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Potential of a mixed extract of ginger, lemongrass, turmeric and black cumin as an immunostimulatory candidate

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Abstract. *Zilhaya U, Sari W, Syifa SB, Chusna SA, Rosnizar. 2025. Potential of a mixed extract of ginger, lemongrass, turmeric and black cumin as an immunostimulatory candidate. Nusantara Bioscience 17: 11-20.* Many Indonesian herbal plants show potential as immunostimulants, capable of enhancing the immune system's activity. Some synthetic immunomodulators are known to cause side effects, and create demand for safer and be more effective alternatives. Ginger (*Zingiber officinale*), lemongrass (*Cymbopogon citratus*), turmeric (*Curcuma longa*), and black cumin (*Nigella sativa*) are four plants recognized for their potential as immunostimulants. These plants contain metabolites such as alkaloids, terpenoids, flavonoids, phenolics, and tannins, which can enhance immune activity. This study aims to evaluate the effectiveness of mixed extracts from these plants as immunostimulants, particularly in boosting immune responses weakened by pathogens. The research employed a completely randomized design, utilizing phytochemical tests, in vitro assays, and in vivo evaluations against *Staphylococcus aureus*. The results demonstrated increased activity and phagocytic capacity of macrophages following treatment with extract solutions at various doses. In vitro tests with 100 ppm and 1000 ppm concentrations, as well as in vivo tests at 650 mg/kg body weight, significantly improved macrophage phagocytic activity and capacity. The mixed extract effectively enhanced the immune system.

Keywords: Black cumin, ginger, immunostimulant, lemongrass, macrophages, turmeric

Abbreviation: EGLTB: Mixed extracts of rhizome ginger, lemongrass, turmeric, and seed black cumin

INTRODUCTION

The human body has a natural defense mechanism against diseases caused by viruses and bacteria, known as the immune system. This system enables the body to combat pathogens by rejecting or destroying various foreign substances (antigens) that enter the body (Irianto 2012). The cellular innate immune system, responsible for neutralizing antigens, includes T cells, B cells, Natural Killer (NK) cells, and macrophages. The activity and development of these cells can be enhanced by immunomodulators (Faradilla and Iwo 2014; Sukmayadi et al. 2014).

Immunomodulators are compounds that can enhance the immune system's function, helping protect the body from disease (Rosida and Handojo 2019). These compounds boost the body's defense mechanisms, both specific and non-specific, and regulate cellular and humoral responses. Immunomodulators can act as immunoregulators (to balance the immune system), immunostimulants (to activate the immune system), or immunosuppressants (to suppress an overactive immune response). A variety of synthetic, recombinant, and natural materials can serve as immunomodulators to maintain immunity. When the body's resistance weakens, pathogens such as bacteria and viruses can invade more easily, which is why immunostimulants are essential. Immunostimulants enhance macrophage activity and capacity, allowing them to better phagocytize invading pathogens. Macrophages play a crucial role in the immune system by engulfing and digesting foreign particles (Aldi et al. 2015). The immune system can be modulated by immunosuppressants or enhanced by immunostimulants. Natural compounds that can boost immunity include curcumin, limonoids, vitamins C and E, flavonoids, and catechins (Alquraisi et al. 2021).

Several chemical immunomodulators, such as prednisone, hydrocortisone, and dexamethasone, are commercially available for treating inflammatory diseases. However, these drugs often cause side effects. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) frequently lead to gastric and intestinal mucosal damage, while corticosteroids can cause bone marrow depletion and skin fragility. Therefore, safer and more effective alternatives are needed. Natural products remain a valuable source of new, safe anti-inflammatory agents (Yuandani et al. 2021).

Various herbal ingredients are traditionally used to boost immunity. Ginger is widely used in herbal medicine due to its active compounds-gingerol, beta-carotene, capsaicin, curcumin, and salicylate-which have anti-inflammatory and antioxidant properties. Turmeric is valued for its antioxidant, antibacterial, antiviral properties, and its ability to boost immunity (Kusumo et al. 2020). Black cumin seeds are also commonly used to strengthen the immune system. According to Gunawati et al. (2020),

black cumin seeds are known for their potential to treat asthma, bronchitis, and diabetes, and have antihistamine, antioxidant, antitumor, and antibacterial. Lemongrass offers antimicrobial, antifungal, antioxidant, antihyperlipidemic, and anticholesterol benefits, as well as detoxifying and insomnia-relief properties. According to research by Ernis et al. (2021), lemongrass can enhance lymphocyte proliferation in mice.

The impact of using herbal plants to enhance the immune system can be tested both in vivo and in vitro. In vivo testing is conducted directly on living test animals, allowing researchers to observe the biological or natural responses of the animals. In contrast, in vitro tests are performed on cells or tissues outside of living organisms, where the conditions can be controlled. In vivo testing can serve as a validation for in vitro or other laboratory models. In vitro studies allow for early-stage risk assessment, offering a more direct evaluation of an extract or drug, as they focus on its absorption. However, in vivo testing may introduce complications due to the indirect nature of the approach (Polli 2008; Rohr et al. 2016). In this study, *Staphylococcus aureus* was used as it is a common pathogenic bacterium found on human skin and mucosal surfaces. It is also capable of infecting healthy individuals, with an infection rate of 30-50%, making it likely to invade body tissues (Mutmainnah et al. 2020).

