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# The Effect of a combination of *Andrographis Paniculata* Extract, *Nigella Sativa* αnd Propolis αs Immunomodulators on the Immune Response in Rats Infected with *Mycobacterium Tuberculosis*

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**BSTRACT**

A combination of *Nigella sativa*, *Andrographis paniculata*, and propolis extracts was investigated for its immunomodulatory effects on rats infected with *Mycobacterium tuberculosis*. Thus, this study evaluated the immunomodulatory potential of active compounds in silico, assessed their ability to inhibit bacterial growth in vitro, determined an effective dose, and analyzed their impact on organ health in infected rats. This study involved several tests, including in silico testing using the Molegro Virtual Docker application, in vitro testing to analyze the potential of the extract against bacterial growth in MODS (Microscopic-observation drug-susceptibility) medium, as well as in vivo testing which included measurement of body weight in rats, white and red blood cell counts, IL-6 and IL-10 examination using ELISA, multiplex PCR test for *Mycobacterium* species, and histopathological examination of the spleen, lungs, and liver. Molecular docking showed strong receptor binding for andrographolide (-63.66 kcal/mol), thymoquinone (-61.72 kcal/mol), and quercetin (-59.88 kcal/mol). In vitro, the extracts inhibited *M. tuberculosis* growth at concentrations below 25.5 mg/ml. In vivo, the combination extract (450 mg/kg body weight) improved blood parameters, reduced IL-6 and IL-10 levels,

and mitigated histopathological damage in the lungs, spleen, and liver. The combination extract of formula C (a combination of *Nigella sativa*, *Andrographis paniculata*, and propolis extracts) at a dose of 450 mg/kg body weight has the potential to exhibit the best activity as an immunomodulator and anti-tuberculosis agent than the other two formulae in this study. Further research may validate its therapeutic application.

## 1. Introduction

Tuberculosis (TB) remains a major global health challenge, caused by the bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*)[1]. Effective TB management requires a prolonged treatment regimen of six to nine months[2]. Upon infection with *M. tuberculosis*, the host immune system activates a series of immune mechanisms, including an inflammatory response, which plays a critical role in controlling the infection[3]. However, excessive or uncontrolled inflammation can contribute to disease progression and tissue damage. Immunomodulators play a vital role in mitigating inflammation and promoting tissue repair in TB-infected individuals[4].

TB is a contagious disease that disproportionately affects developing countries, contributing significantly to morbidity and mortality. The identification of *M. tuberculosis* as the causative agent of TB dates back to the seminal work of Hermann Heinrich Robert Koch, who was awarded the Nobel Prize in Medicine in 1905 for his discovery. Despite the availability of effective anti-TB drugs, the extended duration of treatment often leads to poor patient adherence, increasing the risk of drug resistance[3]. The World Health Organization (WHO) has highlighted drug-resistant TB as a pressing global health issue. Indonesia, ranked as the second-highest TB burden country after India, has committed to eliminating TB by 2030, aiming to reduce the incidence rate to 65 cases per 100,000 population and the mortality rate to 6 per 100,000 population[4,5].

Immunomodulators, which can be of natural or synthetic origin, modulate the immune system by regulating the production of pro-inflammatory and anti-inflammatory cytokines[6]. Key cytokines such as IFN-γ, TNF-α, IL-6, and IL-1, along with chemokines like CCL5, CCL9, CXCL10, and CCL2, play a pivotal role in recruiting immune cells to the site of infection and facilitating pathogen clearance. Immunomodulatory agents target various immune cells, including neutrophils, macrophages, lymphocytes, and natural killer (NK) cells, to enhance immune responses against *M. tuberculosis*[7].

The host immune response to *M. tuberculosis* infection is characterized by a complex interplay between innate and adaptive immunity[8]. Innate immunity involves physical barriers, phagocytic cells, and circulating proteins such as complement, while adaptive immunity generates specific responses to microbial antigens[9]. Hematopoietic stem cells in the bone marrow give rise to two primary lineages: the lymphoid lineage, producing B cells, T cells, and NK cells, and the myeloid lineage, generating granulocytes and monocytes, which differentiate into macrophages and dendritic cells. These immune cells are distributed throughout the body, with a high concentration in lymphoid tissues such as the thymus, spleen, and lymph nodes[10,11].

Recent research has explored the immunomodulatory potential of various natural compounds, including *Andrographis paniculata*, *Nigella sativa*, and propolis[12]. These natural extracts contain bioactive compounds such as flavonoids, alkaloids, terpenoids, polysaccharides, and saponins, which exhibit immunomodulatory properties. *Andrographis paniculata* contains andrographolide, a diterpenoid lactone with anti-inflammatory effects, while *Nigella sativa*'s active component, thymoquinone, has demonstrated antibacterial and anti-TB properties[13,14]. Propolis,

a resinous substance produced by bees, is rich in phenolic and flavonoid compounds known for their immune-enhancing effects[15].

Studies have shown that andrographolide modulates both innate and adaptive immune responses, reducing TNF-α expression in TB-infected rats and inhibiting the production of pro-inflammatory cytokines[15–18]. Thymoquinone has been reported to suppress inflammatory cytokines and macrophage activation while enhancing adaptive immune responses[19–21]. Propolis has been found to activate peritoneal macrophages and upregulate Toll-like receptor (TLR) expression, contributing to improved immune function. However, while individual studies have examined the immunomodulatory effects of these extracts, research on their combined effects remains limited[22–25].

This study aimed to evaluate the immunomodulatory effects of a combination of *Andrographis paniculata*, *Nigella sativa*, and propolis extracts on the immune response in a TB-infected animal model. Specifically, the study assessed the effects of this combination on IL-6 and IL-10 expression, complete blood count profiles, histopathological changes in the spleen, lungs, and liver, and *M. tuberculosis* detection through multiplex PCR. The findings will contribute to a better understanding of the potential synergistic effects of these natural immunomodulators in TB treatment and management.

## 2. Materials And Methods

### 2.1. Plant material

*Andrographis paniculata* (Burm.f) Nees, a herb from the Acanthaceae family, and *Nigella sativa* Linn., a seed from the Ranunculaceae family, were obtained from cultivated plants at the Research Institute for Spices and Medicinal Plants (Balitro), Bogor, Indonesia, and have been identified by the curator at the Herbarium Depokensis (UIDEP), Biota Collection Room of the University of Indonesia (RKBUI). Propolis from the *Geniotrigona incisa* bee species, originating from South Sulawesi, was purchased from CV. Nutrima Sehat Alami, Department of Products and QC, Bogor, West Java, Indonesia.

### 2.2. Extraction Method

The dried *Andrographis paniculata* dan *Nigella sativa* seeds were ground using a grinder to obtain dry powder. The resulting powder was then sieved using a number 80 mesh sieve to obtain uniform particle size. The fine powder obtained was stored in a tightly closed container, protected from light, moisture, and contamination, until ready to be used for the extraction process. Propolis was prepared by cutting it into small pieces measuring approximately 3–5 cm, then placed into a maceration container.

*Andrographis paniculata* dan *Nigella sativa*, and propolis was re-macerated using 70% ethanol solvent (1:10) and filtered every 24 hours. The re-maceration was performed three times, then filtered and evaporated using a rotary evaporator to calculate the crude extract yield. The extract was then freeze-dried, and the yield of the dry extract was calculated.

### 2.3. Treatment of experimental animals

The experimental animals used in this study were female Sprague Dawley white rats, aged 2–3 months, with a body weight ranging from 150 to 207 grams and white in color. The Animal Health Certificate (SKKH) number: 114/524.3/VI/2024/PKH, was issued by the Department of Agriculture and Food Security, Maros Regency Government. The rats were acclimatized for 2 weeks to allow adaptation to the new environment. They were housed in cages under controlled conditions with a temperature range of 23–25°C, humidity of 55% ± 5%, and a 12-hour light/12-hour dark cycle. The rats were fed a mixture of pellets and corn, and provided with tap water ad libitum.

