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Original Research Article

Phytochemical Profile and *In vivo* Immunomodulatory Potential of Galing Plant (*Cayratia trifolia* L. Domin) Extract on various Immunological Parameters in Tuberculosis infection

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ABSTRACT

Pulmonary tuberculosis (TB) remains a significant global health concern due to its impact on immune system function and its association with high mortality rates in numerous countries. In efforts to reduce its prevalence, the bioactive natural products of galing plant (*Cayratia trifolia* L. Domin) are reported to have immunostimulatory effects with the potential of being developed into an immunomodulatory agents for pulmonary TB. This study aimed to assess the immunomodulatory potential of galing extracts on various immunological parameters in an *in vivo* model of tuberculosis infection and to evaluate their phytochemical profile. A total of 28 Wistar rats were allocated into groups: normal, negative control, positive control, and treatment groups receiving leaf and stem extracts at doses of 400, and 500 (mg/kg body weight), administered orally for seven days prior to infection with ESAT-6 TB antigen. The immunomodulatory activity was assessed by evaluating leukocyte subpopulations, macrophage phagocytic activity, cluster of differentiation 14, and interleukin-12 levels using ELISA. The chemical composition of the extracts were analyzed via Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS). Immunological data were statistically analyzed using one-way ANOVA followed by Tukey's post hoc test. The extract-treated groups demonstrated a significant increase in immunological parameters compared to the negative control group ($p<0.05$). LC-HRMS profiling identified several potentially novel bioactive compounds that may contribute to the immunomodulatory activity observed however, further validation is required. These findings provide new scientific evidence supporting the potential of galing extracts as natural immunomodulators for enhancing immune function in pulmonary TB.

Keywords: *Cayratia trifolia,* Immunomodulator, Immunological parameters, Phytochemical profile, Pulmonary tuberculosis

Introduction

Pulmonary tuberculosis (TB) is a globally prevalent infectious disease, affecting approximately 0.5% to 4% of the world's population, and frequently progresses into a chronic condition. It remains one of the leading causes of morbidity and mortality among infectious diseases[1]. Despite the availability of treatment, TB continues to pose a significant public health challenge due to suboptimal therapeutic outcomes, as reflected by persistently high incidence and morbidity rates worldwide. Annually, it is estimated that 10 million individuals are affected by TB, with over one million TB-related deaths reported. According to the Global Tuberculosis Report 2023, Indonesia ranks as the country with the second highest TB burden globally, following India and China[1].

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The number of TB cases increases by approximately 17% each year, largely due to the inability of the host immune system to effectively eliminate *Mycobacterium tuberculosis* (Mtb).[2] A compromised immune system facilitates the progression of Mtb infection, increasing the risk of severe TB and the development of complications, particularly in the presence of comorbidities such as diabetes mellitus and other infectious diseases.[3,4]

The immune system's efforts to overcome the invasion of Mtb pathogens are by destroying them through the phagocytosis activation pathway played by macrophage cells.[5,6] Macrophages infected with Mtb respond by secreting pro-inflammatory cytokines such as interleukin-12 (IL-12).[7] Furthermore, they can activate natural killer (NK) cells directly or through stimulation by IL-12 produced by macrophages. Activated NK cells produce the cytokine interferon-γ (IFN-γ) as a stimulator for macrophages in increasing the eradication of Mtb bacteria through phagocytic activity.[8,9] Previous research has reported several issues related to impaired immune responses in TB, particularly macrophages/cluster of differentiation 14 (CD14) - the first line of defense against TB infection, impaired secretion of IL-12 and IFN-γ cytokines - which act as effectors of non-specific immune cell activation, and failure to present TB antigens to lymphocytes for a specific immune response.[8,10] This highlights the importance of strengthening the immune system in TB patients with immunomodulatory compounds to accelerate healing and prevent complications from other infectious diseases.

The absence of effective immunomodulators remains a significant barrier to overcoming impaired immune system function in tuberculosis

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patients. In response, ongoing research is focused on identifying novel immunomodulatory agents with targeted mechanisms of action. One promising avenue involves the exploration of natural bioactive compounds, such as those derived from the galing plant (*Cayratia trifolia* L. Domin).[11,12] Several studies have proposed the use of galing as an immunostimulant, antidiabetic, antiinflammatory, and antioxidant agent, attributed to its diverse array of secondary metabolites. Traditionally, it has also been used empirically to treat conditions such as typhoid fever, postpartum infections, and to maintain general wellbeing.[13,14] Pharmacological investigations have demonstrated that galing exhibits a wide range of biological activities, including antioxidant, antiinflammatory, antidiabetic, antimicrobial, and immunostimulatory effects. These bioactivities are linked to various plant parts, all of which contain compounds such as flavonoids, tannins, steroids, and alkaloids.[11-15] Research by Yusuf *et al*.[11] reported its effectiveness as an immunostimulant in bacterial infections through enhancement of macrophage phagocytic activity. Other studies have shown that leaf extracts of galing possess significant anti-diabetic, anti-cancer, and antioxidant properties, supported by the presence of secondary metabolites such as flavonoids, alkaloids, tannins, triterpenoids, and saponins.[16-18] The most recent investigation by Ilyas *et al*.[12] revealed that ethyl acetate extracts of galing exert immunostimulatory activity by increasing interferon-gamma (IFN-γ) levels and CD14 expression in macrophages in an in vivo breast cancer model.

Despite previous evidence of immunomodulatory activity of galing plant, detailed research on the investigation of immunomodulatory potential with various immunological parameters in TB disease, as well as the profile of phytochemicals that support the immunomodulatory effect has not been reported. Therefore, it is important to do this, to reveal new scientific facts that support the further development of galing plants as potential immunomodulatory agents in pulmonary TB disease. Hence, this reserach is aimed at evaluating the phytochemical profile and *in vivo* immunomodulatory potential of galing plant extract on various immunological parameters in tuberculosis infection.