Herbal plants can be used in combination for improved efficacy. Kusumo et al. (2020) found that the combination of turmeric and ginger boosts the immune system and helps prevent microbial transmission, including bacteria and viruses. Akrom et al. (2015) demonstrated that ethanol extract from black cumin seeds (*Nigella sativa* L.) enhances immunity by increasing phagocytic activity and the secretion of Reactive Oxygen Intermediates (ROI) by macrophages in Swiss strain mice infected with *Listeria monocytogenes*. Furthermore, Ernis et al. (2021) concluded that lemongrass essential oil (*Cymbopogon citratus* (DC.) Stapf.) promotes lymphocyte cell proliferation in mice. The testing of herbal plant combinations aims to assess their effectiveness and impact on the immune system. Based on the evidence of the benefits of ginger, turmeric, lemongrass, and black cumin seeds as immunostimulators, a study was conducted to examine the combined effect of ethanol extracts from ginger (*Zingiber officinale* Rosc.), lemongrass (*C. citratus*), turmeric (*Curcuma longa* L.), and black cumin seeds (*N. sativa*) as potential immunostimulatory agents.

MATERIALS AND METHODS

Tools and materials

This research was conducted at the Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Syiah Kuala (USK), Banda Aceh, Indonesia. The equipment used included analytical scales, blenders, glass maceration jars, vials, test kits, animal cages, drinking bottles for test animals, sterile surgical instruments (scalpels, scissors, tweezers), trays, oral probes, autoclaves, cuvettes, spectrophotometers, light microscopes, cameras, micropipettes (20-200 µL), magnetic stirrers, hemocytometers, needle tubes, slide boxes, incubators, Laminar Air Flow (LAF) cabinets, Bunsen burners, Rotary Evaporators (RE), Ohaus scales, dye jars, glass slides, 2.5 mL cuvettes, vortex mixers, tally counters, and other glassware.

The materials used consisted of male Balb/C strain white mice (*Mus musculus* Linnaeus, 1758), aged 6-8 weeks and weighing 25-30 grams, mouse feed, bark, lemongrass rhizome (*C. citratus*, ginger rhizome (*Z. officinale*), turmeric rhizome (*C. longa*), black cumin seeds cumin (*N. sativa*), *Staphylococcus aures* ATCC 25923 and NaCl (0,9 %). Other materials included microtubes, pipette tips, 96% ethanol, 70% alcohol, methanol, 0.2 M Na_2EDTA solution, Phosphate Buffered Saline (PBS) solution (pH 7.8), 0.4% trypan blue solution, 10% Giemsa solution, dimethyl sulfoxide (DMSO), Nutrient Agar (NA) media, Nutrient Broth (NB) media, Stimuno (Imboost Force), immersionion oil, plastic wrap, aluminum foil, and filter paper.

Procedure

Herbal extraction and sample preparation

Ginger rhizomes, turmeric and lemongrass fronds were obtained from farms located in Aceh Besar and Banda Aceh, black cumin was obtained from Bandung, Indonesia. The identification process was carried out by the research team using the identification guidebook in the herbarium of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala and have been confirmed by expert.

After identification, each herbal material was peeled and cleaned. The samples were then cut into small pieces and air-dried for seven days (Rosnizar et al. 2022). Dried seeds were used for the black cumin sample. The ginger, lemongrass, turmeric rhizomes, and black cumin seeds were ground into a fine powder. The sample from each plant were then weighed 500 grams in a ratio of 1:1:1:1. Each powdered sample was macerated with sufficient 96% ethanol in sealed glass maceration jars. The entire sample was submerged and raised to a height of ±2 cm above the surface of the samples. The jars were left to stand for 3×24 hours, with occasional stirring. The mixture was then filtered using filter paper to separate the filtrate from the residue. The resulting filtrate was evaporated at a temperature of 55°C to yield a thick extract, which was then tested for metabolite content using phytochemical tests for alkaloids, steroids, terpenoids, saponins, and flavonoids (Chusna et al. 2024).

Phytochemical test

Herbal extracts were tested for their secondary metabolite content using phytochemical tests. Phytochemical tests include:

Alkaloid phytochemical analysis. Alkaloid content analysis was done using 2 mL extract solution with 1 mL of 2 N HCl and 6 mL of distilled water. Then heated for 2 minutes cooled and filtered. Alkaloid compounds were tested using Mayer and Wagner reagents. A total of 4 mL

of filtrate was put into a test tube, then 1 mL of Mayer reagent was added. The alkaloid test is declared positive if a white or cream-colored precipitate is formed. The addition of the Wagner reagent states that the alkaloid test is positive if an orange to reddish-brown precipitate is formed (Illing et al. 2017).

Steroids and terpenoids phytochemical analysis. Phytochemical analysis of steroids and terpenoids was carried out using the Liebermann-Burchard test. A sample of ± 2 mL of extract was put into a test tube. The steroid test shows a positive result if it produces a green or blue color. Terpenoid test shows positive results if it produces red or purple color (Illing et al. 2017).

Flavonoid phytochemical analysis. 2 mL of extract was added to hot water at 90°C and then filtered. The filtrate was added with Mg powder and 1 mL of concentrated HCl then shaken. A positive flavonoid test was indicated if a red, yellow, or orange color was formed (Illing et al. 2017).

Saponin phytochemical analysis. 2 mL of extract was added to hot water at 90°C and a little concentrated HCl was added. The saponin test was positive if permanent foam was formed for ±15 minutes (Illing et al. 2017).

Tannins and phenolic compound phytochemical analysis. 2 mL of extract was added a few drops of H_2SO_4 reagent and gelatin. The tannin test is positive if it shows a white precipitate. Phenolic compound testing is carried out by adding $FeCl_3$ reagent to 2 mL of extract. Positive results of phenolic compounds are indicated by the appearance of a blackish-blue color in the solution (Koparde et al. 2019).