The rats were divided into six groups: the normal group, the negative control group, the positive control group, and the groups receiving combination extract Formula A, B, and C. Each group consisted of 5 rats (n=5). The rats were infected with a bacterial dose of *Mycobacterium tuberculosis* using 0.5 ml of bacterial suspension (1 ml of suspension contain-

**Table 1.** Extraction yield result

| No | Extract | Weight (g) | Viscous extract yield (%) | Yield after freeze-drying (%) |
|---|---|---|---|---|
| 1 | 70% Ethanol Extract of *Andrographis paniculata* (Sambiloto) | 5100 | 15.88 | 13.3 |
| 2 | 70% Ethanol Extract of Black Cumin (*Nigella sativa*) | 5690 | 17.93 | 11.8 |
| 3 | 70% Ethanol Extract of Propolis | 2054 | 57.94 | 15.6 |

**Table 2.** Phytochemical content of extract

| **No** | **Extract** | **Phenol** | **Flavonoid** | **Alkaloid** | **Tannin** | **Quinone** | **Saponin** | **Steroid** | **Terpenoid** |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 70% Ethanol Extract of *Andrographis paniculata* (Sambiloto) | **+** | **+** | **+** | **+** | **-** | **+** | **+** | **+** |
| 2 | 70% Ethanol Extract of Black Cumin (*Nigella sativa)* | **+** | **+** | **+** | **+** | **+** | **+** | **+** | **+** |
| 3 | 70% Ethanol Extract of Propolis | **+** | **+** | **+** | **+** | **+** | **+** | **-** | **+** |

Description: (+) : contains; (-) : does not contain

ing 1.5 x 10^8 bacteria, equivalent to 0.5 McFarland standard) with intraperitoneal. After being infected with tuberculosis for 11 days, the rats were treated according to their respective intervention groups for 14 days, with the Ethics Committee Approval Number No. 037/KEPK-FFUP/XI/2024.

### 2.4. The concentration of extract

The concentration given to the experimental female Sprague Dawley rats were a combination of *Andrographis paniculata* extract + *Nigella sativa* extract + propolis at the following concentrations: Formula A at 50 mg/kg body weight (1:1:1); Formula B at 150 mg/kg body weight (1:1:1); Formula C at 450 mg/kg body weight (1:1:1). The dose of Rifastar was 39.6 mg/100 g body weight, administered orally once daily.

Formula A was administered orally to the experimental animals at a dose of 50 mg/kg body weight. It consisted of a combination of *Andrographis paniculata* (sambiloto), *Nigella sativa* (black cumin), and propolis extracts in a 1:1:1 ratio. The dose was calculated based on each rat's body weight daily prior to treat-

ment, and the administration volume was adjusted accordingly using an oral syringe. The extracts were dissolved or suspended in 0.5% sodium CMC solution before administration. The same procedure was applied for Formula B, Formula C, and the Rifastar tablet.

**2.5. Phytochemical screening**

*2.5.1. Alkaloid test*

The alkaloid test used Bouchardat reagent that formed black-brown precipitate, while the reaction with Mayer's reagent would form a white precipitate, and the reaction with Dragendorf reagent would form an orange-brown precipitate.

*2.5.2. Flavonoid test*

The flavonoid test was conducted by adding 100 mg of magnesium powder, followed by concentrated HCl and 2 ml of amyl alcohol solution could produce a reddish-yellow color.

*2.5.3. Tannin test*

In the tannin test, when 1% iron (II) chloride solution was added, a violet-green color was formed.

*2.5.4. Saponin test*

In the saponin test, when distilled water was added and vigorously shaken for 10 seconds, foam was formed. When 2N HCl was added, the foam did not disappear.

*2.5.5. Phenolic test*

In the phenolic test, by adding 1% ferric chloride solution, a blue or dark green color was formed.

*2.5.6. Quinone test*

In the quinone test, by adding NaOH, a red color was formed.

**Table 3.** Docking analysis results on protein receptor 1T64 with ligand TSN_386 (A)

| **Compound** | **Test Compound** | **MolDoc score** | **Rerank score (RS)** | **H-Bonds** |
|---|---|---|---|---|
| RGFP966 N-(2-((4-(trifluoromethyl)phenyl)thiazol-2-yl)thio)phenyl)acetamide. | Positive control | -110.585 | -86.06 | -4.94382 |
| *Andrographis paniculata* (Sambiloto) | Andrographolide | -106.389 | -63.6623 | -2.5 |
| | Neoandrographolide | -94.8858 | -75.8273 | -2.89927 |
| | 14-Deoxy-11,12-didehydroandrographolide | -93.8293 | -73.6992 | -2.40546 |
| | 14-Deoxyandrographolide | -97.5241 | -67.8218 | -0.328199 |

***Table 3, continued***

| | | | | |
|---|---|---|---|---|
| Propolis | Kuersetin | -87.955 | -61.7207 | - 8.12281 |
| | Kaempferol | -85.9983 | -59.842 | - 6.04255 |
| | 3',4'-Dimethoxyacetophenone | -83.4092 | -68.4302 | -1.10093 |
| | Myricitin | -88.7355 | -59.882 | - 8.95301 |
| Black Cumin (*Nigella sativa*) | Thymoquinone | -62.952 | -53.7743 | - 2.40109 |
| | 9,12 Octadecadienoic acid (Z-Z) methylester | -91.4934 | -67.3247 | 0 |
| | 10,13-Eicosadienoic acid methyl ester | -94.7878 | -71.5351 | -2.83929 |
| | E,E,Z-1,3,12-Nonadecatriene-5,14-diol | -110.601 | -85.6308 | -2.69062 |

*2.5.7. Triterpenoid and steroid test*

In the triterpenoid and steroid test, by adding Liebermann-Burchard reagent (3 drops of anhydrous acetic acid and 1 drop of H2SO4), a green color indicated the presence of sterols, while a red or purple color indicated triterpenoids[26,27].

**2.6. Molecular docking**

Before performing docking, each compound such as andrographolide (the compound from *Andrographis paniculata* extract), thymoquinone (the compound from *Nigella sativa* extract), quercetin (the compound from propolis extract), and RGFP966 (N-(2-((4-(trifluoromethyl)phenyl)thiazol-2-yl) thio)phenyl)acetamide) as a comparative control were drawn for their chemical structures using the 2D ChemDraw 18.1 program. Then, the structures were optimized to their most stable form using the Chem 3D 18.1 application. The stable structures were then docked using Molegro Virtual Docker 5.5, with a computer specification of Windows 10, 8 GB RAM, and AMD Radeon software. The PDB protein used was protein 1T64 (Crystal Structure of human HDAC8 complexed with Trichostatin A) (downloaded from www.pdb.org), with native ligand TSN_386 (A). Each test compound and control were saved in mol.2 format. The docking process is considered complete when the MolDock Score, Rerank Score, Hbond, and RMSD (Root Mean Square Deviation) values appear. The RMSD value indicates the closeness of the drug molecule to its receptor.

**2.7. Bacterial growth activity test of *Mycobacterium tuberculosis* using MODS (in vitro)**

*2.7.1. Preparation of MODS liquid culture medium*

Dissolve 5.9 g of 7H9 powder medium, then add 3.1 ml of glycerol and 1.25 g of casitone to 900 ml of distilled water. Shake the mixture until homogeneous, then autoclave at 121-124ºC for 15 minutes. Afterward, allow it to cool, incubate at 37ºC for 48 hours, and store at 2-8ºC in tightly sealed tubes[28]. b)

Preparation of *M. tuberculosis* H37Rv 0.5 McFarland suspension: First, take 1-2 loops of bacterial colonies from a solid Ogawa slant tube. Then, transfer the bacterial colonies into a boiling tube containing 3 ml of PBS Tween. Vortex the mixture until homogeneous and let it stand for 15 minutes. Afterward, transfer 1-2 ml of the solution into a McFarland tube containing 3 ml of PBS Tween, and measure the concentration using a densitometer until it reaches 0.5 McFarland.

*2.7.2. Immunomodulator activity test on rats infected with M. tuberculosis in vivo*

Before conducting the experiment using animal subjects, an ethical review was performed, and approval was obtained from the ethics committee (Approval No. 037/KEPK-FFUP/XI/2024). The study design was experimental, involving a series of tests on the animals, including: body weight measurement, interleukin-6 (IL-6) and interleukin-10 (IL-10) levels post-infection and post-treatment, complete blood count (CBC) analysis, and histopathological examination of the spleen, lungs, and liver tissues.

