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Immunomodulatory Activity Test of Syrup Dosage Form of Combination *Phyllantus niruri* Linn. And *Sterculia quadrifida* R.Br. Extract

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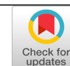
ABSTRACT



Meniran (*Phyllantus niruri* Linn.) is used generally as an immunomodulator and hepatoprotector. Empirically, bark of faloak (*Sterculiaqudrifida* R.Br.) is used as a medicinal plant to cure diseases such as hepatitis, gastroentritis, and diabetes. The combination of meniran extract and bark of faloak extract has the potential as a natural immunomodulatory agents. Immunomodulators are compositions of either recombinant, synthetic, or natural, which can restore the immune system. Therefore it is necessary to optimize the optimal formula in obtaining syrup preparations which have optimal immunomodulatory activity. The aim of this study was to find out the optimal syrup combination of meniran extract and faloak bark extract to produce optimal response values in the in vitro immunomodulatory activity test with TNF-α and NF-κB parameters, and immunomodulatory activity tests in vivo with activity parameters macrophages. The extract was obtained through reflux extraction. The results of the extract were formulated and approved to determine their ability to induce TNF-α and NF-κB, and in the test of mice balb/b (*Mus musculus* L.) orally for 21 days. The lymph organs were isolated, rinsed with an RPMI-1640 solution, and analyzed using ELISA reader 450 nm. The determination of optimal formulas is done by using Rstudio v.3.5.3 software with ANOVA test parametric test and using Design Expert v.11 software using the Simplex Lattice Design (SLD) method. The results showed that the formula for the preparation of combination syrup to produce results induced TNF-α and NF-κB, and macrophage activity, namely the formula using meniran and faloak 0.96993: 0.0300704.

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INTRODUCTION

Traditional medicine used by Indonesian people since has been going on since ancient times, considering that Indonesia is a country rich in the second largest biodiversity in the world after Brazil (Hendarto *et al.*, 2018). Drugs which traditional medicine or chemical drugs used by people can't separable from disease (Rollando and Prilianti, 2018). The disease is caused by 3 factors, pathogen, host, and environment. In the environment, there are many pathogens such as viruses, bacteria, protozoa, fungi, and microbes (Rollando *et al.*, 2018b). This situ-

ation shows that pathogens are widespread in the environment, making it difficult to avoid (Yuniati and Rollando, 2018). One way to avoid pathogens is by increasing the host's immune system (Rollando, 2018; Rollando *et al.*, 2018a). Increased host immune system can increase macrophage activity and capacity. Macrophages are one of the cells that play an important role in phagocytosis of pathogens (Yuniati *et al.*, 2019b). One way to improve the body's immune system by consuming substances that function as immunomodulators (Playfair, 2013).

Therapy with natural ingredients products that have immunomodulatory activities widely used in medicine includes the treatment of autoimmunity, inflammatory disorders, and cancer. Immunomodulatory activities from natural materials have become attractive in recent years because of their relatively cheap price and lower toxic effects than chemical therapeutic products (Rollando, 2018). Immunomodulators are agents that have the ability to increase or suppress the excess immune response used as prophylaxis in a therapeutic combination (Yuniati and Rollando, 2018).

One of the plants that has been used as an immunomodulatory agent is meniran (*Phyllantusniruri* Linn.) (Yuniati *et al.*, 2018). Meniran has been used to treat health problems such as jaundice, wounds, diabetes, diuretics, abdominal pain, and hepatitis. Another Indonesian plant that can be used as a medicinal plant is the faloak tree (*Sterculiaquadrifida*R.Br). The skin from the stem of the faloak tree (*Sterculiaquadrifida*R.Br) is used by the people of NTT as medicinal plants. Decoction of the faloak bark (*Sterculiaquadrifida*R.Br) is used to cure hepatitis, gastroentritis, diabetes, and rheumatoid arthritis (Siswadi *et al.*, 2013). The combination of both extracts of meniran and faloak plants was formulated into syrup preparations.

MATERIALS AND METHODS

Preparation of raw materials

Faloak tree (*Sterculiaquadrifida*R.Br) used in this research is a tree which has a diameter minimal 30 cm. Bark of faloak tree cleaned of dirt by using clean water. Bark of faloak tree dried in room temperature until dry. Powders of bark of faloak obtained after being ground with hammer mill and dried with an oven at temperature 50°C until the moisture content is less then 10%. This procedure used on meniran herbs too.

