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Optimization Formula of Functional Drinks Mixture of *Garcinia xanthochymus*, *Zingiber officinale* and *Curcuma domestica* as Antioxidant and Anti-inflammatory in Mice (Mus musculus)

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***Abstract*—** *The purpose of this study was to obtain an optimal formulation of functional drinks from the mixture of G. xanthochymus, red ginger, and turmeric as well as to see the effectiveness of antioxidants and antiinflammatory functional drinks in vitro. This study consists of 2 stages. The first stage of making functional drinks, to obtain the optimal formulation of functional drinks using the students Response Surface Methodology method with the design of Box-Behnken Design. The second stage was to examine the effect of functional beverage products of G. xanthochymus, red ginger and turmeric mixture as antioxidants and anti-inflammatory in vivo, using male mice (Mus musculus) which were induced by 1% carrageenan by subplantar. optimization of functional beverage formulas can be obtained G. xanthochymus 59.81%, red ginger 9.92%, and turmeric 30.41%. Antioxidant activity ranged from 103.50-175.13 ppm, while the total phenol value ranged from 16.8006 -33.0012 mgGAE /g. The effectiveness of the functional beverage mixture of G. xanthochymus , red and turmeric ginger on the expression of TNFα cytokines decreased, IL-6 decreased and IL-10 increased so that the inflammation that occurred in mice after carrageenan injections decreased significantly and SOD increased significantly.*

***Keywords*—** *Anti-inflammatory, Antioxidants, Functional Drinks, RSM.*

I. INTRODUCTION

Inflammatory is the body's normal protective response to injury or tissue injury caused by physical trauma, hazardous chemicals and microbiological agents (1). Inflammation, when left without treatment, will result in autoimmune or autoinflammatory disorders, neurodegenerative diseases or cancer. Antiinflammatory is available in various kinds, including aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), and various kinds of anti-inflammatory drugs which are still being developed, one of which is traditional medicine that uses local plants. Medicines that come from plants are of minimal risk and do not disturb the defense and immune system (2).

Indonesian has the second largest wealth of biological resources after Brazil with more than 28,000 plant species. However, only about 1,000 plant species are registered with BPOM that have been used to produce functional food, especially for herbal medicine. Functional drinks can be used as probiotic drinks so they can reduce the risk of disease in the body. This abundant natural resource is one of Indonesia's comparative advantages, especially for developing functional food products (3).

Candis acid (Garcinia xanthochymus) is a plant from the Clusiaceae family originating from India. In Indonesia, it is often found in Sumatra and Kalimantan as the main cooking spices (4). People still do not know the benefits of candis acid in terms of health so that the existence of this plant is considered normal. Candis acid contains active compounds such as xanton and flavonoids so that it can produce varied biological and pharmacological activities such as cytotoxic, anti-inflammatory, antimicrobial, antifungal and antioxidants (5).

Turmeric rhizome (Curcuma domestica) contains several chemical components, including essential oils, starch, bitter substances, resins, proteins, cellulose, and several other minerals. Turmeric contains a high phenolic component and acts as an antioxidant. According to Jurenka (6) turmeric has an active substance called curcuminoid. Phenolic compounds in curcumin can act as anti-cancer compounds.

The Ginger rhizome (Zingiber officinale) contains starch, essential oil, fiber, a small amount of protein, vitamins, minerals, and proteolytic enzymes, ginger also contains phenolic compounds. Some bioactive components in ginger extract include (6) -gingerol, (6) -shogaol, diarylheptanoid. Red Ginger Extract contains 3-7% of phenolic compounds such as flavonoids and alkaloids. Ginger is known to have antioxidant activity that will help neutralize free radicals (7).

Antioxidants work by donating one electron to an oxidant compound so that the activity of the oxidant compound can be inhibited (8). Various possibilities can occur due to the work of free radicals, such as cell function disorders, damage to cell structures and modified molecules that cannot be recognized by the immune system (9).

