves Extract, MP = Methyl prednisolone
NYLE/NOLE= nanosuspension of young/old cocoa leaf extract, MP= methyl prednisolone,
BN = Base of nano (sodium lauril sulfat), * = ($p<0.05$)

immunosuppressant with a phagocytic index of 0.51. Then, the extract showed an immunosuppressant effect with an elimination rate that was smaller than that of the control. Both YL and OL cocoa are active immunosuppressants. The crude extract and nanosuspension forms have immunosuppressant activity compared with the respective normal controls. In NOLE, the 100 mg/kg bw dose was statistically significantly different ($p<0.05$).

Organ Index assessment is carried out as an additional parameter for the effects caused by extract on organs that play a direct role in the immune system. Kupffer cells found in the liver are crucial for controlling cell proliferation and differentiation [30]. The spleen, comprised of T, B, and dendritic cells, is the primary filter for infections and antigens [31], and the organ in which T lymphocytes mature is the thymus.

Thus, the phagocytic index is calculated using the liver, spleen, and thymus as factors. A rise in their respective weights may indicate an increase in immune cell proliferation in the liver and spleen.

The specific immune response is slower because it requires sensitization by the antigen but has better protection against the same antigen. B lymphocytes and T lymphocytes, which originate from lymphoid progenitor cells, play this immune system. In the specific immunomodulator test results, the overall antibody value shows that both crude extract and nanosuspension have lower titer values when compared to the control. Then, as the dose increases, the titer value becomes lower. The higher the titer number, the greater the specific humoral immune response in mice, and vice versa. Then, YL and OL also showed lower titer numbers than normal controls. Low titer numbers correlate with fewer antibodies when compared with normal controls.

In immunological reactions, many substances in the form of hormones and growth factors are released by T and B lymphocytes and other cells, which function as intercellular signals that regulate cell activity, these substances are known as cytokines [32]. IFN-γ and IL-2 are types of cytokines secreted by immune cells that can induce pro-inflammatory and anti-inflammatory responses [33,34].

Based on the data obtained on IFN-γ levels, it can be seen that the levels of Methylprednisolone as an immunosuppressant are much lower compared to normal controls. Likewise, YL and OL had lower levels than normal controls. When compared with nanosuspension, it shows lower rates as an immunosuppressant. NYLE 200 mg/kg bw and NOLE 50 mg/kg bw showed significantly different statistical results ($p<0.01$).

Based on the IL-2 level data obtained, it can be seen that the comparison immunosuppressant methylprednisolone has much lower levels when compared to normal controls. YLE and OLE also showed lower results than normal controls. The nanosuspension form also showed lower IL-2 levels when compared to the BN control and crude extract form. The 50 mg/kg bw dose in NOLE showed a significant difference ($p<0.05$).

The nanosuspension of cocoa leaf extract in this study showed quite good potential as an immunomodulator, specifically as an immunosuppressant. In the results of testing both specific and nonspecific immune responses, both gave promising results when compared with negative controls. Suppose we are talking about a comparison with a crude extract at a dose that is not much different. In that case, a deeper study is needed, as well as regarding the efficiency of making the nanosuspension with various methods, as well as additional tests related to the bioavailability and stability in biological systems.

4. CONCLUSION

Based on the data from this research, the cocoa plants in the OL and YL sections have the potential to be candidates for immunosuppressant substances in autoimmune conditions. Likewise, the form of cocoa leaf nanosuspension affects the immunosuppressant activity, which is not very different from the form of the extract. Tests are needed to determine various methods of nanosuspensions and their bioavailability. Conducting studies and tests to obtain specific compounds that act as immunomodulators and apply tests on autoimmune animal models is necessary.

5. MATERIALS AND METHODS

5.1 Study design

This research was conducted on a crude extract form of cocoa leaves, tested for its in vivo activity as an immunomodulator. Non-specific immunomodulatory testing showed a phagocytic index <1. Meanwhile, specific immunomodulator tests showed lower levels of IFN-γ and IL-2 than controls. These results were also shown in the form of cocoa leaf extract nanosuspension. Therefore, cocoa leaves, in this case, ethanol extract, are likely to be used as a candidate for treatment or maintenance in autoimmune conditions.