Extraction of Bark of Faloak and Meniran

The extraction used in this research is reflux method, which used putifies water with comparison 1;5. Powders $\pm$25 gram and mixed with putified water 250 mL.Mixed mixture put in a round bottom flask and mounted to a series reflux extraction device. Reflux extraction is done during 8 hours at temperature 70°C. Filtrate obtained was dried with a rotary evaporator at temperature 80°C. Concentrate filtrate evaporated with a water bath at temperature 80°C until a thick extract obtained.

Formulation of Oral Syrup

The syrup preparation formulation was started by inserting purified water into a 500 mL container and heated to 90°C. Thenmethyl paraben was added to the heated and stirred purified water until the methyl paraben dissolved completely. Methyl paraben solution was added of sucrose and stirred for 10 minutes, and the temperature of the solution was 40°C.In another container, purified water were added, and sorbitol was added. Sirupus simplex solution into sorbitol mixture and homogenized using Ultraturax for 5 minutes. The temperature of the mixture is maintained at 40oC. Thick extract of Sterculiaquadrifida R. Br is inserted and homogenized with Ultraturax for 5 minutes. Thick Phyllantusniruri Linn extract. Inserted into the mixture and homogenized with Ultraturax for 15 minutes. Sodium benzoate added and tutty fruity flavor 50377C and homogenized with Ultraturax for 5 minutes. Purified water was added to 1000 mL and homogenized with Ultraturax for 5 minutes.

Immunomodulatory Activity Test *in vitro*

In vitro immunomodulatory testing of TNF-α and NF-κB was taken from peritonial macrophages measured by supernatant using reactions with Greiss reagents. The collection of TNF-α and NF-κB from the supernatant formed was added RPMI-1640 and Griess reagents (1% sulfonamide and 0.1% N-1-naphtilendiamin hydrochloride in 5% phosphoric acid) in comparison (1: 1) and incubated at room temperature for 24 hours. Added to each series of concentration formulations (500μL) that were to be tested, then measured using LabTech spectrophotometry using sodium nitrate (NaNO) with each parameter. Equipment used such as Petri dishes, Erlenmeyer, test tubes, pipette volume, a tip of the micropipette, and materials such as SDA media sterilized using an autoclave at a temperature of 121°C at a pressure of 1 atm for 15 minutes. The percent calculation of TNF-α and NF-κ induction can be calculated by the following formula.

$$\%\ induction = \frac{absorbance\ sampel - absorbance\ blank}{absorbance\ blanko} \times 100\%$$

Immunomodulatory Activity Test *in vivo*

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The procedure for handling mice is carried out based on the ethical clearance of mice handling. Male mice are placed at a temperature and temperature maintained ($25^oC \pm 1^oC$), with a humidity of 50-60% and 12 hours cycle of light/darkness by being fed and drinking. After 7 days of adaptation, mice were randomly divided into 3 groups, each group consisting of 3 mice. The first group of mice was a control group without treatment. Mice in the second group were positive controls using the original Stimuno flavor. Mice in the third group were negative control mice given preparations without extract (placebo). Mice in the fourth group were mice treated with sample preparations (Utami and Syukur, 2016). Mice treated will be given 3 series of sample concentrations.

Mice treated will be given the drug orally for 21 days. After being treated, the mice were killed using chloroform and calculated the lymph index value. Mice weight is weighed during the experimental period; the weight of vital organs (spleen) can be known after surgery.

The equipment needed in this study were cages for rats, rats, 5 cc syringes, feeding tubes, masks, glove, surgical equipment, tweezers, ependroff, micropipette, RPMI-160 reagent, heparin.

Dosage of meniran extract *Phyllantusniruri* Linn. based on the conversion of adult humans to mice, the human dose multiplied by 0.018 (Febriana, 2015). Dosage of *Phyllantusniruri* Linn. in adults is 150 mg/day so that the dose of white mice is 2.7 mg/day.