Superoxide Dismutase (SOD) is an enzymatic antioxidant which is classified as a primary antioxidant that comes from the body (intracellular) and can work by preventing freezing of new free radicals and is the most powerful natural antioxidant enzyme that becomes a defense for cells exposed to oxygen to prevent cell damage due to free radicals (10). The

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renewal of this study resulted in functional drinks of Candis acid, red ginger, and turmeric as antioxidants and anti-inflammatory using the RSM method of BBD design. The inflammatory response in the body can be observed through cytokines that play an important role in inflammation, where *tumor necrosis factor* (TNF-α) and *Interleukin* (IL) -6 are proinflammatory while the cytokines *Interleukin* (IL) -10 are anti-inflammatory. So that the use of traditional medicine is one alternative that can be used in inflammatory treatment which is considered safer in terms of side effects and toxicity (11).

II. Materials and Research Methods

A. Location and Time

This research was conducted at the Laboratory of Food Chemistry and Biochemistry, Food Nutrition Laboratory, Faculty of Agricultural Technology, Biomolecular Laboratory of FMIPA, Brawijaya University, and Pharmacology Laboratory of the University of Muhammadiyah Malang, from October 2018 to December 2018.

B. Research Materials

The materials used in this study included powder from candis acid (obtained from the village of Sayut, Kapuas Hulu,), powder from red ginger, and powder from turmeric. Chemicals used: focal ciocalteau, gallic acid, Na2CO3 solution, 1,1-diphenyl-2pycril hidrazil (DPPH) reagent. In vivo test materials: male / Mus musculus mice weighing 20-33 grams, carrageenan, diclofenac Na, FCS in PBS 2%, antibodies (TNFα, IL-6, IL-10) and SOD. The tools used include the infusion pan, spectrophotometer (Unico UV-210), plestimometer, and Calibur FACS Calibur flow cytometer.

C. Research Methods

This study consisted of two stages, namely: The first stage of making functional drinks, to obtain the optimal functional beverage formulation using the Response Surface Methodology (RSM) method with Box-Behnken Design design. The second stage examines the effect of functional beverage products as an antioxidant and anti-inflammatory in vivo.

1. Research Procedure

Stage I: physicochemical preparation and testing Functional drinks of candis acid mixture, red ginger, and turmeric. Preparation of the main raw materials for beverage formulations (candis acid, red ginger, and turmeric).

The research design in this experiment uses RSM (Response Surface Method) with the help of Design Expert 7.0 software BBD (Box-Behnken Design) method to obtain modeling that can explain the interaction between the variable candis acid ratio, red ginger and turmeric to the response in this study namely total phenol and total antioxidant or IC50. Research variables and responses in the modeling and optimization of RSM (Response Surface Method). presented in Table 2.1.

TABLE 2.1. Research variables and responses in RSM modeling and optimization

Research variable	Desired response
Percentage of candis acid	Antioxidant Activity
Percentage of red ginger	Total Phenol
Percentage of turmeric	

The initial formulation of the drink was determined using a minimum and maximum reference ingredient from candis acid, red ginger and turmeric which may be added to the drink based on antioxidant activity and phenol content of each raw material, lower 50% candic acid limit, 5% red ginger and turmeric 20% while the upper limit of candis acid is 70%, red ginger is 15% and turmeric is 40%.

Stage II: Testing the anti-inflammatory activity of functional drinks in a mixture of candis, red ginger and turmeric mixtures in vivo.

Research design :

The research was designed using True Experimental Design: Post Test Only Control Group Design. The selection of objects using a completely randomized design (ANOVA) with 5 treatment groups, as follows:

1. Negative control (P1): Mus musculus is healthy
2. Positive control (P2): Mus musculus healthy and carrageenan injection 1% at a dose of 0.05 ml/kg BB at the bottom of the left foot (subplantar)
3. Drug control (P3): Mus musculus healthy injected with 1% carrageenan at a dose of 0.05 ml/kg BB and the drug diclofenac sodium at a dose of 0.019 mg / 20gr BB
4. Treatment group (P4): Mus musculus healthy and injected carrageenan 1% at a dose of 0.05 ml/kg BB, given functional drinks dose of 0.52 ml / 20 mg BB optimization (dose 1)
5. Treatment group (P5): Mus musculus healthy and injected carrageenan 1% at a dose of 0.05 ml/kg BB, given functional drinks dose of 1.04 mg / 20 gr BB (dose 2).