**Table 4.** Binding of test compound's amino acid residues with the protein after docking

| **Compound** | **Test Compound** | **Bonds with amino acid residues after docking between the ligand and protein** |
|---|---|---|
| RGFP966<br>N-(2-((4-(trifluoromethyl)phenyl) thiazol-2-yl)thio)phenyl)acetamide. | Positive Control | Phe 207; **Met 274**; Phe 208 ; **His 180** |
| *Andrographis paniculata* (Sambiloto) | Andrographolide | **His 180**; Phe 152; **Met 274**; Cyc 153; Trp 141; Gly 151; His 142; Asp 267; Gly 304, Tyr 306; Asp 178; Asp 101 |
| | Neoandrographolide | His 143, Gln 263, Trp 141, Cyc 153, Gly 304, Tyr 306, His 142, Asp 267, Asp 178, **Phe 208**, Phe 152, **His 180**, **Met 274** |
| | 14-Deoxy-11,12-didehydroandrographolide | **Phe 208**, His 143, Tyr 306, Gly 206 |
| | 14-Deoxyandrographolide | Asp 178, His 143, Tyr 306, **His 180**, Gly 304, Phe 152, Trp 141, Cyc 153, Asp 101 |
| Black Cumin (*Nigella sativa*) | Thymoquinone | Tyr 306 ; **His 180** ; Gly 151 |
| | 9,12 Octadecadienoic acid (Z-Z) methylester | Phe 152 ; Gly 151 ; **Met 274** ; **His 180**; His 142 ; Tyr 306 |

***Table 4, continued***

| | | |
|---|---|---|
| | 10,13-Eicosadienoic acid methyl ester | **His 180**, Gly 206, **Phe 208**, Tyr 306, Gly 151, Asp 101 |
| | E,E,Z-1,3,12-Nonadecatriene-5,14-diol | Tyr 306, Asp 101, **His 180**, **Met 274**, **Phe 208** |
| Propolis | Quercetin | Asp 101; **Met 274**; Cys 153; Gly 151; Trp 141; His 142; Tyr 306; **His 180** ; Gly 304; Asp 178 |
| | Kaempferol | **Met 274**, Phe 152, Tyr 306, Phe 208, Gly 151, Trp 141, His 143, Gly 304, **His 180**, Gln 263, His 142, Asp 178, Gly 140 |
| | 3',4'-Dimethoxyacetophenone | Phe 208, His 143, Gly 151, Phe 152, Tyr 306, **His 180**, Asp 178 |
| | Myricitin | **His 180**, Trp 141, His 142, Asp 267, Asp 178, Ohe 152, Cys 153, Tyr 306, **Met 274**, Asp 101 |

*2.7.3. Hematological examination*

The hematological examination, which includes red blood cells and white blood cells, was performed using the Licare CC-3200Vet Auto Hematology Analyzer**.** The red blood cell examination includes the following parameters: Red Blood Cell (RBC), hemoglobin (HGB), hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelet (PLT), Mean Platelet Volume (MPV), Platelet Distribution Width (PDW), and Procalcitonin (PCT). The white blood cell examination includes the following parameters: White Blood Cell (WBC), lymphocytes, monocytes, and granulocytes.

*2.7.4. Analysis of IL-6 and IL-10*

The IL-6 and IL-10 analysis was performed using an ELISA reader, following the Quantikine® ELISA protocol for Rat IL-6 Immunoassay from R&D Systems, USA. The procedure for analyzing Rat IL-6 (Catalog Number R6000B, SR6000B, PR6000B) and Rat IL-10 (Catalog Number R1000, SR1000, PR1000) was adhered to as per the kit instructions[29,30].

*2.7.5. PCR analysis of Mycobacterium species*

The analysis begins with DNA extraction, followed by sample preparation. For preparation, 200 µL of blood sample is added into a 1.5 mL Eppendorf tube along with PBS until the total volume reaches 200 µL, then 20 µL of proteinase is added. The mixture is incubated at 60°C for 5 minutes. Next, cell lysis is performed by adding 200 µL of GSB Buffer, vortexing, and incubating the sample at 60°C for another 5 minutes. For DNA binding, 200 µL of ethanol is added, vortexed for 10 seconds, and the mixture is trans-

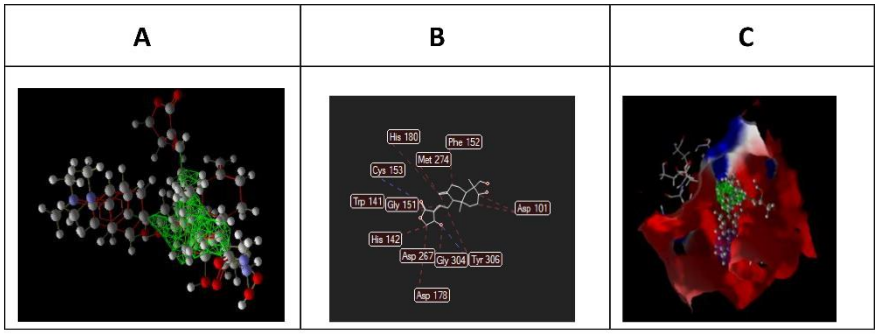


***Figure 1**. Histone deacetylase ligand-receptor binding; B. Amino acid residues after docking; C. Position of ligand in cavities*

ferred to a GS Column placed in a 2 mL collection tube. The sample is centrifuged at 14,000 – 16,000 rpm for 1 minute, and the liquid in the collection tube is discarded. The sample is washed by adding 400 µL of W1 buffer and centrifuging at 14,000 – 16,000 rpm for 30 seconds. The liquid in the collection tube is discarded. Then, 600 µL of wash buffer is added, and the sample is centrifuged at 14,000 – 16,000 rpm for 30 seconds. A new collection tube is placed, and centrifugation is repeated at 14,000 – 16,000 rpm for 3 minutes. Finally, the GS Column is transferred to a sterile Eppendorf tube, and 100 µL of pre-heated elution buffer is added. The sample is centrifuged at 14,000 – 16,000 rpm for 30 seconds, and the GS column is discarded. The liquid remaining in the Eppendorf tube is the DNA product, which is ready for PCR. For PCR, a gel electrophoresis is prepared by adding the DNA sample and primer sequences into the gel medium, and the sample is then processed using a PCR machine[31].

*2.7.6. Histopathology of lungs, spleen, and liver*

Each tissue sample from the lungs, spleen, and liver of the rats was prepared using hematoxylin and eosin (HE) staining. The readings were performed using a light microscope Olympus CX23 at magnifications of 10x and 40x. The observation based on the distribution of tissue damage was scored as follows: Score 1: if tissue damage is <25%, Score 2: if tissue damage is 25–50%, Score 3: if tissue damage is 50.1%–75%, Score 4: if tissue damage is 76%–100%. The observations were based on lesions related to tuberculosis-induced inflammation. In lung tissue, the analysis focused on the presence of pneumonia, nodules, lymphoid follicles, fibrosis, and hemorrhage. In spleen tissue, the analysis included splenonecrosis, hemosiderosis, and hyperemia. In liver tissue, degeneration and hemosiderosis were analyzed. The extent of tissue damage was scored according to the same system

*2.7.7. Statistical analysis*

Statistical analysis was performed using a one-way ANOVA and multiple comparison tests to determine differences between treatment groups. Data analysis was conducted using SPSS software version 26. A p-value of <0.05 was considered statistically significant.

## 3. Results and Discussion

### 3.1. Results of maceration extraction

From the extraction process using maceration followed by freeze-drying, the yields obtained were 13.3% for *Andrographis paniculata* extract, 11.8% for *Nigella sativa* extract, and 15.6% for propolis extract (Table 1). In the phytochemical screening analysis, *Andrographis paniculata*, *Nigella sativa*, and propolis extracts were found to contain secondary

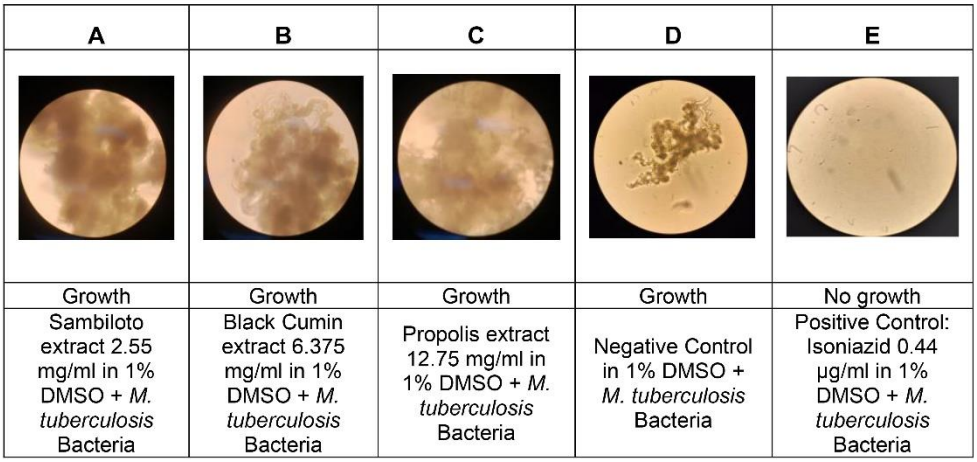


***Figure 2**. Minimum concentration of extract that allows growth of M. tuberculosis bacteria in MODS medium*

**Table 5.** Concentration of extracts in inhibiting the growth of *M. tuberculosis* bacteria

| No | Concentration of Extract (in 1% DMSO) and *M. tuberculosis* Bacteria | Sambiloto extract | *Nigella sativa* extract | Propolis extract |
|---|---|---|---|---|
| 1 | 0.255 mg/ml | + | + | + |
| 2 | 1.275 mg/ml | + | + | + |
| 3 | 2.55 mg/ml | + | + | + |
| 4 | 6.375 mg/ml | - | + | + |
| 5 | 12.75 mg/ml | - | - | + |
| 6 | 25.5 mg/ml | - | - | - |
| 7 | 50.0 mg/ml | - | - | - |
| 8 | 100 mg/ml | - | - | - |

(+) = Growth ; (-) No growth

metabolites, including phenols, flavonoids, tannins, alkaloids, saponins, quinones, steroids, and terpenoids. These secondary metabolites are known to have immunomodulatory properties. Compounds derived from plants (such as curcumin, resveratrol, epigallocatechin-3-gallate, quercetin, colchicine, capsaicin, andrographolide, and genistein) have shown strong effects on both cellular and humoral immune functions[32] (Table 2).