Test animal care

The test animals, male Balb/C strain mice aged 8-12 weeks with a body weight of 25-35 grams were housed in the Vivarium of the Department of Biology, FMIPA, USK. A total of 60 mice were placed in cages lined with husks and covered with wire. Mice were fed 'All Feed-4' type pellets and water were given ad libitum or as needed, and the husks were replaced twice a week. Prior to the experiment, the mice were acclimatized for one week. This research received ethical approval from the Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Syiah Kuala (No: 282/KEPH/XII/2023).

Preparing the sample test

The test solution was prepared by weighing the extract according to the specified dose and multiplied by the body weight of the mice. Weighed extracts are diluted with distilled water with the help of a magnetic stirrer. extract solution is made stock solution for 14 days of oral administration to mice. According to Dewi et al. (2021) the extract solution is given as much as 0.5 mL/20 gBW.

Culturing of Staphylococcus aureus *bacteria*

S. aureus bacteria used in the study were re-cultured on NA media and incubated for 24 hours at 37°C. After incubation, the bacterial colonies were transferred to NB media and incubated again for 24 hours at 37°C. Following incubation, 1 mL of the NB media containing bacteria was mixed with 9 mL of fresh NB media. The bacterial culture was then centrifuged at 2500 rpm for 25 minutes. The resulting pellet was resuspended in 1 mL of PBS. The bacterial cell density was determined using a spectrophotometer at 580 nm, targeting a transmittance of 25%, corresponding to a cell density of 10^9 cells/mL (Perdana 2022).

In vitro testing

To obtain intraperitoneal fluid, the mice were euthanized via cervical dislocation. The abdomen of mice was cleaned with 70% alcohol. If intraperitoneal fluid was insufficient, 3 mL of sterile PBS was injected into the peritoneal membrane, gently stirred for 1-3 minutes, and collected using a micropipette. The macrophage cell density was determined using a hemocytometer to achieve a concentration of 2.5 x 10^6 cells/mL. Viability was assessed using 0.4% trypan blue solution, with viable cells remaining.

The phagocytosis test was conducted by mixing 200 µL of bacterial suspension, 200 µL of macrophage cells, and 200 µL of test solution at concentrations of 0.1 ppm (P2), 1 ppm (P3), 10 ppm (P4), 100 ppm (P5), and 1000 ppm (P6). As a negative control (P0), 200 µL of bacterial suspension, 200 µL of macrophage cells, and 200 µL of PBS (without the test extract) were mixed. As a positive control (P1), 200 µL of bacterial suspension, 200 µL of macrophage cells, and 200 µL of Stimuno solution were mixed (Kirana et al. 2023). Samples were incubated at 37°C for 30 minutes (Handayani et al. 2018), followed by the addition of 50 µL of 0.2 M Na2EDTA solution and homogenized. Three replicate preparations were made for each treatment, then fixed with methanol for 5 minutes. Staining was done using 10% Giemsa solution (Hariyanti et al. 2015) for 5-20 minutes. The samples were then observed under a 1000x magnification and the activity and phagocytosis capacity of macrophages were calculated.

In vivo testing

Administration of the test extract was carried out once a day for 14 days. The test solution was administered according to the specified doses, which were adapted from the research by Masniah et al. (2021). The doses used in this study were 150 mg/kgBW (P2), 300 mg/kgBW (P3), 450 mg/kgBW (P4), and 600 mg/kgBW (P5). The negative control group (P0) received distilled water, while the positive control group (P1) received a commercial immunostimulant solution.

The test solutions were given orally using a sonde as much as 0.5 mL/20 gBW. On the 15th day, mice were intraperitoneally injected with syringe as much as 0.5 mL of a bacterial suspension (Dewi et al. 2021). After the injection, the mice were left for 1 hour. To collect intraperitoneal fluid, the mice were euthanized by cervical dislocation, followed by dissection of their abdomens. The peritoneal fluid was extracted using a syringe.

Three slides were prepared for each treatment group, intraperitoneal fluid was dripped on a glass slide and smears were made with a tilt angle of 30°C and fixed with methanol for 5 minutes. The glass slides were stained with 10% Giemsa solution for 5-20 minutes. The smear

preparations were rinsed using running distilled water and air dried. After drying, the slides were examined under a microscope at 1000x magnification.

Calculation of macrophage phagocytic activity and capacity values

Macrophage cell activity and phagocytosis capacity were then calculated. Phagocytosis activity value is the percentage of macrophage cells that are active in the phagocytosis process per 100 macrophage cells. Phagocytosis capacity was determined based on the total number of test bacteria in 50 active macrophage cells. The calculation values of macrophage phagocytosis activity and capacity were obtained through the following formula (Dewi et al. 2021):

$$\text{Phagocytosis Activity} = \frac{\text{number of activated macrophage cells}}{\text{100 macrophage cells}} \times 100\%$$

Phagocytosis Capacity = Total number of test bacteria (in 50 activated macrophage cells)

Data analysis

The results of the macrophage cell counts and phagocytic activity were analyzed using Analysis of Variance (ANOVA), assuming normal distribution, with a 95% confidence level and a 5% error rate (α=0.05) (Dewi et al. 2021). To further assess differences between treatment groups, a Duncan post-hoc test was conducted (Manurung and Mose 2019).

RESULTS AND DISCUSSION

Phytochemical test

Based on the phytochemical testing of the ethanol extract mixture of ginger, lemongrass, turmeric, and black seed (EGLTB), alkaloids, terpenoids, flavonoids, phenolic compounds, and tannins were identified, as shown in Table 1. The results of the phytochemical tests show that the metabolite compounds identified in the mixed rhizome and seed extract (MRE) are alkaloids, terpenoids, flavonoids, phenolics, and tannins. Alkaloids were derived from ginger (Dhanik et al. 2017), lemongrass (Asif and Khoadadi 2013), and black cumin (Ouattar et al. 2022). Terpenoids were found in turmeric (Sun et al. 2017) and lemongrass (Asif and Khoadadi 2013). Flavonoids were present in turmeric, ginger (Dhanik et al. 2017), lemongrass (Asif and Khoadadi 2013), and black cumin (Ouattar et al. 2022). Phenolic compounds were identified in turmeric (Dhanik et al. 2017), ginger (Sun et al. 2017), and black cumin (Ouattar et al. 2022), while tannins were found in turmeric (Dhanik et al. 2017) and lemongrass (Asif and Khoadadi 2013).