D. Analysis of Data

Quantitative data analysis uses a variant of the Tukey ANOVA analysis with a confidence level of 95% using MINITAB software to determine the effect of each parameter. Optimization of functional beverage formulations using the Response Surface Methodology (RSM) method, data analysis was performed using the help of the D.X Design Expert trial software 7.5.1 (Stat-Ease Inc., Minneopolis, MN, USA).

III. Results and Discussion

A. Analysis Material

The main ingredients used in making these functional drinks are candis acid powder, red ginger powder, and turmeric powder. Powder analysis was carried out to determine the chemical-physical characteristics of the material which included analysis of water content, total phenol, and antioxidants.

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TABLE 3.1 Characteristics of physicochemical of powder

Parameter	Acid Candis Powder	Red Ginger Powder	Turmeric Powder
Water content (%)	9,14±0,77	8,98±0,57	9,73±0,85
Phenol (mgGAE/g)	17,09±0,52	25,07±0,25	17,33±33,68
IC_{50} (ppm)	121,07±4,81	137,56±11,34	101,71±7,42

Description: Data from the analysis are the average of 3 replications ± standard deviation

B. Formulation and Optimization of Functional Drinks

In this study, the value of the independent variables studied in the optimization of functional beverage formulations using the RSM method with BBD design is presented in Table 3.2.

TABLE 3.2. Nilai Variabel Independen dan Respon Desain RSM

	Variabel Dependen			Variabel Indenpenden	
Std	Acid Candis (%)	Red Ginger (%)	Turmeric (%)	IC_{50} (ppm)	phenol (mgGAE/g)
1	50	5	30	172,33	17.9555
2	70	5	30	162,23	20.2508
3	50	15	30	148,93	17.4838
4	70	15	30	143,83	17.1277
5	50	10	20	159,36	18.1907
6	70	10	20	158,94	16.8006
7	50	10	40	140,24	19.6696
8	70	10	40	168,94	18.7157
9	60	5	20	154,32	18.5945
10	60	15	20	162,11	18.3294
11	60	5	40	147,11	20.5899
12	60	15	40	175,13	20.0051
13	60	10	30	110,38	32.9663
14	60	10	30	103,50	33.0012
15	60	10	30	105,07	32.0513
16	60	10	30	118,61	31.5298
17	60	10	30	118,94	32.7449

Table 3.2. shows the response of antioxidant activity and total phenol obtained from the BBD experimental design. There were 17 experimental designs with 5 replications at the center point. Table 3.2 also shows synergism between compounds in material formulations. Synergism is an effect when extracts from several plants show a greater effect than when the extract consists of only one type of plant.

C. Modeling and Analysis of Antioxidant Responses

The results of the analysis of the response of antioxidant activity indicate the suggested quadratic model. The quadratic model has the lowest p-value and is less than 0.05, which is 0.0022. Model selection can be seen from the adjusted R2 and predicted R2 maximum values (12). In the summary statistical model, the quadratic model has the highest adjusted R2 and predicted R2 values compared to the linear and 2FI models which are 0.6955 and -0.7993 respectively. The equation and ANOVA for the response of antioxidant activity are shown in Table 3.3.

TABLE 3.3. Equations and ANOVA in quadratic models with responses to antioxidant activity

Respon	R^2	Model		*Lack of fit*	
		P-value	Ket	*P-value*	Ket
Activity Antioxidants	0,8668	0,0220	Signifikan	0,0526	Not signifikan

Description: x_1 = acid candis powder , x_2 = red ginger powder , x_3 = turmeric powder.

Based on Table 3.3. The parameters used resulted in a quadratic model that was significant at the 95% level to predict the response of antioxidant activity (p = 0.0220). This model has a value of R2 0.8668 which approaches the value of 1. A large and insignificant lack of fit *P-value* implies that the model used is good enough so that it is expected (12) so that the mathematical model can be used to explain the effect of the independent variables in the experiment and predict the optimum conditions to obtain the expected response value(13). The surface response image for antioxidant activity is shown in Figure 3.1.