Additionally, several previous studies have quantitatively measured the active compounds from *Nigella sativa* Linn extract, namely thymoquinone[33], 9,12-Octa- decadienoic acid (Z-Z) methyl ester, 10,13- Eicosadienoic acid methyl ester, and E,E,Z- 1,3,12-Nonadecatriene-5,14-diol[34]. Meanwhile, the active compounds from propolis extract include 3- Prenyl cinnamic acid allyl ester, caffeic acid phenethyl ester, farnesol, isomaltrose, arginine, and phenylpropionic acid[34,35], Kuersetin[35]. The active compounds in *Andrographis paniculata* Nees extract are andrographolide[9], 13-dien-15,16,19-triol Ent-labdane, 8α-methoxy-14-deoxy-17β-hydroxyandrographolide

**Table 6.** Average body weight of rats across groups

| Group | Period | Mean ± SD | Asymp Sig (2-tailed) | Keterangan |
|---|---|---|---|---|
| Normal Control (uninfected) | Normal Control | 162 ± 2.41 | 0.59 | Given that the Sig (2-tailed) value is 0.59 > 0.05, it can be concluded that there is no significant difference between the body weight of the rats post-infection and post-treatment. |
| | Normal Control | 167 ± 0.75 | | |
| Negative Control (infected, no treatment) | after infection | 157 ± 1.67 | | |
| | after infection | 159 ± 0.55 | | |
| Positive Control (infected, treated with Rifastar tablet) | after infection | 167 ± 2.12 | | |
| | after treatment | 164 ± 0.55 | | |
| Formula A | after infection | 189 ± 2.70 | | |
| | after treatment | 190 ± 0.52 | | |
| Formula B | after infection | 191 ± 3.63 | | |
| | after treatment | 194 ± 3.88 | | |
| Formula C | after infection | 170 ± 1.87 | | |
| | after treatment | 169 ± 0.75 | | |

Ent-labdane, andrographolactone, andrograpanin[10], panaculoside, flavonoids, andrographonin, panicalin, neoandrographolide, apigenin 7-4-dimethyl ether[35], 14-deoxyandro- grapholide, 14-deoxy 11,12-didehydroandro- grapholide, and 19-O-β-D-glucopyranosyl-ent- labda-8[36].

### 3.2. Docking results

Rerank score (RS) or Binding Energy indicates the amount of energy required to form a bond between a ligand and its receptor. The lower the binding energy, the more stable the bond. A more stable ligand-receptor interaction is predicted to result in greater activity[37]. Therefore, a compound with a low RS value implies a stable ligand-receptor interaction and is predicted to have higher activity[38]. Docking analysis is performed to computationally evaluate the potential of compounds such as andrographolide, thymoquinone, and quercetin in providing immunomodulatory activity (Tables 3 and 4, Figure 1). In this study, the protein used is 1T64, which is the crystal structure of human HDAC8 complexed with Trichostatin A. This approach aims to identify interactions between these compounds and the target protein, providing insights into their potential immunomodulatory activity.

In the study conducted by Campo et al.[39] it was stated that RGFP966, an HDAC3 inhibitor, not only has a direct effect in killing *M. tuberculosis*, BCG, and M. avium, but also modulates the macrophage inflammatory pathways. In contrast, RGFP966 did not inhibit growth of several other intracellular and extracellular bacteria. We also found that RGFP966 modulated macrophage pro-inflammatory cytokine secretion in response to Mtb infection with decreased IL6 and TNF secretion[39]. Study by Kumbhar et al.[40] based on structure, function, and chemical conservation, 18 mammalian HDAC isomers are classified into Class I (HDAC 1, 2, 3, and 8), Class II (HDAC 4, 5, 6, 7, 9, and 10), Class III (Sirtuin 1–7), and Class IV (HDAC 11).

Therefore, in the docking process conducted by the researchers, the histone deacetylase (HDAC) protein

**Table 7.** Laboratory results of white blood cell count and its derivatives

| **Group** | **WBC** | **Lymphocytes** | **MID (Monocytes)** | **Granulocytes** |
|---|---|---|---|---|
| Normal Values | 2.90 -15.3 $10^9$/ L | 2.60 – 13.5 $10^9$/ L | 0.0 – 0.50 $10^9$/ L | 0.4 -3.20 $10^9$/ L |
| | Mean ± SD | | | |
| Normal Control | 9.70±4.35 | 4.98±1.83 | 0.42±0.23 | 4.30±2.31 |
| Negative Control | 11.76±4.06 | 6.24±2.30 | 0.56 ± 0.21 | 4.96±1.63 |
| Positive Control | 9.42±2.67 | 4.90±1.38 | 0.48±0.13 | 4.04±1.23 |
| Formula A | 11.04±3.58 | 5.98±1.75 | 0.58±0.15 | 4.48±1.78 |
| Formula B | 10.84±3.28 | 5.80±1.75 | 0.54±0.17 | 4.50±1.54 |
| Formula C | 11.38±3.13 | 5.80±1.63 | 0.58±0.13 | 5.00±1.43 |

**Table 8.** Average red blood cell count in rats groups

| **Red Blood Cell** | **Normal Values** | **All group rats Mean ± SD** | **Sig. Oneway ANOVA** |
|---|---|---|---|
| RBC | 5.6 -7.89 $10^{12}$/L | 6.43 ± 0.72 | 0.569 |
| HGB | 12.0 – 15.0 g/dL | 11.82 ± 1.34 | 0.793 |
| HCT | 36 – 46 % | 39.82 ± 3.90 | 0.401 |
| MCV | 53.0 – 68.8 fL | 62.23 ± 3.31 | 0.000 |
| MCH | 16.0 – 23.1 pg | 18.35 ± 0.75 | 0.000 |
| MCHC | 300 -341 g/L | 296,0 ± 10.41 | 0.229 |
| PLT | 100 – 1610 $10^9$/L | 821,0 ± 201 | 0.029 |
| MPV | 3.8 – 6.2 fL | 9.18 ± 0.27 | 0.045 |
| PDW | 5 – 20 | 12.65 ± 1.69 | 0.189 |
| PCT | 0.100 – 0.500% | 0.75 ± 0.17 | 0.046 |

*RBC = Red Blood Cell; HGB = hemoglobin; HCT =hematokrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular hemoglobin; MCHC = Mean corpuscular hemoglobin concentration; PLT = Platelet; MPV = Mean Platelet Volume; PDW = Platelet Distribution Width; PCT = Procalcitonin*

was used. Considering that the structure of HDAC3 is not yet available in the Protein Data Bank (https://www.rcsb.org/structure), the researchers used the 1T64 protein, which represents the structure of HDAC8, as an alternative in this study. This approach allows for the exploration of interactions and the potential of compounds toward a relevant target, even though a different homolog is used. Therefore,

**Table 9.** Comparison of interleukin 6 levels post *Mycobacterium tuberculosis* infection and post treatment using Wilcoxon statistical test

| Group | Intervention | Period | Mean + SD (pg/ml) | Asymp Sig (2-tailed |
|---|---|---|---|---|
| Normal Control | Without *M. tuberculosis* infection, only given Na.CMC 0.5% | Normal Control | 8.76 ± 9.07 | 0.028 |
| | | Normal Control | 3.08 ± 3.08 | |
| Negative Control | *M. tuberculosis* infection only | after infection | 36.13± 40.29 | |
| | | after infection | 4.19 ± 3.23 | |
| Positive Control | *M. tuberculosis* infection + Rifastar 4 FDC Tablet anti-tuberculosis drug | after infection | 59.78± 16.29 | |
| | | after treatment | 3.92 ± 3.92 | |
| Formula A | *M. tuberculosis* infection + combination of extracts at a concentration of 50 mg/kg body weight (1:1:1). | after infection | 23.33 ± 1.98 | |
| | | after treatment | 0.84 ± 0.84 | |
| Formula B | *M. tuberculosis* infection + combination of extracts at a concentration of 150 mg/kg body weight (1:1:1). | after infection | 19.27± 13.81 | |
| | | after treatment | 5.59 ± 3.77 | |
| Formula C | *M. tuberculosis* infection + combination of extracts at a concentration of 450 mg/kg body weight (1:1:1). | after infection | 35.36± 41.72 | |
| | | after treatment | 3.22 ± 2.17 | |

HDAC3 belongs to the same class as HDAC8.