Research by Sun et al. (2017) indicated that turmeric contains terpenoids, flavonoids, phenolic compounds, organic acids, anthocyanins, tannins, and other organic compounds. According to Yuandani et al. (2021), the major compounds in turmeric rhizomes are polyphenols and terpenoids, with curcumin, a key polyphenol, demonstrating immunomodulatory, antioxidant, anti-inflammatory, and antitumor activities. Shabana et al. (2020) also reported that turmeric improves immune function by reducing proinflammatory cytokine levels in diabetic rats.

Besides its immune-boosting properties, the in vivo administration of CEERPB extract can produce pharmacological effects. Peng et al. (2019) found that alkaloids can reduce inflammation and mitigate colonic damage. Tanfil et al. (2023) demonstrated that alkaloids prevent significant increases in total cholesterol, triglycerides, Low Density Lipoprotein (LDL) cholesterol, Very Low Density Lipoprotein (VLDL) cholesterol, and the atherogenic index, while increasing High Density Lipoprotein (HDL) cholesterol levels. Belete (2019) also explained that alkaloids exhibit antibacterial properties by inhibiting bacterial growth and destruction.

Terpenoids also act as immunostimulants, with pharmacological effects demonstrated in vivo. Retnowati et al. (2011) noted that terpenoids interact with porins (transmembrane proteins) in the bacterial cell wall, forming strong polymer bonds that damage the porins. This reduces bacterial cell wall permeability, depriving the bacteria of essential nutrients, which ultimately inhibits bacterial growth or causes cell death. Research by Sari (2016) showed that terpenoids help synthesize organic substances and aid in cell recovery.

Phenolic compounds enhance the immune system and provide pharmacological benefits. According to Diniyah and Lee (2020), phenolics bind free radicals, decompose oxidation products, and chelate harmful metal ions. Puspitasari et al. (2016) found that phenolic compounds also serve as antibacterials by destroying cell walls and precipitating microorganism proteins, leading to cell dysfunction.

Table 1. Phytochemical screening results of mixed ethanol extracts of ginger, lemongrass, turmeric rhizome, and black cumin seeds

Metabolite content	Reagent	Results	Information
Alkaloids	Mayer	+	A white precipitate formed
	Wagner	+	A brown precipitate formed
	Dragendorff	+	A red precipitate formed
Steroids	Liebermann-Burchard test	-	No green color formed
Terpenoids	Liebermann-Burchard test	+	A red color formed
Saponin	Shuffling	-	No foam formed
Flavonoids	HCl and Mg Metal	+	A purple color formed
Phenolic	$FeCl_3$	+	A blue color formed
Tannin	Gelatin + H_2SO_4	+	A white precipitate formed

Flavonoids improve immune response and provide various pharmacological effects. These effects include protecting the intestinal mucosa, optimizing nutrient absorption (Mistiani et al. 2020), and acting as anti-inflammatories by inhibiting COX-2 and nitric oxide synthase enzymes (Rosa et al. 2001). Wang et al. (2011) found that flavonoids reduce asthma symptoms. They also act as anticancer agents by inducing apoptosis, regulating carcinogen metabolism, and inhibiting cancer development (Khoirunnisa and Sumiwi 2019).

Tannins, too, boost the immune system and offer pharmacological effects. According to Farha et al. (2020), tannins have been found to inhibit bacterial growth using various mechanisms of action including iron chelation, inhibition of cell wall synthesis, disruption of cell membranes, and inhibition of fatty acid biosynthetic pathways. Fauziah et al. (2021) demonstrated tannins' antioxidant capabilities, as they neutralize free radicals.

Both flavonoids and alkaloids play critical roles in improving immune function. Sholikhah and Rahayungsih (2015) explained that these compounds act as immunomodulators by increasing IL-2 (interleukin 2) activity and lymphocyte proliferation. Activated Th1 cells influence the Specific Macrophage Arming Factor (SMAF), including IFNγ (interferon gamma), which activates macrophages. When an antigen enters the body, T lymphocytes and macrophages collaborate to eliminate the bacteria. Macrophages phagocytize the bacteria, while T lymphocytes differentiate into CD4+ and CD8+ cells. CD4+ cells further differentiate into Th1 cells, producing IFNγ and TNFα cytokines, which activate Natural Killer cells. CD8+ cells also produce IFNγ cytokines, which activate macrophages, leading to the production of nitric oxide, a compound that kills bacteria.

In vitro test

The research began with a macrophage cell viability test to determine the number of live and dead cells, as well as the number of cells eligible for further testing. The Trypan Blue (TB) method is a very common assay to evaluate cell viability where dead cells absorb TB into the cytoplasm due to loss of membrane selectivity, while live cells remain unstained (Avelar-Freitas et al. 2014). Macrophage cell density was also measured using a hemocytometer to ensure that the number of macrophage cells was sufficient for the phagocytosis test. The percentage of viability and macrophage cell density are presented in Tables 2 and 3, respectively.

Based on the observations from five viability tests, the average cell viability was 96.61%. Only cell cultures with a viability of at least 95% were used for further experiments. This viability value should not be less than 95% (Chairul et al. 2009). The macrophage cell density measured over five repetitions averaged 4.632×10^6, exceeding the minimum required macrophage population density (2.5×10^6).