The mice lymph organ samples obtained were then weighed. The lymph organs are divided into two using surgical scissors and tweezers then washed with the RPMI-1640 reagent. Macrophages were obtained from each formula cultured (1.5×10^6 cells / mL) in CO_2 incubators at a temperature of 20^oC for 16 hours. After the incubation period was completed, the media was collected for testing IL-1 and IL-6 (n = 5 samples for each time point). Testing of IL-1 and IL-6 using ELISA kits according to existing protocols. Blood sample preparation was carried out by dissolving in PBS pH 7.4, homogenized, and centrifuged at 5000 rpm for 10 minutes at 4^oC. Supernatants used in testing IL-1 and IL-6. Samples are read the absorbance at a wavelength of 450 nm (EPOCH, Bio Tek Instruments Inc., Highland Park, USA). Sample data is compared with standard data. A comparative standard used by M27 Biovista Macrophages.

RESULTS AND DISCUSSION

Extraction of Bark of Faloak and Meniran

The average yield of meniran extract from the six times extraction replication was obtained 12,334 $\pm$ 4,880%. The average yield of meniran extract from the three times extraction replication obtained 6.3077 $\pm$ 0.628%. The difference in yield of meniran extract and faloak bark extract due to simplicia powder is reduced in particle size from herbs (roots, stems and leaves) so that the primary metabolites and secondary metabolites are far more than bark of faloak extracts taken from the skin of the faloak tree stem with a diameter of 30 cm.

Formulation of Oral Syrup

The preparation is made by mixing each ingredient according to the formulation procedure. The following are the results of the formulation from the organoleptic test. The taste are sweet, and the smell every formula has the characteristic of the extract. Tests for syrup preparations were only carried out by organoleptic tests. Tests for dosage viscosity are not carried out because when poured, the preparation is easy to pour.

Immunomodulatory Activity Test *in vitro*

Percentage induction calculations with TNF-α and NF-κB parameters are seen as percent induction values obtained in 10^3 dilution series. 10^3 dilutions are chosen because the value obtained is still close to 100% even though the value obtained is above 100%. If seen from the three replications, the data shows that the first replication of formula 8 is known to have the highest percent value of induction that is 294.3301%, which indicates that formula 8 is able to increase TNF-α. In the second test replication, the highest percent induction was obtained in formula 3, which was 523.7379%, indicating that formula 3 was able to increase TNF-α. The third test replication obtained the highest value of percent induction of TNF-α in formula 5, with a percent value of induction of 904.3956%. The mean percent value of TNF-α induction in the three replications of the test is known that the highest percent value of induction in formula 5.

Percent data of TNF-α induction (Figure 1) obtained from the results of statistical tests using Shapiro-Wilk are normally distributed (Table 1). The normality test uses Shapiro-Wilk because the number of samples is less than 50. Variance analysis test (ANOVA) is done to calculate the mean (mean) of each group, then calculate the total mean of all groups and calculate the deviation of the mean in the group and the total mean the whole group. Parametric ANOVA analysis, where it is known that the