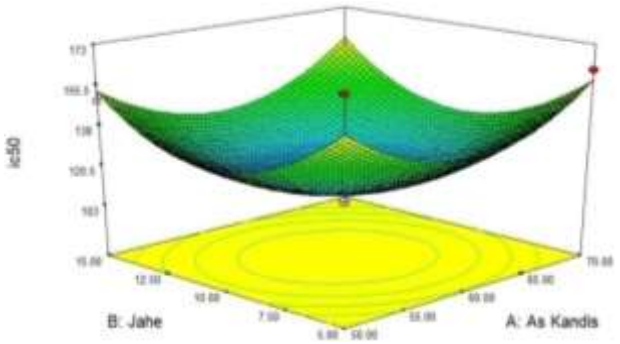

Figure 3.1 Surface response of antioxidant activity

The quadratic equation obtained for the antioxidant activity is :

$Y_1 = +1364,27189 -27,90875X_1 -24,01812X_2 -19,94386X_3 +0,025037X_1X_2 +0,072810X_1X_3 + 0,10115X_2X_3 +0,21365X_1^2 +0,96659X_2^2 +0,24204X_3^2$

Description: x_1 = acid candis powder , x_2 = red ginger powder , x_3 = turmeric powder.

D. Modeling and Analysis of Phenol Response

Analysis of the total phenol response shows the suggested quadratic model. Based on the sum of squares sequential model test, the quadratic model has the lowest p-value and is less than 0.05, which is <0,0001. In the summary statistical model, the quadratic model has the highest adjusted R^2 and predicted R^2 values compared to the linear and 2FI models, which are 0.9838 and 0.9213, respectively. This illustrates the prediction of a small error. The equation and ANOVA for the total phenol response are shown in Table 3.4.

TABLE 3.4. Equations and ANOVA in quadratic models with total phenol response

Respon	R^2	Model		*Lack of fit*	
		P-value	Ket	*P-value*	Ket
Phenol	0,9929	<0,0001	Signifikan	0,1892	Not signifikan

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Description: x_1 = acid candis powder, x_2 = red ginger powder, x_3 = turmeric powder.

Based on Table 3.5, it produces a quadratic model that is significant at the 95% level to predict the total phenol response (p = <0,0001). This model has a value of R^2 0.9929 which is close to the value 1. P-value lack of fit is large and not significant so that it is as expected (12). This model can be used to explain the effects of the independent variables in the experiment and predict the optimum conditions to obtain the expected response value (13). The image of the surface response for total phenol is shown in Figure 3.2.

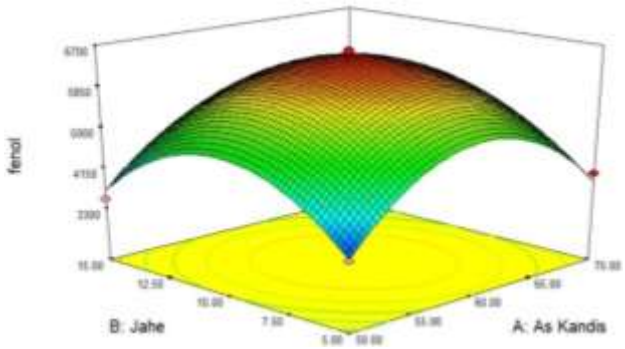

Figure 3.2 Surface response to total phenol

The quadratic equation obtained for the total phenol is :

Y_1 = -67025,57657 + 1853,73840X_1 + 1203,94963 X_2 + 784,13118 X_3 –2,65150 X_1 X_2 + 0,21811 X_1 X_3 – 0,31970 X_2 X_3 – 15,28983 X_1^2 – 52,87462 X_2^2 – 12,93930 X_3^2

Description: x_1 = acid candis powder , x_2 = red ginger powder , x_3 = turmeric powder.

E. Optimization of Total Formulations of Phenols and Antioxidants

This experiment aims to obtain the best input variable that produces the response of antioxidant activity and optimal total phenol in functional beverage formulations. The recommended optimal formulation (prediction on RSM) is confirmed and compared with real testing, can be seen in Table 3.5.

TABLE 3.5 The optimum formula suggested with predicted and observed response values.

Independent Variables			Response Value			
X_1 (%)	X_2 (%)	X_3 (%)	Response	Prediction	Actual	% Error
59,81	9,92	30,41	Antioxidants	111,31	112,44	1,02
			Phenol	32,4894	33,1202	1,94

Based on Table 3.6, the recommended formulation is the proportion of candis acid 59.81%, red ginger 9.92%, and turmeric 30.41%. This formulation produces an optimal prediction of response, namely antioxidant activity (IC50) 111.31 ppm and total phenol value of 32.4894 mgGAE / g with a fairly good desirability value approaching the value 1, which is 0.929 which can be seen in Figure 3.3.