Based on the calculation results of the rerank score for the test compounds, the values were found to be lower than the reference compound, although still showing negative values, indicating the proximity between the ligand and the protein. Based on the rerank score results, *Andrographis paniculata* (Sambiloto) **>** Propolis **>** *Nigella sativa* (Black cumin) showed the closest ligand-receptor binding. After the docking process, the researchers identified amino acid residues that interacted, some of which showed similarities in the bonds formed with the reference compound. This suggests that the test compounds have the potential to possess immunomodulatory properties.

### 3.3. Results of in vitro testing using the MODS method

This test was conducted to analyze the potential of the extracts in inhibiting bacterial growth in the MODS medium. The effective concentration of the extract in inhibiting bacterial growth will be used as the basis for dose calculation in in vivo testing. In the in vitro test, bacterial growth of *M. tuberculosis* was observed. At extract concentrations between 0.255 to 12.75 mg/ml, *M. tuberculosis* growth was detected in the MODS medium (Figure 2). The positive control (isoniazid) showed no bacterial growth, while the

**Table 10.** Comparison of interleukin 10 levels post *Mycobacterium tuberculosis* infection and post treatment using Wilcoxon statistical test

| Group | Intervention | Period | Mean + SD (pg/ml) | Asymp Sig (2-tailed) |
|---|---|---|---|---|
| Normal Control | Without *M. tuberculosis* infection, only given Na.CMC 0.5% | Normal Control | 173.67 ± 19.10 | 0.917 |
| | | Normal Control | 191.77 ± 11.40 | |
| Negative control | *M. tuberculosis* infection only | after infection | 194.98 ± 29.40 | |
| | | after infection | 197.47 ± 22.90 | |
| Positive Control | *M. tuberculosis* infection + Rifastar 4 FDC Tablet anti-tuberculosis drug | after infection | 216.85 ± 40.78 | |
| | | after treatment | 213.69± 39.10 | |
| Formula A | *M. tuberculosis* infection + combination of extracts at a concentration of 50 mg/kg body weight (1:1:1). | after infection | 172.04 ± 12.60 | |
| | | after treatment | 166.16± 20.10 | |
| Formula B | *M. tuberculosis* infection + combination of extracts at a concentration of 150 mg/kg body weight (1:1:1). | after infection | 163.42 ±8.40 | |
| | | after treatment | 177.11± 11.30 | |
| Formula C | *M. tuberculosis* infection + combination of extracts at a concentration of 450 mg/kg body weight (1:1:1). | after infection | 335.78± 234.20 | |
| | | after treatment | 187.11 ± 17.50 | |

negative control exhibited a significant amount of *M. tuberculosis* growth (Table 5).

The concentrated ethanolic extract, obtained after complete evaporation of the ethanol solvent, was free of residual ethanol. For bacterial growth inhibition assays, the extract was re-dissolved in 1% DMSO (dimethyl sulfoxide). DMSO was chosen because it can dissolve both polar and non-polar compounds, and it does not exhibit antibacterial activity at low concentrations (≤1%), ensuring that the observed effects are solely due to the extract.

Mycobacteria have unusually high lipid content in their cell wall. Such lipids include mycolic acids and other saturated and unsaturated fatty acids. Mycolic acids are branched, long chain fatty acids present in the cell wall of a limited number of genera; they exhibit the maximum length in the genus Mycobacterium. The analysis of the lipid content of the mycobacterial cell wall has been widely used for identification purposes. The various techniques used

**Table 11.** Results of multiplex PCR examination for Mycobacterium species

| No | Group | Results of Multiplex PCR with Mycobacterium spp. Primers | | |
|---|---|---|---|---|
| | | **16S rRNA gene All mycobacterial species (506 bp)** | ***rv0577* All *M. tuberculosis* complex (705 bp)** | **RD9 *M. tuberculosis* (369 bp)** |
| 1 | Normal Control | Negative | Negative | Negative |
| 2 | Negative Control | Negative | Negative | Positive |
| 3 | Positive Control | Negative | Negative | Positive |
| 4 | Formula A | Negative | Negative | Positive |
| 5 | Formula B | Negative | Negative | Positive |
| 6 | Formula C | Negative | Negative | Positive |

**Table 12.** Primer sequences *multiplex PCR Mycobacterium species*

| Set Genetic target | Primer sequences (forward/reverse) | Organism Target (s) | Expected product size |
|---|---|---|---|
| 16S rRNA gene | 5' GAGATACTCGAGTGGCGAAC 3'<br>5' CAACGCGACAAACCACCTAC | All mycobacterial species | 506 bp |
| 2 *rv0577* | 5' ATGCCCAAGAGAAGCGAATACA 3'<br>5' AATGTCAGCCGGTTCCGCAA 3' | All *M. tuberculosis* complex | 705 bp |
| RD9 | 5' GTGTAGGTCAGCCCCATCC 3'<br>5' GTAAGCGCGTGGTGTGGA 3' | *M. tuberculosis* | 369 bp |

are based on the physical partitioning between two phases (stationary and mobile) of single lipids present in the mycobacterial cell wall[41]. MODS indirectly exploits the physical and morphological properties that arise from the unique lipid content of the M. tuberculosis cell wall. The characteristic observed "cording morphology" is due to the composition of the cell wall lipids, including mycolic acids.

Microscopic-observation drug-susceptibility (MODS) is a low-cost and simple tool for the high-performance detection of tuberculosis (TB). The MODS test is based on three principles: 1) *Mycobacterium tuberculosis* grows faster in liquid media compared to solid media; 2) The growth of *M. tuberculosis* can be detected earlier microscopically in liquid media compared to the macroscopic colony growth on solid media, and this growth pattern is characteristic of *M. tuberculosis*, which allows it to be distinguished from atypical mycobacteria or fungal and bacterial contaminants; 3) Drugs such as isoniazid and rifampicin can be incorporated into the MODS test to enable direct detection of multidrug-resistant TB (MDR-TB)

**Table 13.** The score lesion of lung damage of rats dosed with a combination extract of *Andrographis paniculata*, *Nigella sativa*, and propolis against Mycobacterium tuberculosis infection

| Group | Pneumonia | Nodule | Lymphoid folicle | Fibrosis | Hemosiderosis | Overall score of lung lesion |
|---|---|---|---|---|---|---|
| Normal Control | 1 | 0 | 0 | 0 | 0 | 1 |
| Negative Control | 2 | 1 | 2 | 0 | 0 | 2 |
| Positive Control | 2 | 1 | 1 | 0 | 1 | 2 |
| Formula A | 3 | 0 | 1 | 1 | 0 | 3 |
| Formula B | 3 | 0 | 1 | 0 | 0 | 3 |
| Formula C | 0 | 0 | 1 | 0 | 0 | 1 |

***Score 1 (minimal)****: The distribution of tissue damage is ≤25%;* ***Score 2 (mild)****: The distribution of tissue damage is 26% – 50%;* ***Score 3 (moderate)****: The distribution of tissue damage is 51% – 75%;* ***Score 4 (severe)****: The distribution of tissue damage is ≥75%.*

simultaneously, eliminating the need for subculture to perform drug susceptibility testing indirectly[42]. MODS is based on the visual identification of the typical cord pattern of *M. tuberculosis* colonies during their growth in liquid phase[43]. This method relies on observing the formation of the characteristic cords of *M. tuberculosis*, which can be seen microscopically in liquid media using an inverted microscope.

The advantages of using MODS are as follows: a) The sensitivity for detecting *M. tuberculosis* is 97.8% for MODS compared to 89.0% for Automated Mycobacterial Culture and 84.0% for Löwenstein-Jensen medium (P<0.001); b) The median turnaround time is 7, 13, and 26 days for MODS, Automated Mycobacterial Culture, and Löwenstein-Jensen medium, respectively (P<0.001); c) The sensitivity for detecting *M. tuberculosis* using MODS is 97.8% compared to 89.0% for Automated Mycobacterial Culture and 84.0% for Löwenstein-Jensen medium (P<0.001); d) The MODS method is fast, inexpensive, sensitive, and specific for detecting *M. tuberculosis* and performing drug susceptibility testing[44]. Therefore, the MODS (liquid media) method is faster in the growth of *Mycobacterium tuberculosis* compared to solid media.