Materials and Methods

Chemicals, Reagents, and Equipment

The materials used in this study were galing plants, male white mice, TB antigen ESAT-6 / *Early Secreted Antigenic Target 6 kDa* (PepMix™), EDTA (ethylenediaminetetracetic acid), ethanol 96%, alcohol 70%, sulfuric acid 1%, $BaCl_2$ 1%, IL-12 ELISA kit, CD14 mouse anti-rat moAb (BioLab™), 0.5% Na-CMC, physiological NaCl, chloroform, distilled water, and commercial meniran® extract. The tools used were vacuum rotary evaporator (Stuart RE300,USA), hematology analyzer (Mindray BC-2600®, China), autoclave (Daihan Lab Tech®), analytical balance (Precisa®), oven (inaco®), incubator (Memmert®), laminar air flow (LAF) (E-Scientific®), electric microscope (Leica®), centrifuge (Boeco®), water bath (Stuart®), 1 cc, 3 cc syringes (Onemed®), and ELISA reader instrument (iMark™).

Plant collection, identification and determination

Fresh galing plants were collected from Kambu sub-district, Kambu district, Kendari City, Southeast Sulawesi Province, Indonesia (GIS coordinates: 4º5'25'S"122º33'41'E) in October 2024. The plant material was identified and determined as *Cayratia trifolia* L.Domin or galing plants in the Research Laboratory of the Faculty of Pharmacy, Universitas Halu Oleo.

Preparation and extraction of plant material

A total of 700 g of powdered galing leaves and 3600 g of stems were extracted using ethanol 96% (1500 mL and 7200 mL, respectively) by maceration at room temperature for 72 hours. The extracts were filtered repeatedly until complete exhaustion. The ethanol extracts were then concentrated using a vacuum rotary evaporator (Stuart RE300,USA) at 50°C under reduced pressure to obtain crude ethanol extracts.[4,19]

Acclimatization and Grouping of Experimental Animals

Preparation of experimental animals began with acclimatization. Acclimatization is the care of experimental animals with the goal of adapting to a new environment. The white mice used were first acclimatized for 7 days to adapt to the new environmental conditions (the white mice's cage). During this acclimatization period, the mice were fed pellets and water (ad libitum) and housed in cages at a temperature of 23°C (±30°C). This acclimatization was carried out to allow the mice to adjust to the new environment.[19] The grouping of experimental animals was calculated using the Federer formula. A total of 28 animals were divided into 7 treatment groups: normal control, negative control, positive control, and 4 animals each receiving galing leaf and stem extracts at doses of 400 and 500 mg/kg BW.[12] The guidelines for animal treatment follows the regulation of the Food and Drug Administration (BPOM) RI number 18 of 2021 concerning guidelines for preclinical pharmacodynamic testing of traditional medicines. This study has received approval from the Health Research Ethics Committee of the UHO LPPM (National Institute of Health Research) under the number: 0999/UN29.20.1.2/PG/2024.

Extract test preparations: extract test preparations were made by suspending each crude leaf and stem extract i.e. 1.8 g and 3.12 g (doses of 400 and 500 mg/kg BW) in 100 mL each in 0.5% Na-CMC with the volume of administration adjusted to the body weight of the test animal.[5,6]

Comparator Preparation: The comparator preparation used was commercial meniran® extract, suspended with 0.5% Na-CMC according to the effective oral human dose of 50 mg/kg BW. A total of 0.169 grams was suspended in 105 mL of 0.5% Na-CMC, adjusted to the dose administered to the experimental mice.[4,6]

Immunomodulator Test

The treatment was administered orally once daily for 7 days according to the dose volume. On the 8th day, each test group was infected with the TB ESAT-6 antigen intraperitoneally.[4,6] A total of 28 mice were used in this study, divided into 7 groups of 4 mice each. The distribution of the treatment groups is described as follows:

KN: normal control group without extract and not infected with ESAT-6 TB antigen
K-: negative control group given 0.5% Na-CMC + infected with ESAT-6 TB antigen
K+: positive control group with commercial meniran® extract 0.922 mg/kg BW + infected with ESAT-6 TB antigen
K1: test group with 400 mg/kg BW leaf extract + infected with ESAT-6 TB antigen
K2: test group with 500 mg/kg BW leaf extract + infected with ESAT-6 TB antigen
K3: test group with 400 mg/kg BW stem extract + infected with ESAT-6 TB antigen
K4: test group with 500 mg/kg BW stem extract + infected with ESAT-6 TB antigen

The choice of leaf and stem extract dosage was based on previous studies by Yusuf *et al*.[11] and Ilyas *et al*.[12] reported that galing extracts at doses of 400 and 500 mg/kg BW effectively increased the phagocytic activity of macrophage cells in bacterial infections and were proven to be immunostimulators in breast cancer by increasing IFN-γ cytokines and macrophage cell expression (CD14) *in vivo*.

Blood samples from all groups were taken 24 hours after TB antigen infection via intracardiac route. Blood samples were collected in EDTA vacutainer tubes and centrifuged after separation for 15 minutes at 3.500 rpm.[4,5]

Leukocyte Count and Expression Test

The total and expression counts of leukocytes (lymphocytes, monocytes, neutrophils, eosinophils, and basophils) were determined using a hematology analyzer (Mindray BC-2600®, China).[20,21]

Macrophage Phagocytic Activity Test

On the eighth day, each mouse (except the normal group) was infected with 0.2 mL of ESAT-6 antigen intraperitoneally and left for 24 hours. They were then anesthetized with ketamine HCl and then dissected using sterile surgical scissors and forceps. When a small amount of

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peritoneal fluid was found in the abdomen, 1 mL of sterile phosphate-buffered saline (PBS) pH 7.8 was added, gently agitated, and the peritoneal fluid was collected with a sterile syringe. Peritoneal fluid was smeared on a glass slide and fixed with methanol for 5 minutes, then stained with 10% Giemsa stain, left for 20 minutes, and rinsed with running water. After the preparation was dry, it was viewed under a microscope (Leica DM500®/Leica Microsystems/Jerman) using immersion oil at a magnification of 100x–1000x.[5,6]