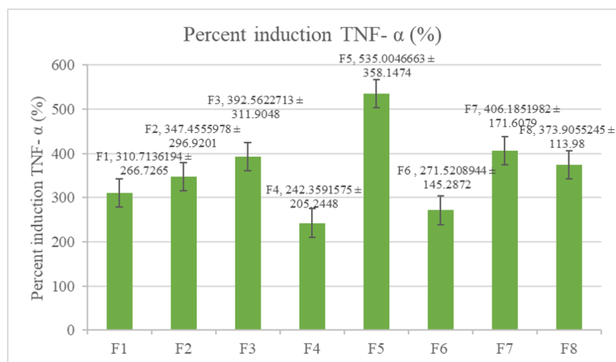


Figure 1: Percent Induction TNF-α

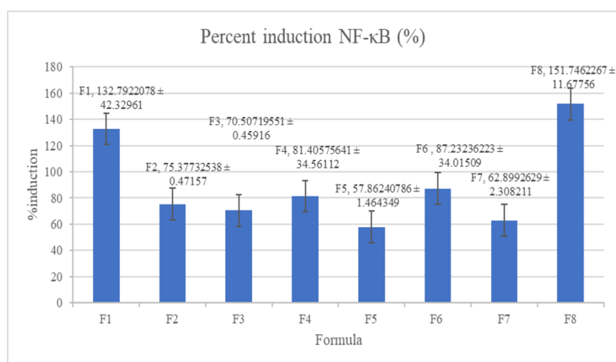
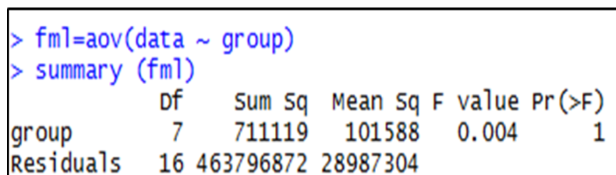


Figure 2: Percent Induction NF-κB



```
> fml=aov(data ~ group)
> summary (fml)
           Df    Sum Sq  Mean Sq F value Pr(>F)
group       7    711119   101588   0.004      1
Residuals  16 463796872 28987304
```

Figure 3: ANOVA result from Percent Induction TNF-α using R-studio v3.5.3

immunomodulatory test results with TNF-α parameters obtained from each formula, are not significantly different from p-value> 0.05 (Figure 3).

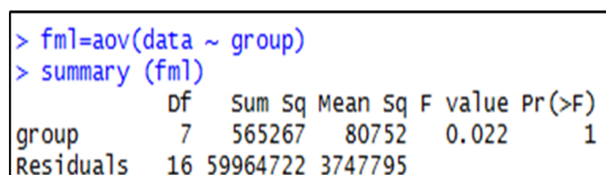
Table 1: The Result of Statistic Shapiro-Wilk Normality Test from Percent Induction TNF-αusing R-Studiov.3.5.3

Formula (F)	p-value
F1	0,4201
F2	0,3004
F3	0,2307
F4	0,2349
F5	0,3515
F6	0,2827
F7	0,3868
F8	0,4065

The normality test was also carried out on percent induction results (Figure 2) from the *in vitro* immunomodulatory test parameters NF-κB. The results of normality testing on the data are intended so that data distribution is seen from the p-value. From Table 2., it can be concluded that the percent NF-κB induction data obtained from the results of statistical tests using Shapiro-Wilk are normally distributed. The variance analysis test (ANOVA) in the NF-κB parameter was also carried out to calculate the mean (mean) of each group, then calculate the total mean of all groups and calculate the deviation of the mean in the group and the total mean for the whole group. Parametric ANOVA analysis, where it is known that the immunomodulatory test results with NF-κB parameters obtained from each formula, are not significantly different from p-value> 0.05 (Figure 4).

Table 2: The Result of Statistic Shapiro-Wilk Normality Test from Percent Induction NF-κB using R-Studio v.3.5.3

Formula (F)	p-value
F1	0,3653
F2	0,3185
F3	0,3207
F4	0,3455
F5	0,242
F6	0,3391
F7	0,2565
F8	0,3624



```
> fml=aov(data ~ group)
> summary (fml)
           Df   Sum Sq Mean Sq F value Pr(>F)
group       7   565267   80752   0.022      1
Residuals  16 59964722 3747795
```

Figure 4: ANOVA result from Percent Induction NF-κB using R-studio v 3.5.3

The immunomodulatory test parameters used in the *invitro* immunomodulatory test include TNF-α and NF-κB. TNF-α and NF-κB were selected as immunomodulatory parameters because TNF-α is an NF-κB transcription factor, interferon (IFN), IL-6, IL-8. Activation of transcription of the NF-κB-controlled gene due to activation of Caspase-7 by Caspase-1, which cuts adenosine diphosphate (ADP). These two parameters are related to where TNF-α is a proinflammatory mediator that plays a role in: the deposition of neutrophils and monocytes toward the site of infection for phagocytosis, increased expression of adhesion molecules (selectin and ligand) vascular endothelial cells for leukocytes, stimulating macrophages to secrete

chemokines and chemotaxic induction and leukocyte release, stimulating mononuclear phagocytes releasing IL-1 with effects such as TNF-α, inducing inflammatory cell apoptosis, stimulates the hypothalamus which induces heat, where TNF-α is called an endogenous pyrogen. Fever is caused by the release of prostaglandin after the release of TNF-α and IL-1. TNF-α as well as IL-1 and IL-6 increases the synthesis of certain serum proteins such as amyloid protein and fibrinogen by hepatocytes; TNF-α can cause intravascular thrombosis, mainly due to loss of endothelial anticoagulant properties. Stimulates the expression of tissue factor by endothelium for coagulation activation and prevents tromodulin expression (coagulation inhibitors). Exacerbation of changes in endothelial activated neutrophils which give rise to vascular obstruction.