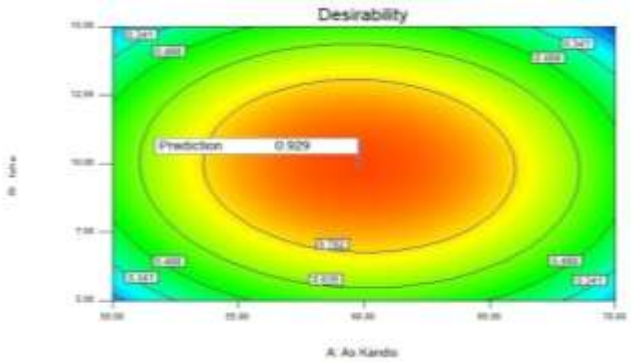

Figure 3.3 Contour graph plot desirability response

F. Organoleptic uses Hedonic Test

In the food industry, consumer satisfaction will be achieved if producers are able to provide the highest quality products (14). One way to get a product with good quality can be done by organoleptic testing through a hedonic test that indicates the choice, acceptance, or acceptance of a product. Test parameters include sensory assessment attributes, namely product appearance attributes, aroma, color and taste (15) using 60 panelists. Control sample "Kiranti Orange" which has been circulating in the market. Organoleptic tests are carried out on a hedonic scale 1-5 (very dislike).

Figure 3.4 Average Panelist Favorite Level based on Hedonic Test.

The results of Friedman's statistical analysis showed that the level of preference of panelists for taste was significantly different (p-value <0.05) while the level of preference of panelists for color, aroma, and appearance was not significantly different due to (p-value> 0.05).

G. Effect of Functional Drinks in In Vivo

Effect of Functional Drinks on *Mus musculus* Induced by Carrageenan. Acute anti-inflammatory effects using the carrageenan induction method (16). Mice induced with carrageenan by sub-plantar injection. After 90 minutes of 1% carrageenan injection with a dose of 0.05 ml/kg BB, the test animals were pressed with functional drinks and Diclofenac Sodium drugs according to the dose of the group treatment. Then the volume of edema was measured per 30 minutes until the 300th minute after carrageenan induction (16). Carrageenan plays a role in edema formation in acute inflammatory models (17). The results of the analysis of the anti-inflammatory activity at the feet of mice with carrageenan-induced edema are shown in **Figure 3.5.**

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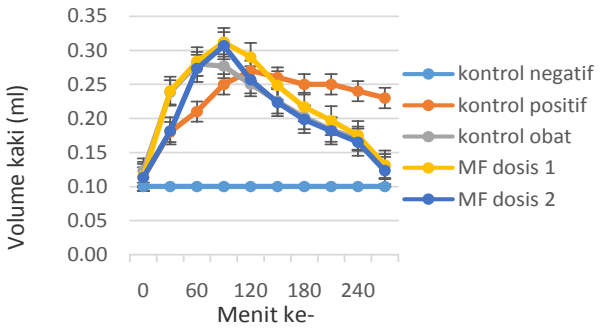

Figure 3.5. Anti-inflammatory activity in carrageenan-induced mice edema feet.

In this study the analysis of edema using a plestimometer. The value of the foot volume is based on time, and the average volume of the foot is taken (18).

TABLE 3.6. Effects of treatment on the percentage of inhibition of edema

No	Treatment	Edema inhibition (%)
1	Negative control	-
2	Positive control	77,58±0,02^{c}
3	Drug control Na. Diklofenak	79,22±0,01^{ab}
4	Treatment group dose 1	77,92±0,01^{bc}
5	Treatment group dose 2	79,77±0,02^{a}

Description: the value is an average ± SD of 6 replications. Fisher's Advanced Test (BNT) 5%

Based on Table 3.6% the highest inhibition of edema was found in the treatment dose 2 drinks but did not differ significantly from the% inhibition of Na edema. Diclofenac. In the positive control group that is not given drugs or functional drinks, inflammation occurs. The mechanism of inflammation begins when mast cells degrade and release chemical ingredients such as histamine, serotonin, and other chemicals. Histamine which is the main chemical mediator of inflammation is also released by basophils and platelets. As a result of this histamine release is vasodilation of blood vessels so that there is an increase in blood flow and an increase in capillary permeability at the beginning of inflammation (19).