Calculating phagocytic activity

The increase in phagocytic activity of peritoneal macrophage cells in test animals was achieved by calculating the percentage of macrophage cells actively phagocytosing Mtb antigens in 100 macrophage cells as in equation 1.[4,6]

$$\text{Phagocytic Activity} = \frac{\text{Number of macrophages active cell}}{\text{Total of macrophages cell}} \text{ x } 100\% \quad (1)$$

Measurement of Interleukin-12 (IL-12) and Cluster of Differentiation 14 (CD14) Using ELISA Method

The levels of IL-12 and CD14 were determined using a sandwich ELISA method, following the specific protocols provided in each kit. The procedure was carried out as follows:

A 96-well microplate was coated with TB antigen at a concentration of 2 µg/mL in 18 µL PBS per well and incubated overnight at 4°C. Each well was washed three times with 450 µL of 0.05% PBST20. The wells were then blocked with 0.5% BSA in PBS (100 µL per well) and incubated for 1 hour at 37°C. Subsequently, 100 µL of diluted plasma (1:4 in PBS) was added and incubated for 2 hours at room temperature. After three washes with 450 µL of 0.05% PBST20, 100 µL of specific isotype antibodies (IL-12 or CD14) were added to each well and incubated for 30 minutes at room temperature. The wells were washed again three times with 450 µL of 0.05% PBST20, followed by the addition of 100 µL of diluted IL-12 or CD14 antibodies (1:5000) to each well, and incubated for 15 minutes at room temperature.

After a final wash (3x) with 300 µL of 0.05% PBST20, 100 µL of TMB substrate was added and incubated for 15 minutes at room temperature. The substrate reacted to produce a color change, which was stopped by adding 100 µL of 2 N H_2SO_4, resulting in a yellow color. The absorbance was then measured at a wavelength of 450 nm using an ELISA microplate reader.[5,12]

Chemical Compound Profiling

The chemical compound profile of the leaf and stem ethanol extract of galing plant was analyzed using Liquid Chromatography–High Resolution Mass Spectrometry (LC-HRMS) employing a Xevo G2-XS QTOF system (Milford, USA). The mobile phase was composed of solvent A (0.1% formic acid in water) and solvent B (acetonitrile with 0.1% formic acid). A 1 µL aliquot of the extract was injected into the LC system. Compound separation was achieved based on polarity and differential retention times, resulting in distinct peaks visualized in the chromatogram. The LC-HRMS data provided detailed information, including peak intensity and corresponding molecular weights of the detected compounds. These data enabled identification and characterization of the chemical constituents in the extract, including molecular mass, structural features, and relative abundance of each component.[4,22]

Statistical Analysis

Statistical analysis was conducted using one-way Analysis of Variance (ANOVA), with a significance threshold set at $p<0.05$ and a 95% confidence interval, under the assumption of normal data distribution. Significant differences among groups were further evaluated using the least significant difference (LSD) post hoc test. All statistical computations were performed using the statistical package for the social sciences (SPSS), version 27 (IBM, USA).

Results and Discussion

Investigation of the Immunomodulatory Potential of Galing Extract
Evaluation of Total Leukocyte Count and Leukocyte Type Expression

The investigation of the immunomodulatory potential of ethanol extract of leaves and stems of galing plant was carried out by evaluating several immunological parameters, namely the number and expression of leukocyte types (lymphocytes, monocytes, eosinophils, neutrophils, and basophils), phagocytic activity of macrophage cells, and IL-12 and CD14 levels in mice as a model of TB infection. The TB infection model was established via intraperitoneal administration of the ESAT-6, a highly studied antigen in TB pathogenesis due to its direct involvement in *Mycobacterium tuberculosis* (Mtb) virulence and its role in modulating host immune responses.[23] Leukocyte quantification and differential analysis were performed using a hematology analyzer, which offers advantages such as minimal sample volume, rapid processing, and no requirement for complex sample preparation.[24] This method enabled the determination of both total leukocyte counts and the relative proportions of specific leukocyte populations. The administration of galing extract at various doses led to a significant increase in both the total leukocyte count and the expression of leukocyte subtypes specifically lymphocytes, neutrophils, monocytes, eosinophils, and basophils in mice challenged with the ESAT-6 antigen. These findings suggest a positive immunostimulatory effect of the extract on host immune function in the context of TB infection. The data regarding leukocyte profile
alterations following extract administration are presented in **Figure 1**.

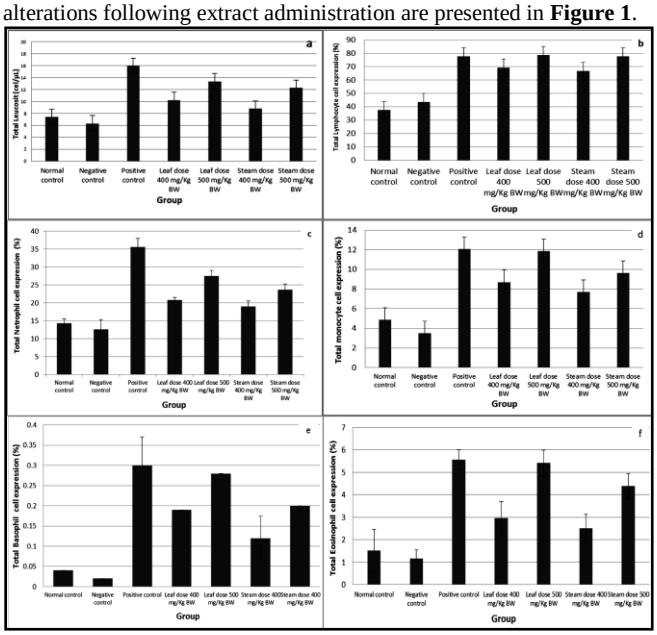

Figure 1: Graph of the total number and expression of leukocyte cell types in each treatment group; a. total number of leukocytes; b. lymphocytes; c. neutrophils; e. monocytes; e. basophils; f. eosinophils