Immunomodulatory Activity Test *in vivo*

The immunomodulatory activity of a compound is not related to the inflammatory response in host cells. Inflammation is the basic response of the immune system, which helps fight pathogens, improve tissue, and heal wounds. Acute and chronic inflammation are the main symptoms of various diseases, such as allergies (atopic), atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischemic perfusion, cardiovascular disease, myocardial infarction, stroke, septic, and aging. The inflammatory response results from proinflammatory mediators such as cytokines (TNF-α, IL-1, IL-6), nitric oxide (NO), prostaglandin E2 (PGE2) and anti-inflammatory cytokines (transforming growth factor β (TGF-β), IL- 10, IL-4). The balance between proinflammatory and anti-inflammatory cytokines, inhibition of COX-2 production, and NO production play a role in reducing inflammation. The immune system is divided into innate and adaptive immune systems, which play a role in protecting invading pathogens. The role of macrophages and macrophage precursors, monocytes act as antigen-presenting cells, reminders, cleansing, T cell activation, and phagocytosis. Fagocytosis is a process that occurs due to the mediation of receptors regulated by cytokines such as IL-10 and IFN-γ. Increased phagocytic activity increases the role of macrophages in clearing dead cells and debris cells from different inflammatory responses.

Immunomodulators help stimulate the immune system to increase host defenses against pathogens and many autoimmune diseases. Many antifungal compounds show immunomodulatory activity with complement activation and cytokine tissue; natural killer cell stimulation; effects on the activation, differentiation, and effector of T cell and B cell functions; and depends on the response of the macrophages (Divate and Chung, 2017). Formerly the spleen was considered a neglected organ, but now the spleen is considered to play a central role in immune regulation through T-cell mediated phagocytosis and B cell-mediated humoral immunity (González-Gallego *et al.*, 2018). In this study, the spleen was used to test the compound to determine the immunomodulatory activity of a syrup combination of meniran extract and faloak bark extract. The known immunomodulatory activity from the lymph, including splenocyte proliferation into B cells and T cells using the RPMI-1640 reagent. The immunomodulatory activity of natural material compounds is inseparable from the secondary metabolites of plants. Meniran extract and faloak bark extract have flavonoids which play a role in the immunomodulatory activity.

Flavonoids are a group of polyphenols found in secondary metabolites of plants, fruits, vegetables, and nuts, commonly found in tea or chocolate (tannins, catechins, and flavonols). Flavonoids which are synthesized from amino acids phenylalanine and tyrosine are generally associated with antioxidants, immunomodulators, anti-inflammatory, and prebiotics. Polyphenols are compounds that are capable of being free radicals and anti-free radicals and anti-inflammatory with NF-κB and signaling pathways associated with the use of systemic inflammation. Related antiinflammatory activities in the immunization and adaptation system through modulation of various cytokines that work by (a) changing the signaling and enzymatic processes involved in inflammation such as tyrosine and serine-threonine protein kinase, which have been studied related where B lymphocytes are carried out and T cell proliferation (pro-inflammatory mediators, NF-κB, inducible nitric oxide synthase (iNOS), proinflammatory mediators using cyclooxygenase-2 (COX-2), mitogen-activated protein kinase (MAPK) and protein kinase-C); (B) by reducing cell secretion which induces inflammation; (c) by protecting oxidative stress by becoming free and inflamed radicals such as superoxide anion antioxidants, hydrogen peroxide; and (d) with inflammatory mediators as cytokines, peptides, arachidonic acid.