Macrophage phagocytic activity and capacity were used as indicators of immune system enhancement. Macrophages, which are phagocytic cells, play a critical role in the non-specific immune response and function as Antigen Presenting Cells (APCs). The immunostimulatory potential of EGLTB was assessed by observing the ability of macrophages to phagocytize *S. aureus*. According to Jensch-Junior et al. (2006), macrophage phagocytosis can be measured by calculating the percentage of phagocytic activity and capacity. The average activity and phagocytic capacity after EGLTB administration are shown in Table 4.

The mean values of macrophage cell phagocytic activity from the negative control to the highest concentration were $37.4^{a}\pm2.191$, $79.0^{e}\pm15.81$, $41.4^{b}\pm3.082$, $53.4^{c}\pm42.19$, $74.0^{d}\pm43.93$, $89.4^{f\pm}2.408$, and $98.6^{g}\pm1.342$, respectively. The mean values of phagocytic capacity of macrophage cells from the negative control to the highest concentration were $365.60^{a}\pm6.656$, $843.60^{e}\pm13.557$, $491.40^{b}\pm5.595$, $646.00^{c}\pm15.110$, $791.40^{d}\pm8.803$, $897.40^{f}\pm10.526$, and $988.00^{g}\pm7.176$, respectively. The highest percentage of phagocytic activity and capacity was found in the P6 treatment, while the lowest was found in the P0 treatment. Statistical analysis indicated that the data were normally distributed ($P>0.05$) and homogeneous ($P>0.05$). An ANOVA test was conducted to evaluate the effect of each treatment on increasing macrophage phagocytic activity and capacity.

Table 2. Macrophage cell viability test results

Test	Living cells	Dead cells	Viability (%)
1	94	3	96.9%
2	93	4	95.87%
3	89	3	96.7%
4	89	2	97.8%
5	91	4	95.78%
Average			96.61%

Table 3. Macrophage cell density in laboratory mice (using a hemocytometer)

Test	Density (cells/mL)
1	4.86×10^6
2	4.8×10^6
3	4.42×10^6
4	4.45×10^6
5	4.63×10^6
Average	4.632×10^6

Table 4. Mean phagocytic activity and capacity of macrophages after administration of EGLTB

Treatment	Cell average	
	Activity (%)	Capacity (cells/50 active macrophages)
P0	$37.4^{a}\pm2.191$	$365.60^{a}\pm6.656$
P2	$41.4^{b}\pm3.082$	$491.40^{b}\pm5,595$
P3	$53.4^{c}\pm42.19$	$646.00^{c}\pm15,110$
P4	$74.0^{d}\pm43.93$	$791.40^{d}\pm8,803$
P1	$79.0^{e}\pm15.81$	$843.60^{e}\pm13.557$
P5	$89.4^{f}\pm2.408$	$897.40^{f}\pm10.526$
P6	$98.6^{g}\pm1.342$	$988.00^{g}\pm7,176$

Note: P0: Negative control (no extract); P1: Positive control (comparative immunostimulant); P2: EGLTB 0.1 ppm; P3: EGLTB 1 ppm; P4: EGLTB 10 ppm; P5: EGLTB 100 ppm; P6: EGLTB 1000 ppm

The increased macrophage cell activity and capacity following EGLTB administration may result from the metabolite compounds present in EGLTB, which induce macrophage cells to become more active and phagocytize more *S. aureus* bacteria. EGLTB contains metabolite compounds such as alkaloids, terpenoids, flavonoids, phenolics, and tannins (see Table 1). The flavonoid content in EGLTB exhibits antioxidant activity, which can improve immune system function. This is because antioxidants can neutralize free radicals produced by the innate immune system, such as neutrophils and macrophages. According to Canton et al. (2021), these two phagocytic cells possess cell membranes bound to the NADPH-oxidase system, enabling macrophages to produce Reactive Oxygen Species (ROS), including hydrogen peroxide, hydroxyl radicals, and superoxide radicals. Additionally, macrophages produce prostaglandins, leukotrienes, interleukin-1, and interferons.

In addition to flavonoids, the phenolic and terpenoid contents in EGLTB can also boost the immune system. Tohma et al. (2017) reported that phenolic compounds in ginger possess antioxidant properties that support immune system improvement. El Gazzar et al. (2006) also noted that terpenoid compounds, such as thymoquinone, can enhance the immune system in patients with bronchial asthma caused by allergies.

The bioactive compounds in EGLTB, which are secondary metabolites, can attach to bacteria and act as chemoattractants for macrophage cells, making it easier for macrophages to phagocytize bacteria. These bioactive compounds can also bind to the macrophage cell membrane and influence signaling pathways (Elmowalid et al. 2007) to form pseudopodia, increasing the cells' activity and phagocytic capacity against bacteria.

Observation of macrophage cell phagocytic activity and capacity against *S. aureus* was performed using a trinocular microscope. Macrophage cells were observed after Giemsa staining, which allowed differentiation of the macrophage cell nucleus, cytoplasm, and *S. aureus* bacterial cells. The macrophage cell nucleus absorbed the stain more intensely than the cytoplasm, while *S. aureus* bacteria appeared as concentrated stains, making them easy to observe. According to Mokobi (2016), Giemsa is a specialized stain used for blood smear examinations to detect parasitic infections, particularly malaria, and can also be employed to observe macrophage cell phagocytic activity. In addition, Giemsa staining is used to distinguish various blood cells such as erythrocytes, platelets, and leukocytes, as well as cellular components such as cell nuclei and cytoplasm. The results of macrophage and *S. aureus* staining are shown in Figure 1.