The total number of leukocytes (Fig. 1a) in the treatment group with all doses of galing leaf and stem extract was higher than the group without extract (negative control), but was not better than the positive control in increasing the number of leukocytes. This indicates the potential of galing to increase the number of leukocytes, where an increase in the number of leukocytes is needed in the early stages of TB infection, while also illustrating the initial immune response of the innate immune system. To determine the increase in leukocyte types, it is necessary to know the types of leukocytes that play a role in fighting TB infection by measuring their expression. The results showed that the number of lymphocyte cells (Fig. 1b), neutrophils (Fig. 1c), monocytes (Fig. 1d), basophils (Fig. 1e) and eosinophils (Fig. 1f) also increased compared to the group without extract treatment (negative control) and the normal control. This proves that the increase in the number of leukocytes is directly related to the increase in the expression of these types of

leukocytes, although it is still higher than the positive control group that is given a standard commercial meniran extract preparation. Types of leukocytes such as lymphocytes, monocytes and neutrophils are very necessary in fighting TB infection through their function as professional phagocytic cells, to prevent the development of chronic TB infection.[25] The observed increase in leukocyte count and subtype expression in the experimental animals was attributed to the presence of bioactive chemical constituents in the galing plant, which are believed to promote leukocyte proliferation and activation. This finding is consistent with previous studies conducted by Yusuf *et al*.[11], and Thawkar *et al*.[14], which identified various secondary metabolites in galing, including alkaloids, tannins, saponins, triterpenoids, and flavonoids. Among these, flavonoids and alkaloids have been reported to enhance the production of interleukin-12 (IL-12), a cytokine known to augment phagocytic and endocytic activity in immune effector cells. IL-12 also enhances the antimicrobial activity of monocytes, lymphocytes, and, neutrophils by stimulating the secretion of lysozyme, granzymes, and other pathogen-destroying enzymes.[26]

Tannin compounds, moreover, have demonstrated the ability to modulate physiological functions by stimulating phagocytic activity, and exhibiting antitumor and antibacterial effects.[27] Several secondary metabolites identified in the current study further support the immunomodulatory role of galing. For instance, the compound (+)-ar-turmerone (Fig. 7a) is known to modulate immune responses by influencing the functional activity of T lymphocytes and macrophages.[28] Likewise, abscisic acid and 12-oxo-phytodienoic acid (Fig. 7b and 7c) have been shown to stimulate IL-12 production in macrophages and other immune cells, while also inhibiting pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) and enhancing anti-inflammatory cytokine IL-10 production, particularly under infectious conditions.[29] Another compound, 2,2,6,6-Tetramethyl-1-piperidinol (TEMPO), exhibited immunostimulatory effects by enhancing the activation and cytotoxic function of natural killer (NK) cells and modulating T lymphocyte activation.[30] Additionally, 4-coumaric acid, a phenolic compound found in galing, was reported to enhance T lymphocyte proliferation and overall leukocyte production *in vivo*.[31]

Evaluation of Macrophage Cell Phagocytic Activity

Macrophage cell phagocytic activity was measured 24 hours after mice were infected with the ESAT-6 antigen. This was done to observe the macrophage's ability to phagocytose the antigen through TLR-2 recognition, initiating the phagocytosis process. Macrophages secrete various cytokines to activate naive macrophages and specific immune cells, such as helper lymphocytes, to aid in the killing of TB antigens.[32] Macrophage phagocytic activity was determined by observing active and inactive macrophages after Giemsa staining. This facilitates the identification of specific cell morphology, produces clear nuclear images, and provides longer shelf life in tropical climates. Giemsa staining shows the nucleus of macrophage cells in bluish purple.[5,6] Phagocytic activity was determined by counting active macrophage cells in 100 macrophage cells expressed in percentage (%), active macrophages will increase in size, where phagosomes, lysosomes, golgi apparatus and endoplasmic reticulum will develop, so that the structure in macrophage cells will appear enlarged, while inactive macrophages are characterized by smaller shapes and sizes, in observations of thin blood smears under a light microscope with a magnification of 1000x.[4,6] The results of observations of phagocytic activity can be seen in Figure 2.

Furthermore, tannins present in galing have demonstrated immunostimulatory effects by augmenting the function of phagocytic cells, especially macrophages, in the context of infectious diseases.[14,15] The findings from the LC-HRMS analysis (**Fig. 7a–f; Table 1**) revealed the presence of several candidate compounds in the galing extract that may enhance the activity of macrophages, monocytes, neutrophils, and lymphocytes by modulating the production of immune-activating cytokines, including IL-12, and IFN-γ. These insights provide further evidence supporting the immunomodulatory potential of galing and its candidacy as a novel agent in adjunctive therapy for tuberculosis.[33–35]

Interleukin-12 Level Evaluation

The sandwich ELISA technique was used to measure IL-12 levels due to its high sensitivity for detecting the presence of target antigens in low-level samples. IL-12 is considered to play a crucial role in TB pathogenesis. A balanced IL-12 level plays a strategic role in enhancing non-specific cellular immune responses, such as macrophages and NK cells, and as an activating cytokine for T lymphocytes and B cells, contributing to specific cellular and humoral immune responses.[8,9] The IL-12 analysis results obtained in each treatment group can be seen in Figure 3.