Antioxidant activity of flavonoids by inactivating reactive oxidative species (ROS), inactivation of chelating trace, and enzymes that form free radicals. From the chemical structure, flavonoids are fairly reactive compounds with the characteristic of having a ring that connects the three-carbon chain with one of the benzene rings. Flavonoids have electron-rich aromatic nuclei π and unstable phenolic groups, -OH, which is a donor container and

electron transfer. Flavones and flavonols display a general affinity for ATP binding proteins. Protein kinases are specifically the main target where catalyzing the transfer of phosphate groups from cofactors ATP to proteins or peptide substrates. Serine/threonine kinase and tyrosine kinase are differentiated based on ligand residues that receive phosphate groups. Catalytic phosphorylation is very important in cell signaling mechanisms, especially those involved in growth, proliferation, survival, and apoptosis. In the inflammatory process, flavonoids increase in number, mobility, lifetime, tissue entry ability, and phagocytic activity of neutrophils (Tekin and Marotta, 2018). The effects of flavonoids on the expression and activity of enzymes involved in the formation of pro-inflammatory mediators such as a nitric oxide or prostanoid and leukotrienes have been widely reported. Nitric oxide (NO) is a molecule that affects many aspects of the inflammatory cascade, ranging from self-production by immunocompetent cells to leukocyte recruitment; in addition, the formation of peroxynitrite from the reaction of NO and superoxide can cause cytotoxicity. NO is produced from L-arginine by three enzymes of nitric oxide synthase (NOS); Endothelial NOS (eNOS), neuronal NOS (nNOS), and induced NOS (iNOS), with the formation of L-citrulline stoichiometric counts (González-Gallego *et al.*, 2018).

Modulation of the immune system results from inflammation. Inflammation that occurs can trigger tumor development. The inate and adaptive immune systems communicate directly with each other or with the help of cytokines. This is expressed in the release of proinflammatory mediators that balance the growth of the tumor. Therefore, a causal relationship is obtained between chronic inflammation and the development of cancer. Phenolic compounds have an important role in every part of the immune system. Cytokines are messenger proteins in the immune system that play a role in paracrine or autocrine so that functioning locally and at a certain distance decreases or increases the immune system. Polyphenols can modulate various cytokines to regulate innate and adaptive immunity but also play a key role in the regulation of extracellular apoptosis. TNF-α is produced by tumor cells or inflammatory cells in the tumor microenvironment, which can increase the survival of tumor cells through gene induction encoding NF-κB-dependent anti-apoptotic molecules. Phenolic compounds can reduce TNF-α transcription by inhibiting the NF-κB pathway (Ghiringhelli *et al.*, 2012).

Extrinsic apoptosis pathways are important in the immune system and involve binding of death ligands to TNF family receptors by trimerization and recruiting adapter proteins such as Fas-associated death Domain (FADD), TNF Receptor-Associated Death Domain (TRADD) to its cytosolic death domain. Suitable receptors and death ligands are FasL / FasR, TNF-α / TNFR1, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) / TRAILR1 or TRAILR2. Phenolic compounds play a role in increasing TRAIL and are able to increase TRAIL-mediated apoptosis in various types of cancer, such as human melanoma. Phenolic compounds inhibit the production of IL-1β. IL-1β plays a role in recruiting intracellular adapters of molecules such as IRAK, which play a role in NF-κB activation. The mechanism of action of phenolic compounds in IL-6 is different for each polyphenol compound. IL-6 plays a role in the occurrence of inflammation and initiation of signal transducer signaling pathways and activator of transcription-3 (STAT-3). Phenolic compounds work on IL-6 by inhibiting the NF-κB and JNK pathways, decreasing IL-6 and TNF-α, inhibiting apoptosis, and inhibiting STAT-3. The inflammatory process is inseparable from the presence of ROS. ROS that is reactive and dextructive can trigger cell damage and trigger cancer. The antioxidant properties of phenolic compounds provide protection against the damaging effects of oxidative stress (Ghiringhelli *et al.*, 2012).