### 3.4. In vivo test results: Immunomodulatory effects on infected with rats *Mycobacterium tuberculosis*

3.4.1. *Body weight of rats*

In this study, it was observed that the body weight of rats infected individually decreased during the post-infection period (Table 6). However, with the paired sample test between post-infection and post-treatment, a Sig (2-tailed) value of 0.591 > 0.05 was obtained, indicating that there was no significant difference in the body weight of the rats between post-infection and post-treatment. Based on the study by Nikonenko et al.[45] it was explained that mice infected with *M. tuberculosis* H37Rv on day 20 consistently lost more than 25% of their body weight, whether treated or not treated. Major et al.[46] explained that lung infiltration by granulomatous pneumonic inflammation was significantly higher in mice with a 10% body weight loss compared to

**Table 14.** Average percentage of spleen damage and average lesion scores of rats dosed with a combination extract of *Andrographis paniculata*, *Nigella sativa* and propolis against *Mycobacterium tuberculosis* infection.

| Group | Average lesion score on spleen tissue | | | Overall lesion score of spleen tissue |
|---|---|---|---|---|
| | Splenonekrosis | Hemosiderosis | Hyperemia | |
| Normal Control | 0 | 0 | 0 | 0 |
| Negative Control | +2 | 0 | 0 | +2 |
| Positive Control | +1 | +2 | 0 | +2 |
| Formula A | 0 | +2 | 0 | +2 |
| Formula B | 0 | +2 | 0 | +2 |
| Formula C | 0 | +1 | 0 | +1 |

those without clinical signs.

3.4.2. *White blood cell count and its derivatives examination*

Based on the results of the hematology examination, the average monocyte values in the groups were as follows: Formula A group = 0.58, Formula B group = 0.54, Formula C group = 0.58, positive control group = 0.48, negative control group = 0.56, and normal group = 0.42 (10^9/L) (Table 7). From these data, it can be observed that the *M. tuberculosis*-infected rats have higher monocyte values than the normal group, indicating that an infection is present in the rats. The normal group, which is uninfected, has normal monocyte values. The increase in innate immunity (monocytes) above normal indicates that the immune system is actively fighting the infection. The extract formulations have higher monocyte levels compared to other intervention groups, suggesting that the extract is capable of modulating the innate immune system to combat the entering bacteria. Monocytes are a general morphological term for cells that rapidly differentiate (within hours) into macrophages or dendritic cells. These are immune cells that not only phagocytize invasions but also process them for presentation to the adaptive immune system. Macrophages are found in circulation and tissues, known as alveolar macrophages in the lungs. They have surface receptors rich in mannose and fructose, which nonspecifically recognize components commonly found on pathogens, and more specific receptors capable of recognizing unique microbial components such as LPS from Gram-negative bacteria. They also possess receptors that recognize antibodies and complement proteins[8].

The response of granulocytes in infected rats is part of the acute inflammatory response. Among the granulocyte series, the most active cells are polymorphonuclear neutrophils (PMNs). These cells have a characteristic nucleus with several lobes and cytoplasmic granules that contain lytic enzymes and antimicrobial substances, including peroxidase, lysozyme, defensins, collagenase, and cathepsin. PMNs have surface receptors for antibodies and complement and are active phagocytes. In addition to digestive enzymes, PMNs have both oxygen-dependent and oxygen-independent pathways to kill microorganisms, unlike macrophages[3]. Many immune cells circulate in the blood as white blood cells. In addition, the blood also delivers precursors that develop into tissue immune cells. The circulating immune cells include granulocytes (polymorphonuclear leukocytes, PMNs), which consist of neutrophils, eosinophils, and basophils; lymphocytes; and

**Table 15.** Average percentage of liver damage and average score lesion in rats dosed with a combination extract

| Group | Lesion Score on Liver Tissue | | | | Overall score lesion of liver |
|---|---|---|---|---|---|
| | Hepatitis | Necrosis | Degeneration | Hemosiderosis | |
| | 0 | 0 | 0 | 0 | 0 |
| Normal Control | 1 | 0 | 0 | 0 | 1 |
| Negative Control | 1 | 0 | 1 | 0 | 1 |
| Positive Control | 1 | 0 | 0 | 1 | 1 |
| Formula B | 1 | 0 | 0 | 0 | 1 |
| Formula C | 1 | 0 | 0 | 0 | 1 |

monocytes. The immune response in tissues is further strengthened by these cells after extravascular migration, as well as tissue macrophages (derived from monocytes) and mast cells (associated with basophils). Working together, these cells provide a strong defense for the body[47].

3.4.3. *Red blood cell count in rat groups*

Some rats exhibited anemia, as indicated by hemoglobin levels below normal (Table 8). Anemia can be caused by acute blood loss, hemolysis, chronic diseases such as infections, endocrine disorders, kidney dysfunction, bone marrow failure, and infiltrative metastatic diseases in the bone marrow. Patients with tuberculosis (TB) often experience anemia due to the chronic inflammatory response in the body. During the inflammatory process, the body produces pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α). These cytokines can interfere with the production of erythropoietin (the hormone that stimulates red blood cell production) and affect red blood cell production in the bone marrow. They can also reduce iron availability by redirecting iron to body depots (e.g., liver) and inhibiting its use in hemoglobin production. The effect of these cytokines on erythropoietin production, which is induced by hypoxia, can lead to chronic anemia[48].

3.4.4. *The levels of interleukin 6 and interleukin 10 in the rat group*

From the comparison of interleukin 6 levels post *Mycobacterium tuberculosis* infection and post-treatment using the Wilcoxon statistical test, an Asymp Sig (2-tailed) value of 0.028 < 0.05 was obtained (Table 9), which indicates a significant difference in the mRNA expression levels of interleukin in all groups. During *Mycobacterium tuberculosis* infection, there was an increase in IL-6 levels, which is part of the acute inflammatory response that to occurs immediately after infection. IL-6, as a pro-inflammatory cytokine, plays a role in activating immune cells and assisting the body in fighting infection. In addition to innate and adaptive immunity that combats *M. tuberculosis*, the combination of extracts can help modulate the immune system to repair inflamed tissues, thereby reducing IL-6 levels in the blood. In

**Table 16.** The results of HPLC analysis on Formula C

| Formula | Extract | Compound | Concentration (mg/mg) ± SD | Linear regression equation ($r^2$) |
|---|---|---|---|---|
| Formula C 450 mg/kgBB (1:1:1) | *Andrographis paniculata* | Andrographolide | 0.0225 ±0.0002 | y= 21586x – 6613.8 $R^2$ = 0.9998 |
| | *Nigella sativa* | Thymoquinone | 0.00013±0.00001 | y= 77818x – 37352 $R^2$ = 0.9999 |
| | Propolis | Kuersetin | 0.007316± 0.0014 | y = 0.0076x + 0.0348 $R^2$ = 0.9971 |

the mRNA expression of IL-6, all extracts were able to reduce IL-6 levels, meaning that the extract combination acted as an immunomodulator, activating interleukin 6 as a pro-inflammatory effect and interleukin 10 (Table 10) as an anti-inflammatory effect. The increase in IL-6 in serum indicates infection or tissue damage, with macrophages activating IL-6 and recruiting other immune cells as a response to inflammation in the area infected by M. tuberculosis. The specific immune response to antigens requires at least 7 to 10 days to develop; this time is necessary for the proliferation and differentiation of T and B lymphocytes specific to the antigen. During the development of the specific immune response, the pathogen can continue to proliferate in the host or be controlled by innate immunity and inflammatory mechanisms[49].

*Andrographis paniculata* (Sambiloto) can function as an immunostimulator, enhancing the immune response when the body's immunity is weakened. It can also act as an immunosuppressor, reducing immune responses when the immune system becomes overactive beyond normal body conditions. Furthermore, as an immunomodulator, *Sambiloto* is capable of normalizing the body's condition, even in the presence of an infection[18].

Based on the research by Wang et al.[20] andrographolide can modulate both innate and adaptive immune responses by regulating macrophage phenotype polarization and the production of antigen-specific antibodies. The MAPK (Mitogen-Activated Protein Kinase) and PI3K (Phosphoinositide 3-Kinase) signaling pathways are crucial in cells that regulate various processes, including macrophage activation and polarization. These pathways are involved in the mechanism through which andrographolide regulates macrophage activation and polarization[20].

