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Histological Evidence of Enhanced Humoral Immunity by a Polyherbal Formulation in an Animal Immunogenicity Model

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Abstract

Background: *Polyherbal formulations, which combine multiple medicinal plants, may provide enhanced immunomodulatory effects through synergistic interactions among their bioactive constituents.* ***Objective:*** *This study aimed to evaluate the immunomodulatory effects of a polyherbal formulation on splenic white pulp structure and splenic index in mice immunized with tetanus toxoid.* ***Methods:*** *Twenty-five male BALB/c mice were randomly divided into five groups: a normal control group, a vaccine control group, and three treatment groups receiving low, medium, or high doses of the polyherbal formulation for 28 days. Tetanus toxoid was administered to the vaccine control and treatment groups on days 7 and 14. The splenic index was calculated as the ratio of spleen weight (mg) to body weight (g). Splenic white pulp diameter was assessed histologically in hematoxylin and eosin-stained spleen sections at 100× magnification. Data were analyzed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) post hoc test.* ***Results:*** *The polyherbal formulation did not significantly affect the splenic index among the experimental groups ($p = 0.103$), indicating no significant splenic enlargement associated with treatment. In contrast, splenic white pulp diameter increased significantly in all polyherbal treatment groups compared with the vaccine control group ($p < 0.001$). The mean white pulp diameters were 354.2 µm, 436.4 µm, and 518.0 µm in the low-, medium-, and high-dose groups, respectively, demonstrating a dose-related increase in white pulp development.* ***Conclusion:*** *The polyherbal formulation significantly increased splenic white pulp diameter without significantly altering the splenic index. These findings suggest that the formulation may enhance local adaptive immune responses without inducing significant systemic splenic enlargement in tetanus-toxoid-immunized mice.*

Keywords: *Adjuvants, Immunologic; Immunity, Humoral; Spleen; Tetanus Toxoid; Plant Preparations*

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INTRODUCTION

The use of traditional herbal medicines as immunomodulatory agents has attracted growing scientific interest due to their ability to enhance immune defenses through naturally derived bioactive compounds. Numerous clinical and experimental studies have demonstrated that herbal medicines offer several advantages over conventional drugs, including a broader spectrum of biological targets, multi-pathway interactions, and relatively low toxicity profiles [1,2]. Consequently, herbal medicine is increasingly recognized as a viable option for promotive, preventive, and curative interventions against various diseases [3].

To optimize the therapeutic potential of herbal preparations, recent advances have increasingly emphasized evidence-based standardization and novel delivery strategies, including nanotechnology-based herbal formulations and herbal nanoparticles, to improve the solubility, stability, bioavailability, and pharmacological activity of phytochemicals [4,5,6]. Plant-derived natural products also remain important sources for drug discovery and therapeutic development because of their chemical diversity and multi-target biological activities [7,8]. In addition, traditional and complementary medicine continues to be recognized globally as an important health resource, with the World Health Organization emphasizing the need for evidence-based integration, safety, quality, and regulation within health systems [9,10]. These developments highlight the enduring relevance of herbal medicine in modern immunopharmacology, particularly for supporting immune homeostasis and the immune system's natural defense mechanisms [11].

Among recent innovations, the concept of polyherbal formulations, defined as combinations of multiple medicinal plants within a single preparation, has gained particular attention. This approach, rooted in Traditional Chinese Medicine for thousands of years, aims to achieve synergistic therapeutic effects through the complementary actions of different plant constituents [4]. Polyherbal formulations may enhance therapeutic efficacy, reduce the required doses of individual components, and minimize adverse effects [2, 5]. Moreover, using a single polyherbal product simplifies patient compliance and promotes multi-target immune regulation, aligning with the holistic principles of traditional medicine [5].

In this study, the selected polyherbal formulation comprises ingredients well known and widely used in Indonesia as traditional jamu [6], including *Zingiber officinale* var. *amarum* (white ginger rhizome), *Zingiber officinale* var. *rubrum* (red ginger rhizome), *Blumea balsamifera* (sambong leaves), *Mentha arvensis* (mint leaves), *Alstonia scholaris* (pule bark), *Myristica fragrans* (nutmeg seed), *Panax ginseng*, and royal jelly. Each component has documented immunomodulatory properties, contributing to the formulation's expected synergistic effects on cellular and humoral immunity [6,7].

Bioactive compounds from *Zingiber officinale*, such as gingerol, have demonstrated potent immunostimulatory and anti-inflammatory effects, suggesting therapeutic potential for modulating immune-related disorders [6,7]. In an *in vitro* study, gingerol and shogaol significantly enhanced B-lymphocyte proliferation at 50 µg/mL under non-oxidative conditions, indicating a direct role in humoral activation [8].

Blumea balsamifera leaves contain quercetin, a flavonoid that increases circulating B lymphocytes and stimulates antibody production in animal models of inflammation [9,10]. Mentha arvensis has been shown to elevate immunoglobulin M and G levels in heat-stressed broiler chickens when administered as a 2% powder supplement, thereby improving overall hematological and biochemical profiles [11]. Extracts of *Myristica fragrans* regulate hepatic inflammation in high-fat diet-induced mice by suppressing the NF-κB signaling pathway and downregulating the proinflammatory cytokines TNF, IL-6, and IL-1β [12]

Meanwhile, *Panax ginseng* is among the most widely studied herbal immunomodulators, containing active ginsenosides that stimulate both cellular and humoral immune responses. Oral administration of ginsenoside Rg3 for 19 days significantly increased serum IgG levels and the immune organ index in cyclophosphamide-induced immunosuppressed mice [13]. In addition, royal jelly enhances the proliferation of macrophages, B cells, and T cells [14,15], as well as natural killer (NK) cell activity, providing broad immunostimulatory and protective effects in LPS-induced models [16,17].