5.2 Ethical consideration

Ethical Approval: the research was approved by Komite Etik Penelitian Universitas Padjajaran, Bandung, Indonesia. (Nomor: 1135/UN6.KEP/EC/2018)

5.3 Preparation of Test Plants

Cocoa leaves were obtained from Kasomalang Kulon village, Kasomalang subdistrict, Subang district, West Java. Plant determination was carried out at the Badungense Herbarium, School of Biological Sciences and Technology, ITB. Thus, Cocoa leaves are then divided into two parts, namely, YL (leaves 1-4

from the shoot) and OL leaves (leaves 5-8). The cocoa leaves are washed, then sorted wet, chopped into small pieces, dried, sorted dry, and ground to form powder.

5.4 Preparation of Test Extract

Powder from the cocoa leaf was extracted by maceration with 96% ethanol solvent. Maceration was carried out for 24 hours, and stirring was carried out every hour for the first 6 hours. The results were concentrated using a rotary evaporator until a thick extract was obtained. Cocoa Leaf Extract is divided into 2, namely young leaf extract (YLE) and old leaf extract (OLE).

5.5 Characterization and Quality Check of Extract and Powder Tests

The extract and powder obtained were then characterized by determining water content, water-soluble essence content, ethanol-soluble essence content, total ash content, and specific gravity.

5.6 Phytochemical Screening

Phytochemical screening was carried out on the extract obtained, including determining the groups of alkaloids, flavonoids, phenolics, saponins, tannins, quinones, steroids, and triterpenoids.

5.7 Making Nanosuspensions

Cocoa leaf extract was weighed according to the dose, as surfactant SLS 0.5% w/v was used. The suspension was then homogenized using a water bath sonicator, and the particle size was reduced using a probe sonicator. Particle size evaluation was carried out using PSA until a particle size of 200-300 nm was obtained.

5.8 Nonspecific Immune Response Test

5.8.1. Carbon Clearance Test

Phagocytic and organ index assessments are used to evaluate the immunomodulatory potential of cocoa leaf extract in nonspecific immune response tests. Sixteen treatment groups, consisting of the regular group, Levamisole 2.5 mg/kg body weight group, and Methylprednisolone 40 mg/kg body weight group, were randomly assigned to BALB/c female mice, test extract group with three dose levels. Namely 50, 100, and 200 mg/kg body weight, respectively, for YL and OL, extract nanosuspension group with three graded doses each.

Test material was provided to grouped mice for seven days. On the 8th day, mice were treated intravenously with carbon (pelican B17 black ink). Blood is taken via the tail vein (T0). After that, blood was taken from the mice at intervals of 3, 6, 9, 12, and 15 minutes. A spectrophotometer was used to determine the transmittance and absorbance values at a wavelength of 675 nm after mixing 20 µl of blood with 2 ml of 1% acetic acid.

5.8.2. Organ Index

After taking blood in the 15th minute, they were then sacrificed, and surgery was carried out to remove the liver, spleen, and thymus to observe the organ index with the following equation: $\text{Organ Index}(\%) = \frac{\text{Organ (g)}}{\text{Body Weight (g)}} \times 100\%$

5.9 Specific Immune Response Assay

Mice that have been grouped are immunized with 1% sheep red blood cells (SRBC) as much as 0.1 ml/10 g body weight intravenously (day 0). Mice were fed the test material orally for 12 days. On the 5th day after immunization, blood was taken via the tail vein and centrifuged to obtain serum. Antibody titers were determined by hemagglutination using a V-bottom microwell plate consisting of 96 wells divided into 12 rows and eight columns. 50 µL of 0.15 M phosphate buffer pH 7.2 was filled into each well. The test serum was diluted 1:2 as a stock solution. The stock solution was placed in the first row and diluted 1:2 until the 12th. 25 µL of SRBC suspension was added and incubated for 24 hours at 37 °C. The highest dilution value that provides hemagglutination is expressed as the primary antibody titer. Blood was taken on the 12th day, and the antibody titer was determined again using the hemagglutination technique. This hemagglutination value is expressed as a secondary antibody titer. Then, the serum collected on the last day was tested using an ELISA reader to determine the levels of IFN-γ and IL-2.

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Conflict of interest statement: We declare that we have no conflict interest

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