IL-17 stimulates the production of IL-1, IL-6, TNF-α, CXC chemokines, IL-22, and antimicrobial peptides from ILCs, alveolar macrophages (AM), and cellular constituents of the alveolar–capillary membrane. Signaling through receptors that utilize the JAK (Janus kinase)/STAT pathway, IL-6 activates many elements of the acute phase response, including the production of fibrinogen, serum amyloid A protein, and C-reactive protein by the liver. IL-6 also has thrombopoietic activity, both directly (minor effect) and through stimulating thrombopoietin production (major effect), which promotes platelet production, often depleted during serious infections[50]. Anti-inflammatory molecules, including IL-10 and IL-1 receptor antagonists, produced by NKT cells, T cells, exudate macrophages, and myeloid-derived suppressor cells, contribute to the resolution of inflammation[49].

Macrophages can be activated and phenotypically polarized by various stimuli and microenvironments. In general, activated and polarized macrophages can be classified into two main groups: classically activated inflammatory macrophages (M1), which produce large amounts of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, IL-18, and IL-23; focus-

ing on the induction and effects of cellular immune responses. Alternatively activated macrophages (M2) produce other cytokines such as IL-10, IL-1Ra, and IL-18BP. M1 participates in the Th1-polarized response as inducers and effectors, while M2 supports the effects and functions associated with Th2 (T helper 2) involved in tissue regulation and repair, as well as humoral immune responses[15]. IL-10 inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12, and antigen presentation by monocytes or macrophages by downregulating MHC II (molecules important for antigen presentation to CD4+ T cells) and co-stimulatory molecules (molecules like CD80 and CD86, which are needed to provide additional signals to T cells for effective activation)[51].

The research conducted by Slamet et al.[17] explains that the *Andrographis paniculata* extract containing 14.8% andrographolide was shown to reduce the expression of TNF-α (a pro-inflammatory cytokine involved in the immune response to pulmonary tuberculosis) in rats induced with CFA (Complete Freund's Adjuvant). The *Andrographis paniculata* extract also demonstrated a reduction in the number of pulmonary granulomas in rats induced with tuberculosis using CFA. There was a positive correlation between the strong expression of TNF-α and the number of granulomas in the lungs of rats induced with tuberculosis by CFA[12].

The research conducted by Mahmud et al.[14] explains that thymoquinone (TQ) administered at concentrations between 12.5 to 25 μg/mL and 6.25 to 12.5 μg/mL was able to reduce the number of *M. tuberculosis* H37Rv and XDR-TB in RAW 264.7 cells after 72 hours of infection. This indicates that TQ has antibacterial activity against both types of bacteria. Additionally, TQ also reduced the production of nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) in cells infected with H37Rv. This shows that TQ has anti-inflammatory effects by decreasing the level of inflammation caused by the infection[9].

The study by Salem et al.[52] notes that *Nigella sativa* is a complex substance containing more than 100 compounds. The combination of fatty acids, volatile oils, and other compounds is believed to contribute to its effectiveness. Published research frequently highlights the immunomodulatory potential and immunotherapy potential of the active compounds in *Nigella sativa*, particularly thymoquinone (TQ). The efficacy of TQ as an immunotherapy is associated with its antitoxic, antihistamine, and anti-inflammatory properties[52].

The study conducted by Wolska et al.[53] explains that the flavonoids and phenolic acids from propolis compounds, such as caffeic acid (cinnamic acid) phenethyl esters and artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), demonstrate immunomodulatory effects on lymphoid cells or monocytes mediated by kinase signaling pathways regulated by extracellular signal 2 and mitogen-activated protein kinases, as well as by eukaryotic transcription factors: TNF alpha activation and TNF kappa B. Both in vitro and in vivo tests have shown that propolis activates monocytes/macrophages and neutrophils, enhancing their microbicidal activity. Propolis also boosts the lytic activity of natural killer cells against tumor cells[53].

3.4.5. *Mycobacterium species multiplex PCR examination*

Based on the PCR results (Tables 11 and 12), the rats from the positive control, negative control, formulas A, B, and C showed positive results for bacterial DNA through PCR testing, while group F, which was uninfected and untreated, showed negative results. Bacterial infections in rats can lead to significant health disturbances. Testing the combination extract of sambiloto, black cumin, and propolis therapy is expected to demonstrate potential therapeutic effects in addressing such infections. The objectives of this test are: 1) to differentiate between *Mycobacterium tuberculosis* complex (MTBC) and non-tuberculous mycobacteria (NTM) using rv0577 or RD750; 2) to differentiate *M. tuberculosis* (MTB) from *Mycobacterium tuberculosis* complex (MTBC) using RD9[31].

3.4.6. *Histopathological examination of lung condition*

Pneumonia was observed in the normal group,

negative control group, positive control group, as well as in groups A and B, while no pneumonia was found in group C (Table 13). Pneumonia results from the proliferation of pathogenic microorganisms at the alveolar level and the host's immune response to them. The entry of these pathogens occurs through microaspiration of oropharyngeal organisms into the lower respiratory tract. The failure of both innate and adaptive immune systems to combat these microorganisms can lead to the clinical syndrome of pneumoniaioid[54]. Granulomas in *M. tuberculosis* infections are formed as a result of an immune response that activates macrophages, triggered by mediators or cytokines released by macrophages and activated T cells. The characteristic appearance of epithelioid granulomas includes macrophages and epithelioid cells. In *M. tuberculosis* infection, granulomas may be accompanied by the formation of necrotic tissue at the center, resulting from macrophage lysis.

The scoring method applied in this experiment was based on the ordinal method[55,56]. The ordinal method is defined as the distribution of lesions occurred repeatedly in animal tissues such as epithelial necrosis, inflammation and hemorrhage[55,56]. The lesion score is divided into 4 categories such as score +1 (mild), score +2 (mild), score +3 (moderate) and score +4 (severe) found in several fields of observation minimum for 4 observation fields. Since observation of lesion are taken for 4 field of observation, the lesion score may then be determined as an average score. The magnification of observation for the lesions is varied for x40 to x100.

The overall score of lung lesion in rat dosed with the extract combination against tuberculosis infection was between +1 (mild) an +3 (moderate). Pneumonia seen in normal control group seem not to be caused by tuberculosis infection, but due to external factors such bedding of cages and animal house hygiene. Pneumonia and nodules in lungs are commonly regarded as significant pathological changes in tuberculosis infection. The present study showed that pneumonia, nodule and lymphoid follicles were found in all group of animals. Pneumonia and nodule are regarded as a significant lesion of M. tuberculosis infection. These lesions are seen only in negative and positive control groups. Pneumonia can be seen in all groups of animals except the formula C. The formula A, B and C seem to inhibit the development of nodule in lungs of the treated animal, where nodules were not developed in these groups, and pneumonia were still present in formula A and B. The formula C seems to be a better formula to treat M. tuberculosis infection in this study, since both lesions of pneumonia and nodule did not develop in formula C. Histopathologically, each nodule represents a granuloma with central caseous necrosis, surrounded by mononuclear inflammatory cells. These nodules are typically located in the peribronchiolar interstitium. Tuberculomas appear as nodules with well-defined and smooth edges[57].

Lymphoid follicles were found in all groups of animals except for control normal group. The negative control group had a lesion score of +2, higher than other intervention groups. This lesion is due to an in progress chronic inflammation. Lymphoid follicles are the structures of lymphoid tissue consisting of clusters of immune cells. The lymphoid follicles play an important role in the immune response to infections or antigens. Proliferation of lymphoid cells may occur in these follicles. The role of B cells in TB infection is observed from the fact that B cells form follicle-like aggregates (inducible lymphoid follicles / ILF)[58]. The formula C group showed less lung tissue damage compared to other interventions, proving that the combination of extracts can modulate cytokines by reducing IL-6 (pro-inflammatory cytokine) and increasing IL-10 (anti-inflammatory cytokine). The IL-10 examination results for group C post-infection were 335.7782 pg/ml, which decreased to 187.1062 pg/ml after treatment with the extract, and the IL-6 levels also showed a decrease. This indicates that the inflammation is tending to improve.