According to the results presented in Figure 2, the average phagocytic activity of macrophages in the groups treated with galing leaf and stem ethanol extracts at all administered doses demonstrated a notable enhancement in phagocytic function compared to the negative control group (TB-infected without extract treatment). The data further indicate a dose-dependent relationship, whereby increased extract concentrations correlate with elevated phagocytic activity in TB-infected animal models. This enhancement is likely attributed to the presence of bioactive constituents in the extract that potentiate macrophage functionality during antigenic challenge. Previous studies by Yusuf *et al*.[17] have reported that galing ethanol extracts are rich in secondary metabolites, including alkaloids, tannins, saponins, triterpenoids, and flavonoids. Among these, flavonoids and alkaloids are known to stimulate the secretion of cytokines such as interleukin-12 (IL-12), and interferon-gamma (IFN-γ), which serve as critical mediators in macrophage activation and enhancement of phagocytic and endocytic responses against Mtb antigens.[5] These cytokines also improve macrophage antimicrobial mechanisms by upregulating lysosomal enzyme activity, increasing surface receptor expression, and facilitating pathogen recognition and intracellular destruction.[8,32]

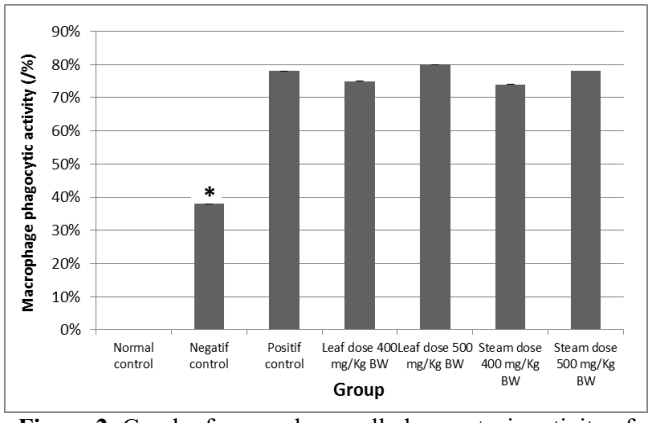

Figure 2: Graph of macrophage cell phagocytosis activity of each treatment group (*p<0.05=post hoc LSD test is significantly different from the negative control group)

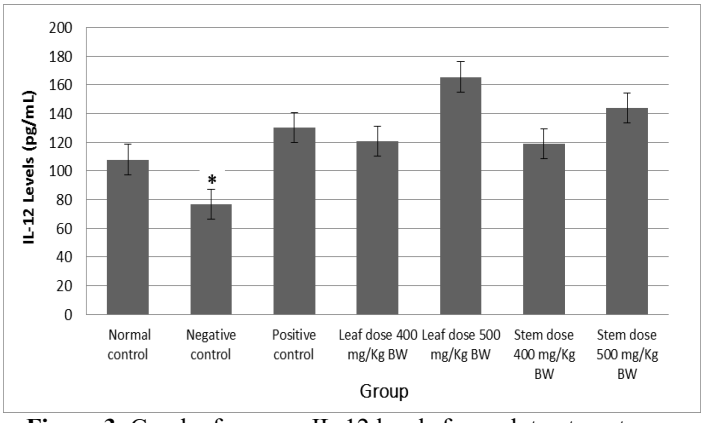

Figure 3: Graph of average IL-12 levels for each treatment group (*p<0.05=post hoc LSD test significantly different from the negative control group)

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Based on the findings in Figure 3, it can be evaluated that there was an increase in IL-12 levels in all groups given galing leaf and stem extract doses, where the highest increase in IL-12 occurred at a leaf and stem dose of 500 mg/kg BW compared to all other treatment groups. This shows that the administration of leaf and stem extracts is indicated as a potential immunomodulator in mice infected with TB based on the increase in IL-12 levels. The results of the one-way ANOVA statistical test and the LSD post hoc test showed that all groups of galing extract doses had a significant difference (*p<0.05) with the negative control (Na-CMC), this explains that the administration of galing extract was able to increase IL-12 levels significantly in TB infection conditions, so it has the potential to be developed as an immunomodulatory agent for TB patients. This increase in IL-12 is certainly influenced by the role of secondary metabolites or bioactive compounds in galing, as previously reported, which have been shown to contain secondary metabolites flavonoids, tannins, alkaloids, terpenoids, and saponins, which are suspected to be potential immunomodulatory agents.[14] The increase in IL-12 secretion by immune cells can be modulated by plant compounds such as flavonoids and alkaloids by stimulating lymphocyte cell proliferation, increasing the number of T lymphocytes and increasing IL-12 activity, increasing the activation of macrophage effector cells and NK cells in carrying out antigen phagocytosis activity. In addition, it triggers the release of IL-12, and IFN-γ cytokines as activators of specific cellular immune responses.[15,27] Previous research by Yusuf *et al*.[11] reported that ethanol extract of galing plants at doses of 400, and 500 (mg/kg BW) was shown to increase the non-specific immune response (innate immunity) through macrophage cell phagocytosis activity in test animals infected with *Staphylococcus aureus* bacteria. Other studies also reported that the galing plant was proven to be an immunostimulator in breast cancer by increasing IFN-γ levels, and CD14 expression in macrophages.[12] Based on the profile of secondary metabolite found in galing (**Fig.7a-f**), several compounds were found to have the potential to act as immunomodulators in TB, as previously reported by researchers this include ar-turmerone which is able to modulate the cellular immune response through the activity of T lymphocytes and macrophages in destroying pathogens. The compound abscisic acid has been shown to influence the production of IL-12 cytokines which is an inflammatory mediators needed at the beginning of TB infection to prevent the worsening of the disease, in addition IL-12 activates lymphocyte cells. Hence, the results of the IL-12 investigation in this study reveal the ability of galing extract to improve the function of the immune system through the regulation of IL-12 in TB disease.[33,34,36]

Evaluation of Cluster of Differentiation-14 Expression in Macrophages

CD14 expression was evaluated based on plasma levels measured using a sandwich ELISA using a FITC mouse CD14 anti-rat kit (Bioassay Technology Laboratory®, China). The evaluation of CD14 expression is important because it is a specific marker of macrophages or monocytes. A measured CD14 count indicates the number of active macrophages or monocytes, which function as innate immunocompetent cells in the phagocytosis process early in TB infection, thus playing a strategic role in preventing more severe infections.[8,12] The results of CD14 measurements in each treatment group are presented in Figure 4. Based on the findings illustrated in **Figure 4**, the average expression levels of CD14 in all treatment groups receiving leaf and stem ethanol extracts of galing at doses of 400, and 500 (mg/kg BW) showed a significant increase compared to the negative control group (TB-infected without extract treatment). The most pronounced effect was observed at the 500 mg/kg BW dose. This was statistically validated through one-way ANOVA followed by LSD post hoc analysis, which confirmed a significant difference (*p<0.05) in CD14 expression between extract-treated groups and the negative control group. These findings indicate that galing extract exhibits immunomodulatory potential by enhancing CD14 expression on macrophages in the context of TB infection. Notably, the 500 mg/kg BW dose of both leaf and stem extracts demonstrated comparable efficacy to the positive control group (treated with a standard commercial *Phyllanthus niruri* extract), suggesting that galing extract can effectively stimulate CD14 expression at this dose.