In vivo test

The calculation of activity values and phagocytic capacity of mice macrophages was conducted to assess the ability of mixed rhizome and seed extracts of ginger, turmeric, lemongrass, and black cumin to enhance immunity. The results of statistical analysis showed that the data for phagocytic activity and capacity were normally distributed ($P>0.05$) and homogeneously varied ($P>0.05$), and were then analyzed using Analysis of Variance (ANOVA). Based on the ANOVA results, it was found that there was a significant effect ($P<0.05$) with a P-value of 0.00 on the activity and phagocytic capacity of macrophages treated with EGLTB. These results indicate that EGLTB acts as an immunostimulant. To further determine the effect of each dose on macrophage activity and phagocytic capacity, a Duncan test was performed. The activity values and phagocytic capacity of macrophages in mice treated for 14 days are presented in Table 5.

Based on the further test results presented in Table 5, the macrophage activity in the negative control (P0) was 31%, in the positive control (P1) it was 45.6%, for the 150 mg/kgBW treatment (P2) it was 37.6%, for the 300 mg/kgBW treatment (P3) it was 49%, for the 450 mg/kgBW treatment (P4) it was 62.6%, and for the 600 mg/kgBW treatment (P5) it was 69%. The treatments P2, P3, P4, and P5 showed a significant increase compared to the negative control, indicating a boost in macrophage activity and capacity as a result of natural immunity. Phagocytic activity reflects the immune response to foreign bodies, carried out by macrophages. This aligns with Coico and Geoffrey (2015), who stated that immunity functions to protect the body from pathogens, and that innate immunity can be considered a natural immune response present from birth. Additionally, Rosales and Eileen (2017) emphasized the key role macrophage cells play in natural immunity through phagocytosis, enabling the body to defend itself against antigens. The P0 treatment reflects the body's natural response to exposure to *S. aureus* bacteria, a common pathogen.

Table 5 also shows that macrophage phagocytic capacity values differed significantly between the negative control (P0), the positive control (P1), and the various treatment groups. Administering EGLTB at 300 mg/kgBW (P3) resulted in activity levels similar to P1 and P2, but the phagocytic capacity differed from the other treatments. EGLTB at 450 mg/kgBW (P4) and 600 mg/kgBW (P5) showed comparable activity levels but significantly different capacity values compared to other doses. The activity value represents the percentage of active macrophages among 100 macrophages, while the capacity value reflects the number of bacteria successfully phagocytosed by 50 active macrophages (Sari et al. 2016).

The differences in these results highlight the immune-boosting effects of EGLTB. Turmeric contains curcumin, which reduces inflammation and oxidative stress in the body, supporting immune function (Hewlings and Kalman 2017), and modulates cytokine production, which regulates immune responses (Aggarwal et al. 2013). Ginger inhibits free radicals and exhibits antitumor, anti-inflammatory, anticarcinogenic, antibacterial, antimutagenic, and antioxidant activities, thereby inhibiting pathogens (Munadi 2018). Lemongrass acts as an immunomodulator, increasing the activity and proliferation of lymphocytes in laboratory mice (Ernis et al. 2021). Black cumin also acts as an immunostimulant by increasing lymphocyte and monocyte numbers, though it does not reduce the numbers of eosinophils and neutrophil stem cells. Black cumin has immunomodulatory activity through both immunostimulant and immunosuppressant mechanisms (Gunawati et al. 2020).

The administration of EGLTB increases the activity and phagocytic capacity of macrophages. The results of macrophage and *Staphylococcus aureus* staining (in vivo test) are shown in Figure 2. Based on Figure 2.C, at a dose of 150 mg/kgBW, there is an increase in the number of bacterial cells phagocytosed by macrophages compared to the negative control (P0). Figure 2.A (negative control, P0) shows fewer phagocytosed bacteria than the other doses. Figure 2.B (positive control) shows almost the same amount of phagocytosis as Figures 2.D (P3), 2.E (P4), and 2.F (P5). The higher the dose, the greater the phagocytic ability of macrophages treated with EGLTB. This suggests that the EGLTB mixture can enhance macrophage function and the phagocytosis of pathogens. According to Hirayama et al. (2018), macrophages are activated by pathogen invasion and present peptide antigens from ingested bacteria to the Major Histocompatibility Complex (MHC), which activates T helper cells.

An increase in macrophage phagocytic activity and capacity indicates an improvement in the immune system. This mixture of ginger, turmeric, lemongrass, and black cumin functions as an immunostimulant. According to Purkon et al. (2021), immunostimulants stimulate the immune response by enhancing the activity of both non-specific and specific immune components against infection. In this study, macrophages act as part of the non-specific immune response, acting quickly as the body's first line of defense. This aligns with Hirayama et al. (2018), who stated that macrophages play an essential role in the innate (non-specific) immune system by recognizing and destroying pathogens. When this system fails to eliminate pathogens, adaptive immunity is activated. Upon recognizing a particle, phagocytes activate several signaling pathways to initiate phagocytosis.

Phagocytosis is the process by which cells engulf and eliminate particles larger than 0.5 µm in diameter, including microorganisms, foreign substances, and apoptotic cells. This process occurs in almost all cell types in multicellular organisms (Uribe-Querol and Rosales 2020). Phagocytic cells eliminate microorganisms and present antigens to lymphocytes, aiming to activate the adaptive immune response (Gordon 2016). The process of phagocytosis consists of several stages: (i) Detection of the particle by phagocytic cells; (ii) Activation of the internalization process; (iii) formation of phagosomes, which are specialized vacuoles; and (iv) maturation of phagosomes. During this process, foreign particles are incorporated into phagosomes, which then mature and fuse with lysosomes to form phagolysosomes. These contain enzymes that destroy and decompose the particles. Phagocytosis can target various cells, including apoptotic cells and microbes (Uribe-Querol and Rosales 2020). The process of antigen presentation is described in Figure 3.