In the control group the drug diclofenac sodium, a high enough % inhibition due to its ability to reduce prostaglandin and leukotriene synthesis (20, 21). This is consistent with Tjay and Rahardja (22), that the mechanism of action of non-steroidal drugs is through inhibition of prostaglandin. This is also supported by Priyanto (23), that diclofenac sodium has an analgesic effect at low doses and is anti-inflammatory at large doses.

In the functional drink group dose, 1 was shown to be an anti-inflammatory activity, although not as high as% inhibition of edema by dose 2 functional drinks. This was due to insufficient concentration and amount to inhibit inflammation caused by carrageenan. This is because carrageenan is a strong chemical used to release inflammatory and proinflammatory mediators such as prostaglandin, leukotriene, histamine, bradykinin, TNF-α, and others (24, 25).

Effect of functional drinks on TNF-α, IL-6, and IL-10

One of the inflammatory mediators is cytokines, which are substances released by leukocytes. Pro-inflammatory cytokines play a role in stimulating macrophages to increase phagocytosis and stimulate the bone marrow to increase leukocyte and erythrocyte production. Anti-inflammatory cytokines include interleukin-4 and interleukin-10 which play a role in reducing the secretion of pro-inflammatory cytokines. In addition, there is also chemokine, a type of cytokine, works as a chemotactic agent that regulates the movement of leukocytes (19). The results of the analysis of variance and further testing of TNF-α, IL-6, and IL-10 can be seen in Table 3.7.

TABLE 3.7. Relative values of TNF-α, IL-6, and spleen IL-10 *Mus musculus*

Treatment	% relative		
	TNF-α	IL-6	IL-10
Negative control	21,85±1,12^{e}	16,53±3,44^{e}	35,15±2,09^{d}
Positive control	71,48±1,43^{a}	72,82±3,62^{a}	26,65±3,52^{e}
Drug control Na. Diklofenak	31,14±1,33^{d}	25,10±4,21^{d}	78,49±3,71^{a}
Treatment group dose 1	46,35±0,85^{b}	40,78±0,53^{b}	41,05±1,23^{c}
Treatment group dose 2	36,322±1,24^{c}	31,06±1,26^{c}	46,89±2,00^{b}

Description: the value is an average ± SD of 6 replications. Fisher's Advanced Test (BNT) 5%

Based on Table 3.7, there were significant changes to proinflammatory cytokines and anti-inflammatory cytokines in carrageenan-induced mice and experienced inflammation. The control group positively showed TNF-α cytokines, IL-6. relatively high compared to other groups, while the lowest IL-10 was found in positive controls. This is because the positive control group is not given a drug or drink that has an anti-inflammatory effect, so the cytokine level remains high. In addition, the functional drink group dose 2 had a relative cytokine approaching drug control. This shows that the dose of metabolite and concentration of functional drinks are optimal as an anti-inflammatory. Whereas in functional drinks dose 1, it has the ability to reduce cytokines but not as significant as dose 2 functional drinks.

Positive control groups high in proinflammatory cytokines such as IL-6 and TNF-α TNF-α at low levels can induce acute inflammation, but at high levels of TNF-α can cause septic shock in the heart, blood vessels and liver (26) These cytokines are released by macrophages. Macrophages have the ability to phagocytosis of large numbers of bacteria, viruses, or other foreign particles in tissues (27).