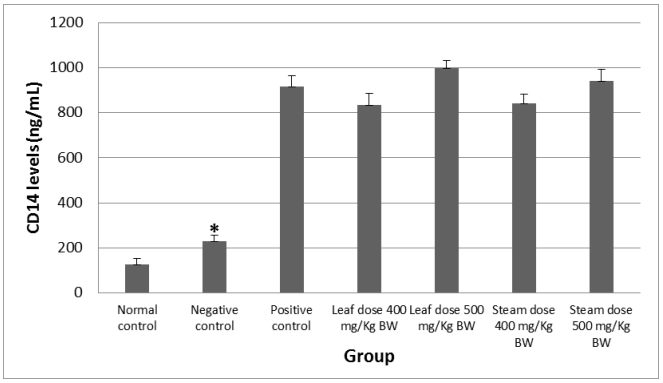

Figure 4: Graph of average CD-14 expression in macrophages in each treatment group (*p<0.05=post hoc LSD test significantly different from the negative control group)

These results support the conclusion that galing possesses promising immunostimulatory activity and may serve as a potential candidate for development as a novel immunomodulatory agent for tuberculosis management. The observed upregulation of CD14 expression in TB-infected mice is likely attributable to the phytochemical constituents of the galing plant. Previous studies have documented that galing contains a rich profile of secondary metabolites, including alkaloids, tannins, saponins, triterpenoids, and a high concentration of flavonoids, which are known to play critical roles in modulating immune responses.[14,27] Flavonoids have been reported to play a significant role in modulating immune responses by stimulating the mitogen-activated protein kinase (MAPK) pathway. This activation promotes lymphocyte proliferation, increases T lymphocyte populations, and enhances interleukin-2 activity, which subsequently augments the activation of effector immune cells such as lymphocytes and macrophages, facilitating the elimination of pathogens.[26,27] Elevated flavonoid levels have also been shown to influence leukopoiesis, particularly enhancing the activity of phagocytic leukocytes, thereby supporting more effective bacterial clearance a crucial mechanism in combating *Mycobacterium tuberculosis* infection. These findings align with previous studies, notably by Yusuf *et al*.[11] who demonstrated that galing extract augmented innate immunity by enhancing macrophage phagocytic activity in *in vivo* bacterial infection models. Consistent results were reported by Ilyas *et al*.[12] who observed that the galing extract significantly increased interferon-gamma (IFN-γ) levels and CD14 expression in macrophages within an *in vivo* breast cancer model, indicating its potent immunostimulatory effects. Further analysis using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) (Table 1; Fig. 7a–j) revealed the presence of several bioactive constituents in galing extract with immunomodulatory potential. One of such compound is, (+)-ar-turmerone, which has been reported to regulate immune responses by modulating the activity of T lymphocytes and macrophages.[37] T lymphocytes, in particular, secrete IFN-γ cytokines, which upregulate CD14 expression in monocytes and macrophages and initiate intracellular signaling pathways that promote *CD14* gene transcription.[38] Another identified compound, (±)-abscisic acid, is known to regulate immune balance by suppressing pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β), while enhancing the production of the anti-inflammatory cytokine interleukin-10 (IL-10). Interleukin-10 indirectly upregulates CD14 expression via activation of NK cells and subsequent IFN-γ secretion.[12,37] The modulation of this cytokine balance is essential to prevent excessive inflammation and maintain immune homeostasis during TB infection. Additionally, 4-Coumaric acid was identified as another immunoactive compound, previously reported to influence both pro-inflammatory (e.g., IL-6, TNF-α) and anti-inflammatory (e.g., IL-10) cytokine profiles.[31] Interlekin-6 and TNF-α enhance CD14 expression in monocytes and macrophages through janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways, while TNF-α further activates the nuclear factor-kappa B (NF-κB) signaling cascade, which is essential in the early immune response to TB infection.[38,39]

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Chemical Compound Profile of Galing Ethanol Extract

The phytochemical profile of the leaf and stem of galing ethanol extract was determined using LC-HRMS. These compounds were separated based on polarity using liquid chromatography, producing a chromatogram of various compounds (Fig. 5). This was then interpreted using a mass spectrometer, which identifies compounds based on their mass-to-charge ratio (m/z). Tandem mass spectrometry (MS/MS) provides more specific detection by splitting ions to provide more detailed information on molecular structure, thus identifying several phytochemicals with higher accuracy (Fig. 6 and 7).[19,22] The LC-HRMS results (Table 1) indicate that the phytochemical profile of galing extract includes various compounds from the secondary metabolite groups flavonoids, terpenoids, triterpenoids, and alkaloids.

The results of phytochemical analysis using LC-HRMS are shown in Table 1. Ten (10) types of compounds were successfully analyzed. The identified compounds are classified as bioactive immunomodulatory compounds based on several previous research. These compounds are reported to have various potentials that support their activity as immunomodulators via modulating various immunological parameters such as the number of leukocytes and increasing the expression of leukocyte types, increasing the phagocytic work of macrophage cells through secreting macrophage cell activator cytokines, significantly influencing the increase in IL-12 cytokine secretion, and increasing the number of macrophage or monocyte CD14 expression, in TB infection conditions.[14,27-29,31,33,35-37] However, further investigation should isolate these bioactive compounds (**Fig. 7**), to prove their individual effects on the various immunological parameters that have been described herein, possibly the isolated compound can serve as a novel immunomodulator in TB.