Table 5. Mean macrophage phagocytic activity and capacity in vivo

Treatment (mg/kgBW)	Mean	
	Activity (%)	Capacity (cells /50 macrophages active)
P0	31.0^{a}±7.6	137.4^{a}±27.94
P1	45.6^{b}±8.3	281.8^{d}±34.01
P2 (150)	37.6^{ab}±7.6	180^{b}±30.27
P3 (300)	49.0^{b}±15.17	219^{c}±24.87
P4 (450)	62.6^{c}±5.4	396.6^{e}±19.85
P5 (600)	69.0^{c}±2.5	537.6^{f}±20.26

Notes: P0: Negative control treatment without extracts; P1: Positive control treatment (Imboost Force 0.975 mg/kgBW); P2: EGLTB 150 mg/kgBW; P3: EGLTB 300 mg/kgBW; P4: EGLTB 450 mg/kgBW; P5: EGLTB 600 mg/kgBW

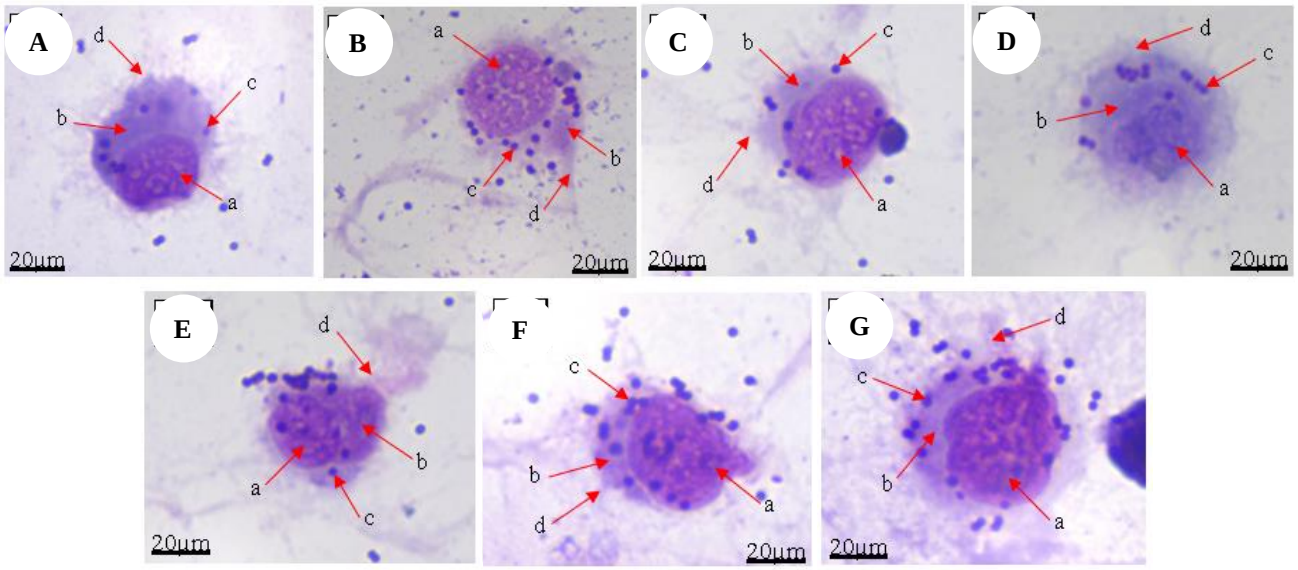

Figure 1. Mice's intraperitoneal macrophage cells after adding EGLTB and *Staphylococcus aureus* bacterial infection. A. Negative control; B. Positive control; C. EGLTB concentration 0.1 ppm; D. EGLTB concentration 1 ppm; E. EGLTB concentration 10 ppm; F. EGLTB concentration 100 ppm; G. EGLTB concentration 1000 ppm. a: Cell nucleus; b: Cytoplasm; c: Phagosome; d: Pseudopodia. Magnification 10x100