The release of cytokines and interleukin production is a sign for antigen-presenting cells (APC) in recognizing antigens and areas that are inflamed. The immune system can recognize antigens in 3 ways, namely through macrophages, dendritic cells, and B lymphocytes. Macrophages can be identified with specific expressions of a number of proteins, including CD14, CD11b (Rollando *et al.*, 2019; Yuniati *et al.*, 2019a). Tumors infiltrating macrophages (TAM) play an important role in the regulation of inflammation through the secretion of cytokines. Macrophages are divided into M1 and M2. M1 macrophages are activated by IFNγ or microbial products, overexpression of TNF-α, IL-1, IL-6, IL-12 or IL-23, the major expression of histocompatibility complex-II (MHC-II) and produce nitric oxide (NO). Mephophage M1 can kill pathogens and the main anti-tumor immune response. M2 macrophages are activated due to the release of low levels of IL-4, IL-10 and IL-13, MHC class II and IL-12 but release IL-10 and express high levels of scavenger receptor A (SRA) and arginase (Ghiringhelli *et al.*, 2012). Phenolic compounds play a role in increasing the phagocytic activity of macrophages by regulating ROS and NO (Siswandi, 2018).

Continuous stimulation of the inflammatory reaction activates the NF-κB pathway, produces reactive oxygen species (ROS), proinflammatory cytokines,

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interferon-γ, and glutamate if excessive damage (Spagnuolo *et al.*, 2018). This is evident from the occurrence of the liver and lymph enlargement (Matsumura *et al.*, 2010). Enlargement of the lymph organs is characterized by the weight of the control group's lymph organs and reddish lymph color, indicating inflammation (Figure 4). Lymph nodes are an important expression in protecting the body by elaborating on the immune system. Normal lymph size of test animals 1 to 169, while if there is a tumor, the scale becomes 1 to 60.

The liver becomes the main place of metabolism of drugs and chemicals that trigger the possibility of liver injury (hepatic injury) due to metabolism. The pathogenesis of hepatic injury is triggered by a toxic agent or bioactivation of reactive metabolites. Flavonoids are reactive metabolites that can give rise to immune responses or protein dysfunction, lipid peroxidation, DNA damage, oxidative stress, and reduction of glutathione. The pathogenesis of hepatic injury involving all cells (hepatocytes, copper, stellate, and endothelial cells) is present in the liver through apoptosis, necrosis, ischemia, and regeneration. The whole process leads to changes in gene expression . In addition to the active ingredient extract as a nutritious compound, additional ingredients used may also have an effect on liver damage. One of the ingredients used in the formulation and the possible influence on liver damage, sorbitol. Sorbitol is sugar alcohol. Sorbitol fatty acid esters are difficult to digest about 12% or less digested by the pancreas during the digestion process. As a result of difficult digestion, animals experience a lack of energy even though it feels more pleasant than xanthan gum solution (Matsumura *et al.*, 2010).

The mechanism of the immune inate occurs due to the introduction of antigens by phagocytic cells (macrophages, dendritic cells). Antigen recognition by phagocytic cells is carried out by the toll-like receptor (TLR), which is a transmembrane receptor. Antigen recognition by TLR and the introduction of antigenic nucleotides with the nucleotide-binding oligomerization receptor (NLR) becomes the initiation of the inflammatory response. There are 13 TLRs identified in humans and mice, except TLR-3, signals through myeloid differentiation response gene 88 (MyD88) as a central protein adator which activates the NF-κB pathway, which acts as the master controller of inflammation. TLR-3 signaling pathway through toll interleukin (IL)-1 receptor domain-containing adapters are inducing IFN-γ (TRIF) and NF-κB activation.

TLR recognizes patterns of microorganisms with the relationship of pathogen-associated molecular patterns (PAMP) and endogenous ligands as damage associated moleculer pattern (DAMP) that initiates immune signals. The relationship of PAMP is generally related to infectious agents such as viruses, bacteria, and fungi. The relationship of DAMP is related to molecular level damage to tissue damage, necrosis, and apoptosis. DAMP includes DNA, RNA, heat shock, and nuclear high cytosolic proteins mobility group protein-1 (HMGB1) and extracellular matrix (fibronektin and fibrinogen).

Liver damage is suspected due to the DAMP pattern where tissue damage and tissue repair from dead cells are detected by TLR of phagocytic cells, capillaries, and fibroblasts. The initiation of the immune inate system signal through TLR induces the release of proinflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6, IL-12, IFN-α, IFN-β, IFN-γ. Cytokines and DAMP are bound to each receptor, such as TNFR and IL-1R. TNF-α plays a direct role in cell damage, and NF-κB plays a role in inhibiting the formation of new cells. Increased expression of TLR-2, TLR-3, TLR-4, and TLR-9 can increase the expression of proinflammatory cytokines such as IFN-α, IFN-γ, IL-4, IL-17, TNF-α, and IL-6.