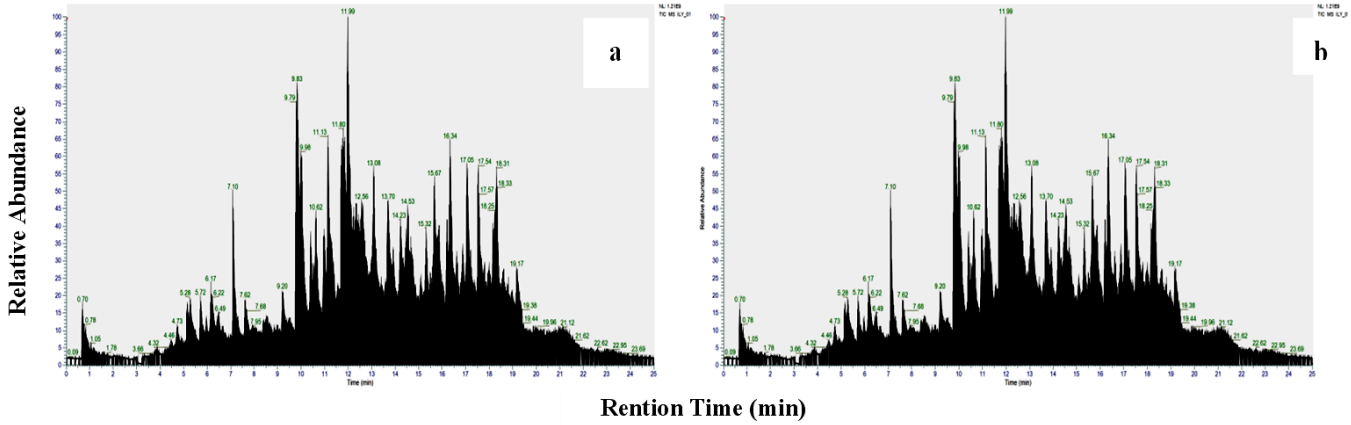

Figure 5: The LC-MS chromatogram/TIC chromatogram of the galing plant of ethanol extract (a. Leafe; b. Stem)

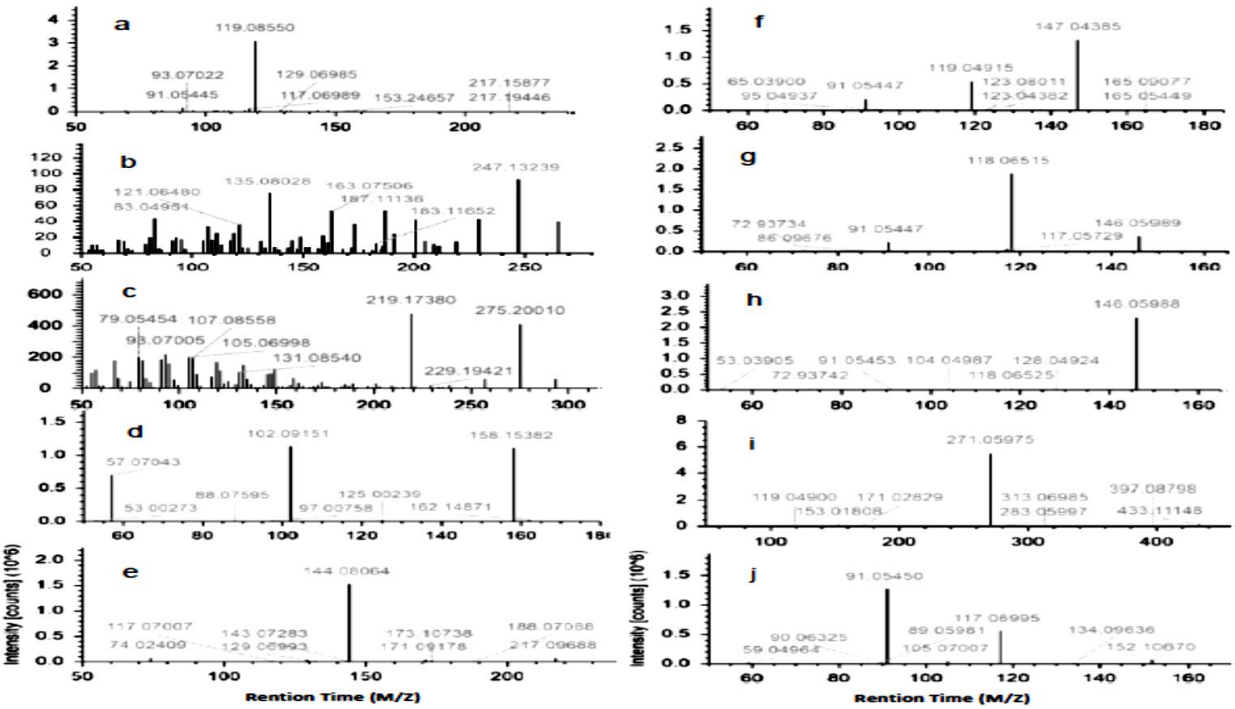

Figure 6: LC-HRMS chromatograms of leaves **(a-e)** and stems **(f-j)** of galing plant of ethanol extract. (a. (+)-ar-Turmerone; b. (±)-Abscisic acid (ABA); c. 12-Oxo phytodienoic acid; d. 2,2,6,6-Tetramethyl-1-piperidinol (TEMPO); e. 2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid; f. 4-Coumaric acid; g. 4-Methoxycinnamic acid; h. 8-Hydroxyquinoline; i. Apigetrin; j. L(-)-2-Amino-3-phenyl-1-propanol)

The phytochemical profile of galing revealed a diverse spectrum of secondary metabolites with promising potential for development as immunomodulatory agents in the treatment of pulmonary tuberculosis. The identification of various bioactive constituents underscores the necessity for advanced isolation techniques to obtain purified compounds with specific biological activities. Among the notable