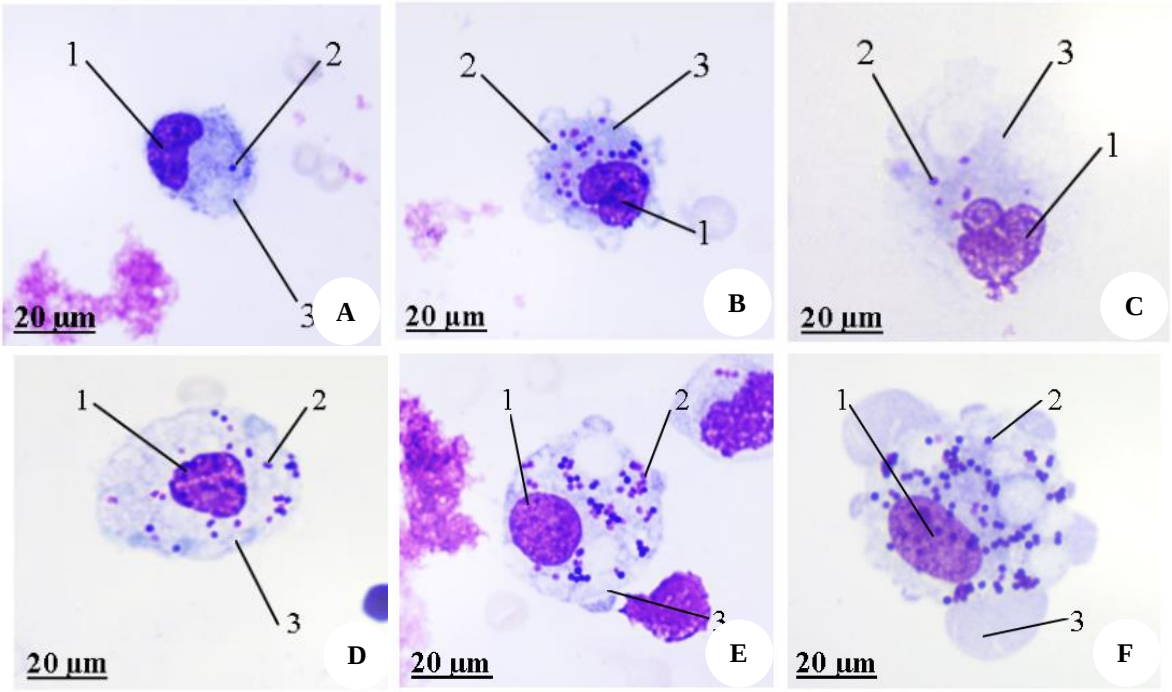

Figure 2. The administration of EGLTB increases the activity and phagocytic capacity of macrophages. A. P0; B. P1; C. P2; D. P3; E. P4; F. P5; 1. Macrophage; 2. Fagosom; 3. Pseudopod

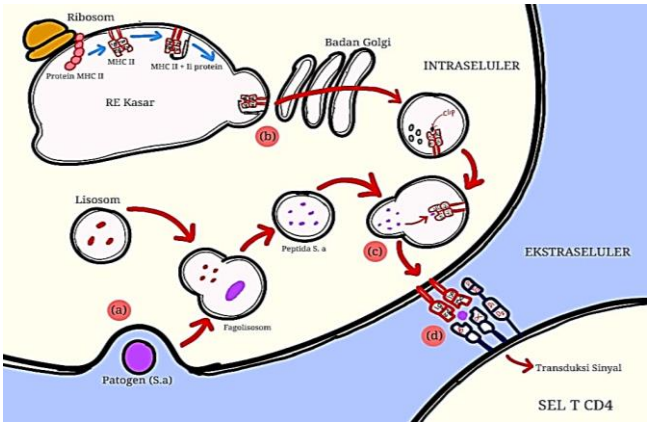

Figure 3. Antigen presentation process. A. Phagocytosis, B. MHC II formation, C. Bacterial peptide fusion with MHC II, D. Signaling of MHC II with bacterial antigen to CD4 T cells

When macrophages phagocytize *S. aureus* (Figure 3.A), lysosomes fuse with phagosomes to form phagolysosomes, producing small bacterial peptides. Meanwhile, the rough Endoplasmic Reticulum (ER) forms major histocompatibility complex II (MHC II) (Figure 3.B). MHC II passes through the Golgi body, producing a clip on MHC II. The MHC II then binds to bacterial peptides (Figure 3.C). The CD4 T cell receptor verifies that the cell has indeed produced MHC II. Upon confirmation, signal transduction occurs, activating CD4 T cells (Figure 3.D). This process is consistent with the findings of Mufidah et al. (2013), who demonstrated that *S. aureus* is phagocytosed and recognized by MHC II, which then presents the peptide antigens to T lymphocytes. The effector CD4+ T cells secrete interferon-gamma (IFN-γ), a cytokine that activates macrophages.

The signaling pathway is initiated when macrophages sense danger signals through Pattern Recognition Receptors (PRRs), which allow immune cells to rapidly respond to various pathogens with common structures, known as Pathogen-Associated Molecular Patterns (PAMPs). For example, components of bacterial cell walls, such as lipopolysaccharide (LPS), and viral double-stranded RNA are produced during infection (Marshall et al. 2018). *S. aureus* has a cell wall composed of lipopolysaccharide (LPS), which is the primary component of Gram-negative bacterial cells and causes septic shock. *S. aureus* has been shown to trigger the secretion of cytokines and chemoattractants (TNF-α, IL-1β, IL-10, IL-12, IL-8, leukotriene B4, complement factor 5a, MCP-1, MIP-1α, and granulocyte colony-stimulating factor) from monocytes and macrophages. Mohammad et al. (2022) explained that *S. aureus* expresses several molecules, including bacterial lipoproteins (Lpps), which play a role in both the immune response and disease pathogenesis. Lpps, the primary ligand for TLR2, are critical for bacterial survival by maintaining bacterial metabolic activity and contributing to host cell invasion during various infections. Nguyen et al. (2017) further explained that the lipid moiety of Lpps in *S. aureus* functions as part of Microbe-Associated Molecular Patterns (MAMPs), alerting the innate immune system via PRR detection, particularly through TLR2 in host cells. This lipid moiety serves as an important danger signal to the host.

In conclusion, the phytochemicals obtained from this study were a mixture of ethanol extracts of ginger rhizome, turmeric, lemongrass, and black cumin seeds, including alkaloids, terpenoids, flavonoids, phenolics, and tannins. The administration of a mixture of ethanol extracts from ginger rhizomes, turmeric, lemongrass fronds, and black cumin seeds positively affects the immune system. This is evidenced by an increase in the activity and phagocytic capacity of intraperitoneal macrophages in mice after induction with *S. aureus*. The enhancement of the immune system corresponds with the dose administered. Optimal doses for increasing macrophage activity and phagocytic capacity are concentrations of 100 and 1000 ppm in vitro tests, and 450 mg/kgBW and 600 mg/kgBW in in vivo tests. The signaling process of macrophages occurs from the activation of CD4 T cells by MHC II. To further explore the immunomodulatory potential of the ethanol extract mixture (EGLTB), additional research is recommended, focusing on test animal organs to evaluate cellular effects.

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