3.4.7. *Observation of the histopathological condition of the spleen in all groups*

Based on the lesion scores of spleens in rats dosed with a combination extract of *Andrographis panicu-*

*lata*, *Nigella sativa* and propolis against *Mycobacterium tuberculosis* infection, all intervention groups of rats received a score of 2 (indicating 25–50% tissue damage), except for the normal group (Table 14). The lesions of spleen tissue consisted of splenonecrosis, hemosiderosis, and hyperemia. The prominent lesions found in spleens were haemosiderosis indicating that systemic haemorrhage were found in the animals. The formula C may reduce hemosiderosis or systemic haemorrhage in rats infected with M. tuberculosis indicating that the lesion score was +1. Immune-responsive cells were found throughout the body, both in circulation and in fixed locations within tissues. These cells are concentrated in lymph nodes and the spleen, forming integrated filtration networks designed as a warning system. In the lymphoid series, cells become mature T cells in the thymus. Therefore, the thymus, spleen, and lymph nodes can be considered as organs of the immune system. Collectively, these organs are referred to as lymphoid tissue[8].

The spleen has three adaptive functions: (a) eliminating bacteria and particles from the blood (the spleen filters blood to remove bacteria, foreign particles, and old or damaged cells); (b) generating immune responses to specific pathogens, where the spleen plays a role in shaping and coordinating the body's immune response to pathogens (viruses, bacteria, etc.); and (c) producing blood cellular components in situations where the bone marrow cannot meet the needs (i.e., extramedullary hematopoiesis). The spleen can take over this function and produce new blood cells[59]. The lesion scores on spleen tissue were similar across all groups.

3.4.8. *Observation of histopathological changes of liver in rats dosed with a combination extract*The liver damages showed that the score lesion of liver were +1 in all groups of animals, the liver showed hepatitis, degeneration of epithelial cells of liver and hemosiderosis. Hepatitis was the prominent lesion found in all groups of animals. Hepatic degeneration was only found in negative control group with score lesion of +1 and hemosiderosis appeared in positive control group with score lesion of +1 (Table 15). Hepatitis may be due to various factors such as infectious diseases, intoxication and heat stress. Since all groups of rats had mild hepatitis, this lesion appeared to be due to an early reaction either to the infection of M. tuberculosis, treatment of animals with extract combination and the internal environment where the animal being kept and raised. Interleukin-6 is secreted by macrophages and mast cells, and possibly also by non-immune cells such as muscle and fat cells. Its primary function is to signal the liver to increase the production of acute-phase proteins, which then enter circulation and cause fever and cachexia. It also triggers the release of new neutrophils from the bone marrow, which is detected as an increase in white blood cell count, or leukocytosis[60]. Many cases of chronic hepatitis are considered immune-mediated attacks on the liver. Evidence that this disorder is mediated by the immune system includes liver biopsy findings showing inflammation (lymphocyte infiltration) in characteristic areas of the liver (e.g., portal versus lobular areas). Additionally, various autoimmune disorders occur with high frequency in patients with chronic hepatitis[61]. The lesion scores on liver tissue were similar across all groups.

3.4.9. *Levels of Active Compounds in the Extract Combination at a Concentration of 450 mg/kg Body Weight*

Among the three tested formulas, Formula C demonstrated the least tissue damage and inflammation in the lungs, spleen, and liver compared to Formulas A and B. This was supported by complete hematological laboratory data, as well as interleukin-6 and interleukin-10 levels.

The combination of black cumin, andrographis (sambiloto), and propolis exhibits a synergistic effect as an immunomodulator. Previous studies have shown that andrographolide significantly inhibits the production of pro-inflammatory cytokines by Mtb-infected macrophages and suppresses the production of IL-6 and TNF-α at concentrations of 25 μM and 50 μM[19]. Furthermore, andrographolide can modulate both innate and adaptive immune

responses by regulating macrophage phenotype polarization and the production of antigen-specific antibodies. The MAPK and PI3K signaling pathways may be involved in the mechanism by which andrographolide regulates macrophage activation and polarization[20]. Similarly, black cumin (thymoquinone) at a dose of 50 mg/kg body weight significantly increased CD68+ macrophages (pro-inflammatory). The CD68/CD163 macrophage ratio (pro- vs anti-inflammatory) was higher in the low-dose thymoquinone group (25–50 mg/kg body weight) compared to the positive control group. It was concluded that thymoquinone may inhibit M2 polarization of pulmonary macrophages in rats infected with Mycobacterium tuberculosis[62]. Consistently, propolis at a concentration of 200 mg/kg body weight increased the expression of TLR-2 and TLR-4 in peritoneal macrophages of rats treated with propolis. The expression of TLR-2 and TLR-4, as well as the production of IL-1β and IL-6, were also activated in splenocytes of propolis-treated rats. It was concluded that propolis initiates immune responses by enhancing TLR expression and pro-inflammatory cytokine production in rats, thereby modulating innate immune mechanisms[63]. Propolis and several of its constituents, such as CAPE, cinnamic acid, and artepillin C, influence the nonspecific (innate) immune system by modulating the activity of neutrophils and monocytes/macrophages[53].

Based on these findings, a synergistic effect may result from the combination of black cumin, andrographis, and propolis in modulating immune responses. Research has shown that the combination at a dose of 450 mg/kg body weight (1:1:1 ratio) exhibits immunomodulatory activity.

The subsequent test involved determining the concentration of active compounds in the extracts used in Formula C using analytical instrumentation namely HPLC instrument: ArcTM HPLC with PDA Detector. HPLC condition: Column: Eclipse plus® C18 (250 x 4.6 mm, 5 µm). Column temperature: 40°C, Solvent: A (0.2 Formic acid in Water) B (Methanol): 0.0 – 5.0 65% B (Hold); 5.0 – 6.0= 65% – 80% B (Gradient); 6.0 – 11.0 80% B (Hold); 11.0 – 15.0; 100 % B (Hold); 15.0 – 18.0 65%B (Hold). Flow rate: 1 ml/min.

## 4. Conclusions

This study demonstrated the potential immunomodulatory effects of active compounds derived from *Andrographis paniculata* (andrographolide), black cumin (*Nigella sativa*, thymoquinone), and propolis (quercetin) using a molecular docking approach. In vitro testing showed no growth of *Mycobacterium tuberculosis* using the MODS method at the minimum inhibitory concentrations of *A. paniculata* extract (6.325 mg/ml), black cumin extract (12.75 mg/ml), and propolis extract (25.5 mg/ml). Furthermore, in vivo experiments revealed that the combined extract formula C (1:1:1 ratio) at a concentration of 450 mg/kg body weight demonstrated significant immunomodulatory activity. This was evidenced by modulation of white blood cell and red blood cell counts, regulation of IL-6 and IL-10 cytokine levels, and histopathological improvements in inflammation of the lung, spleen, and liver tissues of rats infected with *M. tuberculosis*.

This study demonstrated the potential immunomodulatory effects of active compounds derived from *Andrographis paniculata* (andrographolide), black cumin (*Nigella sativa*, thymoquinone), and propolis (quercetin) using a molecular docking approach. In the in vitro testing, no growth of *Mycobacterium tuberculosis* was observed using the MODS method at the minimum concentration of *Andrographis paniculata* extract (6.325 mg/ml), black cumin extract (12.75 mg/ml), and propolis extract (25.5 mg/ml). Furthermore, in the in vivo testing, the combination of extract formula C with a concentration of 450 mg/kg body weight (1:1:1) demonstrated significant immunomodulatory activity. This was based on the examination of white blood cell count, red blood cell counts, regulation of IL-6 and IL-10 cytokine levels, as well as histopathological analysis which demonstrated improvements in inflammation in the lung, spleen, and liver tissues of rats infected with *M. tuberculosis*.

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**Author contribution**

A: concepts or ideas, design, literature search, experimental studies, data analysis, statistical analysis, manuscript preparation, editing, and review. SA: concepts or ideas, design, definition of intellectual content, literature search, data analysis, manuscript preparation, manuscript preparation, editing, and review. YS: concepts or ideas, literature search, experimental studies, manuscript preparation, manuscript editing and review. GA: concepts or ideas, definition of intellectual content, experimental studies, data analysis, manuscript editing, manuscript review. GFS: literature, data acquisition, data analysis, manuscript preparation and editing. Furthermore, all authors have approved the final version of manuscript.

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**Conflict of interest**

none to declare.

**Ethics approval**

Before conducting the experiment using animal subjects, an ethical review was performed, and approval was obtained from the ethics committee (Approval No. 037/KEPK-FFUP/XI/2024).

**Abbreviation List**

MODS: Microscopic-observation drug-susceptibility
IL-6: interleukin-6
IL-10: interleukin-10
CBC: complete blood count
RBC: Red Blood Cell
HGB: hemoglobin
HCT: hematocrit
MCV: Mean Corpuscular Volume
MCH: Mean Corpuscular Hemoglobin
MCHC: Mean Corpuscular Hemoglobin Concentration
PLT: Platelet
MPV: Mean Platelet Volume
PDW: Platelet Distribution Width
PCT: Procalcitonin
WBC: White Blood Cell
HE: Hematoxylin and eosin

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