Release of proinflammatory molecules due to the introduction of antigens by macrophages, which causes the release of leukotriene and prostaglandin. Migration (chemotaxis) stimulated by chemicals in the inflammatory exudate is called chemokine. IL-8 plays a role in collecting monocytes and neutrophils to the site of infection. Phagocytes ingest intracellular antigen, and digestion begin. After pH in the inflamed area becomes more acidic, cellular proteases induce leukocyte lysis. Macrophages come to the site of infection and swallow leukocyte debris and antigens and open up healing of the local inflammatory process.

IL-1 has a role like TNF-α, a proinflammatory mediator in response to infection. The work of IL-1 with TNF-α plays a role in the immune inate. The main source of IL-1 from activation of mononuclear phagocytes. IL-1 plays a role in macrophage proliferation, proliferation, and differentiation of T lymphocytes and B lymphocytes, inducing prostaglandin in the hypothalamus causing fever, muscle proteolysis due to the release of prostaglandins, APP synthesis, and bone reabsorption.

IL-6 plays a role in accelerating the migration of macrophages to the site of infection. IL-6 plays a role in the maturation of young macrophage cells and increases the capacity of phagocytosis to be more efficient. Increased IL-6 induces B cell differentiation into plasma cells that produce immunoglobu-

lins. Increased IL-6 increases the production of antibodies. IL-6 with cerebrospinal fluid stimulates progenitors in the bone marrow to produce neutrophils.

Fagocytosis of antigens by macrophages is the beginning of the inate immune response. The phagocytic antigen will be broken down into antibodies that are united with vesicles containing major histocompatibility complex-II (MHC-II) protein and will present it to CD4 + T cells. T cells modulate B cell function in a number of ways. T cell cytokines from IL-4, IL-5, IL-6, IL2, and IFN increase B cell proliferation and differentiation into plasma cells that produce antibodies. Increased activity and capacity of phagocytosis are expected to also influence the increase in the adaptive immune response.

From the three test responses, it can be concluded that the formula that has the ability to provide the most optimal response after being compared with other formulas is formula 5. Formula 5, with a ratio of 0.25: 0.75, is the most optimal formula because it has a significant influence on immunomodulatory activities when compared with another formula. Formula 5 has an extract ratio (0.25: 0.75) that is capable of inducing TNF-α of 535,0047 $\pm$ 358,1474%, NF-κB of 57,86241 $\pm$ 1,464349%, and increasing the phagocytic capacity of macrophages at doses prevalent at 98.45 $\pm$ 0.23%. The capacity of macrophages of formula 5 is said to be better because of Stimunois able to increase the phagocytosis capacity of macrophages by 92.43 $\pm$ 5.32%.

The test results using Design-Expert version 11 show the optimal optimization formula results. The optimal SLD Formula Optimization results from the desired value. The desired value or equal to 1 is the optimal formula. It can be concluded that the most optimal formula with extract is 0.96993: 0.0300704. From the assessment, it was predicted that the results of the trials obtained from point prediction were TNF-α 394.552%, NF-κB 131.67%, and macrophage activity 91.4673%.

CONCLUSIONS

Syrup combination formula of meniran extract and faloak stem bark extract capable of modulating TNF-α, NF-κB and increasing macrophage capacity is 535,0047 $\pm$ 358,1474%, 57,86241 $\pm$ 1,464349%, and 98.45 $\pm$ 0.23% based on Data analysis was formula 5 with a ratio of meniran extract 0.25: 0.75 extract of faloak bark. From the eight formulas optimized using the Simplex Lattice Design method 2 factors 2 levels obtained the optimal new formula with desirability value (0.993) closest to the value 1, which is a ratio of 0.96993: 0.0300704 with predictions of in vitro immunomodulatory test results with TNF-parameters α 394,552 $\pm$ 81,6683%, NF-κB 131,679 $\pm$ 16,7298% and phagocytic capacity of macrophages 91,4673 $\pm$ 12,6186%.

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