Effect of Functional Drinks on *Superoxide Dismutase* (SOD)

The antioxidant ability to inhibit the formation of free radical compounds is called antioxidant activity. Phenol groups have a role in antioxidant activity, where the higher the content of phenol compounds in a food ingredient, the greater the antioxidant activity (28). The results of the analysis of SOD expression on her *Mus musculus* can be seen in Figure 3.6 below:

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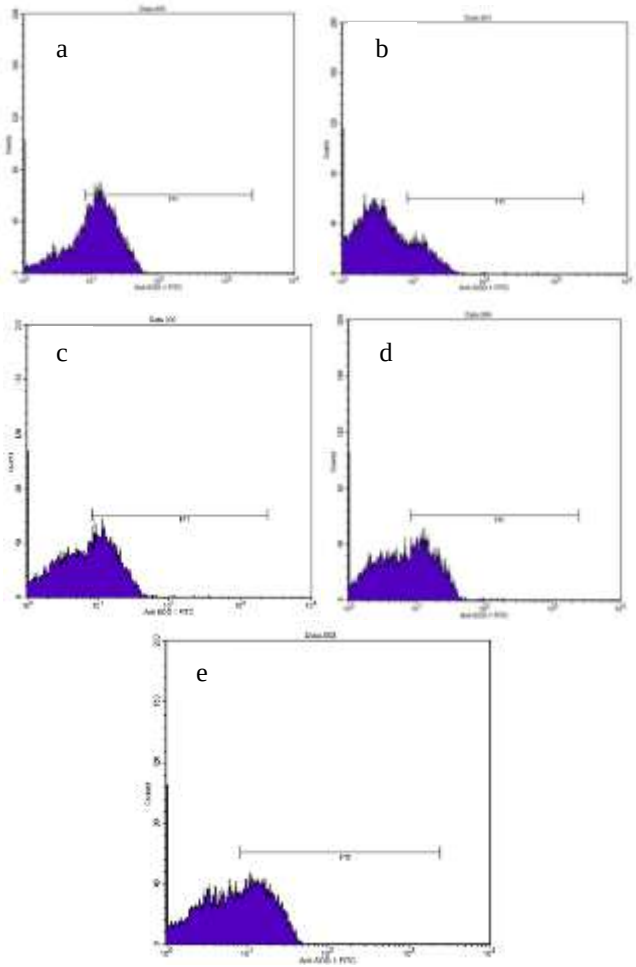

Figure 3.6. Results of SOD analysis in the liver Mus musculus

Description:

- Area M1: liver cells that express SOD
- (a) Negative control, (b) Positive control, (c) Drug control Na. Diklofenak, (d) Treatment group dose 1, (e) Treatment group dose 2

Based on Figure 3.6, the highest% of total SOD was found in the liver of the *Mus musculus* negative control group. While the lowest% of total SOD was found in the liver of positive control group *Mus musculus*. The results of the analysis of variance and further testing of hepatic SOD in mice can be seen in Table 3.8.

TABLE 3.8. % of total liver SOD *Mus musculus.*

No	Perlakuan	% total SOD
1	Negative control	35,82±2,92[a]
2	Positive control	11,48±1,06[e]
3	Drug control Na. Diklofenak	29,10±1,09[b]
4	Treatment group dose 1	19,41±2,04[d]
5	Treatment group dose 2	25,06±1,38[c]

Description: the value is an average ± SD of 6 replications. Fisher's Advanced Test (BNT) 5%

Based on Table 3.8, there was a significant increase in% of total SOD in the positive control group compared to the other groups. While the negative control group is the group with the lowest levels of the cytokine. This is because the positive control group is not given medication or drink which has an anti-inflammatory effect so that the total SOD remains high. In addition, the functional drink group dose 2 has a total% of SOD that approaches drug control.

In the functional beverage group, oxidative stress can be prevented because of the presence of phenols in functional drinks that have antioxidant activity. This is in accordance with the study (29) that curcumin tablets with a dose of 200 mg / kgBB had antioxidant activity so as to reduce liver MDA levels and increase SOD enzyme activity. The mechanism of phenol as an antioxidant is by neutralizing free radicals through electron donors or H atoms, suppressing the rate of oxidation by inhibiting the formation of free radical precursors, being able to act as a scavenger in radical lipid peroxide chain reactions.

IV. CONCLUSION

Based on the results of the study using RSM with BBD design, the optimal formulation of functional drinks was obtained with the composition of candic acid 59.81%, red ginger 9.92%, and turmeric 30.41%. Antioxidant activity ranged from 103.50-175.13 ppm, and total phenol ranged from 16.8006-33.0012 mgGAE / g. The effectiveness of functional beverage formulations on TNFα cytokine expression decreased, IL-6 decreased and IL-10 increased, and SOD increased significantly.

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