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compounds detected are (+)-ar-turmerone, (±)-abscisic acid, 12-oxo-phytodienoic acid, and 2,2,6,6-tetramethyl-1-piperidinol (Fig. 7a–d)—previous studies have documented their capacity to modulate innate immune responses by enhancing leukopoiesis, regulating leukocyte subtype expression, augmenting phagocytic activity, and upregulating key immunological markers such as CD14 and interleukin-12 (IL-12). These compounds have also been associated with activation of T lymphocytes and other innate effector mechanisms.[28,29,31,37] In addition, other detected constituents—such as 2,3,4,9-tetrahydro-1H-β-carboline-3-carboxylic acid, 4-coumaric acid, 4-methoxycinnamic acid, 8-hydroxyquinoline, apigetrin, and L(-)-2-amino-3-phenyl-1-propanol (Fig. 7e-j)—have been reported to possess immunostimulatory properties. These compounds exert their effects through the modulation of pro-inflammatory and anti-inflammatory cytokines, particularly IL-12 and interferon-gamma (IFN-γ), which play critical roles in macrophage and lymphocyte activation during infectious processes.[31,35,36] The enhancement of both innate and adaptive immune responses is crucial in the control and resolution of Mtb infection.

Table 1: Profile of chemical contained of leaves **(a-e)**, and stems **(f-j)** of galing *(Cayratia trifolia)* ethanol extract by LC-HRMS profiling

Component Name	Observed m/z (Mr)	Molecular Weight (Da)	Chemical Formula	Observed Retention Time (minutes)	Compound Group
(+)-ar-Turmerone (a)	99.3	216.15	$C_{15}H_{20}O$	12.969	Flavonoid
(±)-Abscisic acid (ABA) (b)	94.2	264.14	$C_{15}H_{20}O_4$	7.612	Penilpropanoid
12-Oxo phytodienoic acid (c)	92.0	292.20	$C_{18}H_{28}O_3$	12.542	Alkaloid
2,2,6,6-Tetramethyl-1-piperidinol (TEMPO) (d)	92.7	157.15	$C_9H_{19}NO$	9.082	Terpenoid
2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid (e)	99.6	216.09	$C_{12}H_{12}N_2O_2$	3.654	Alkaloid
4-Coumaric acid (f)	93.3	164.05	$C_9H_8O_3$	3.856	Phenolic
4-Methoxycinnamic acid (g)	96.0	178.06	$C_{10}H_{10}O_3$	15.056	Terpenoid
8-Hydroxyquinoline (h)	99.9	145.05	$C_9H_7N_O$	3.378	Penilpropanoid
Apigetrin (i)	99.7	432.10	$C_{21}H_{20}O_{10}$	6.537	Phenolic
L(-)-2-Amino-3-phenyl-1-propanol (j)	99.6	151.10	$C_9H_{13}O$	10.398	Terpenoid

Given the diversity and pharmacological relevance of the chemical constituents identified in this study, further pharmacodynamic investigations, including *in vitro* and *in vivo* assays, are warranted to substantiate the immunomodulatory potential of these compounds and their viability as candidate agents in the development of novel therapeutics for pulmonary tuberculosis.

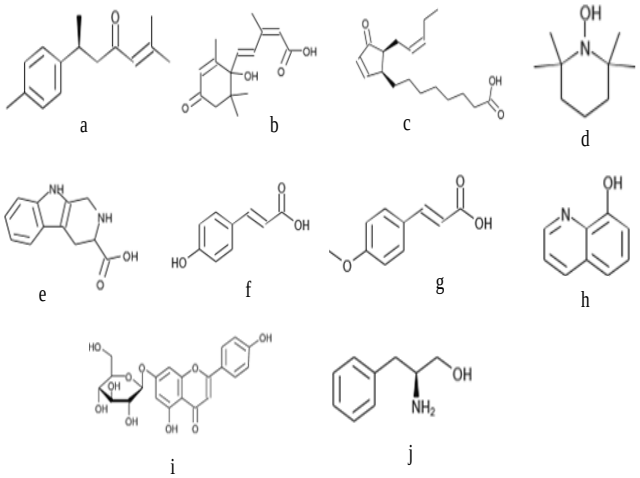

Figure 7: Structure of compounds identified from the LC-HRMS profiling of leaves **(a-f)**, and stems **(f-j)** of the galing plant of ethanol extract. (a. (+)-ar-Turmerone; b. (±)-Abscisic acid (ABA); c. 12-Oxo phytodienoic acid; d. 2,2,6,6-Tetramethyl-1-piperidinol (TEMPO); e. 2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid; f. 4-Coumaric acid; g. 4-Methoxycinnamic acid; h. 8-Hydroxyquinoline; i. Apigetrin; j. L(-)-2-Amino-3-phenyl-1-propanol)

Conclusions

The findings of this study demonstrate that both leaf and stem ethanol extracts of galing (*C. trifolia*) possess significant immunomodulatory activity ($p<0.05$) when compared to the untreated (negative control) group. This conclusion is supported by the observed enhancement in multiple immunological parameters, including the total count and expression of leukocyte subtypes (monocytes, neutrophils, basophils, eosinophils), elevated IL-12 levels, CD14 expression, and increased macrophage phagocytic activity in an *in vivo* model of tuberculosis (TB) infection. Moreover, chemical profiling revealed a wide array of secondary metabolites potentially responsible for these immunomodulatory effects.

Despite these promising results, additional pharmacological investigations both *in vitro* and *in vivo*—are required to confirm and elucidate the mechanisms underlying these immunomodulatory activities. The diverse array of bioactive compounds identified in this study positions *C. trifolia* as a promising natural source for the development of novel immunomodulatory agents in the management of pulmonary tuberculosis. Future research efforts should focus on the targeted isolation, characterization, and mechanism-based validation of the active constituents responsible for the observed biological effects.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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