Collectively, these findings suggest that the selected polyherbal formulation may enhance immune activity, particularly by stimulating B-cell proliferation and antibody synthesis, both of which are key features of humoral immunity. However, although the immunostimulatory potential of individual components has been extensively reported, histological evidence confirming the combined polyherbal effect on lymphoid tissue architecture remains limited.

The spleen is a crucial organ for evaluating immunostimulatory responses, serving as the largest secondary lymphoid organ by filtering antigens from the bloodstream and coordinating adaptive immune activation [18, 19]. Within the spleen, the white pulp is the primary site of humoral immune activity. It comprises three microanatomical regions: the periarteriolar lymphoid sheath (PALS), rich in T cells; lymphoid follicles, where B cells cluster and become activated; and the marginal zone, populated by macrophages, dendritic cells, and B cells [20].

After antigen exposure, mature B cells migrate from the bone marrow to the lymphoid follicles, recognize antigens via B-cell receptors (BCRs), and proliferate to form germinal centers. Within these centers, B cells undergo somatic hypermutation, clonal selection, and affinity maturation, ultimately differentiating into plasma cells that produce immunoglobulins or into long-lived memory B cells [21, 22]. These dynamic changes are reflected in the histological features of the splenic white pulp, making it a valuable tissue for assessing immunostimulant activity.

The integrity and expansion of the white pulp, along with prominent germinal centers, serve as histological indicators of effective humoral immune responses. Conversely, physiological stress or pathological conditions can lead to splenic atrophy and reduced lymphoid follicle development, indicating impaired antibody production [23]. Therefore, quantitative and qualitative assessments of white pulp diameter, follicular density, and germinal center morphology provide critical evidence of adaptive immune activation.

Based on this rationale, the present research focuses on evaluating histological evidence of enhanced humoral immunity in an animal immunogenicity model after administration of the selected polyherbal formulation. It is hypothesized that the synergistic combination of the herbal components, through the actions of gingerols, quercetin, ginsenosides, and other bioactive molecules, can promote splenic white pulp proliferation and germinal center formation, reflecting organized and safe activation of the adaptive humoral immune response.

This histological approach provides not only morphological evidence but also a mechanistic basis for understanding how polyherbal formulations modulate immune architecture. The findings are expected to contribute to the growing scientific foundation for developing safe, effective, and evidence-based polyherbal immunostimulants, particularly in preventive and supportive therapies for infectious and immune-related diseases.

MATERIALS AND METHODS

Materials

The polyherbal preparation used in this study was a commercially available tablet, Antangin JRG plus Jahe Merah® (AJ; BPOM registration number TR152586931; PT Deltomed Laboratories, Indonesia), which served as the polyherbal immunostimulant in tablet form. Each tablet contained 650 mg of test material. According to the formulation description, AJ contains *Zingiber officinale* var. *amarum* rhizome 1000 mg, *Zingiber officinale* var. *rubrum* rhizome 500 mg, *Blumea balsamifera* leaves 425 mg, *Mentha arvensis* leaves 400 mg, *Alstonia scholaris* stem bark 124 mg, *Myristica fragrans* seeds 124 mg, *Panax ginseng* 100 mg, and royal jelly 65 mg. Before administration, the tablet was powdered and suspended in 0.5% CMC-Na and aquabidest. Twenty male BALB/c mice aged 8–12 weeks and weighing 30–40 g were used as experimental animals. Additional materials included Rat Bio feed, drinking water, bedding, tetanus toxoid vaccine (Biofarma, Indonesia; 10 Lf/0.5 mL or 20 µg/0.5 mL), ketamine-xylazine, 4% paraformaldehyde in phosphate-buffered saline (PBS), RNA Stabilization Solution, FavorPrep™ Tri-RNA, chloroform, isopropanol, 70% ethanol, DEPC, Excel RT™ series cDNA reverse transcription kit II, SensiFAST SYBR Lo-ROX, CD32 and GAPDH primers, 10% buffered formalin, graded alcohol solutions, xylol/xylene, liquid paraffin, albumin, poly-L-lysine, hematoxylin-eosin staining reagents, DPX mounting medium, and immersion oil.

Tools

The equipment used in this study included latex gloves, masks, writing utensils, label paper, a ruler, a logbook, a permanent marker, tissues, mouse cages with special ventilation (Individual Ventilated Cages), mouse food and water containers, 1-cc syringes, a minor set, microtubes, capillary microhematocrit tubes, an autoclave, a clean bench, an Eppendorf tube, a micropipette, D1000, D200, and D10 tips, an analytical balance, a 96-well PCR plate, coverslips, microscope slides, cover glasses, a light microscope, a cryostat, a staining jar, an incubator, a centrifuge, a Nanodrop, a thermal cycler, and an ABI 7500 RT-PCR machine.

Method

Experimental Animals and Grouping

Twenty-five male BALB/c mice (8–12 weeks old; 30–40 g) were used in this study. Animals were acclimated for 7 days under standard laboratory conditions. After acclimation, mice were randomly assigned to five experimental groups, including normal control (NC), vaccine control (VC), and three treatment groups (T1, T2, and T3). The experimental design and workflow are illustrated in Figure 1.

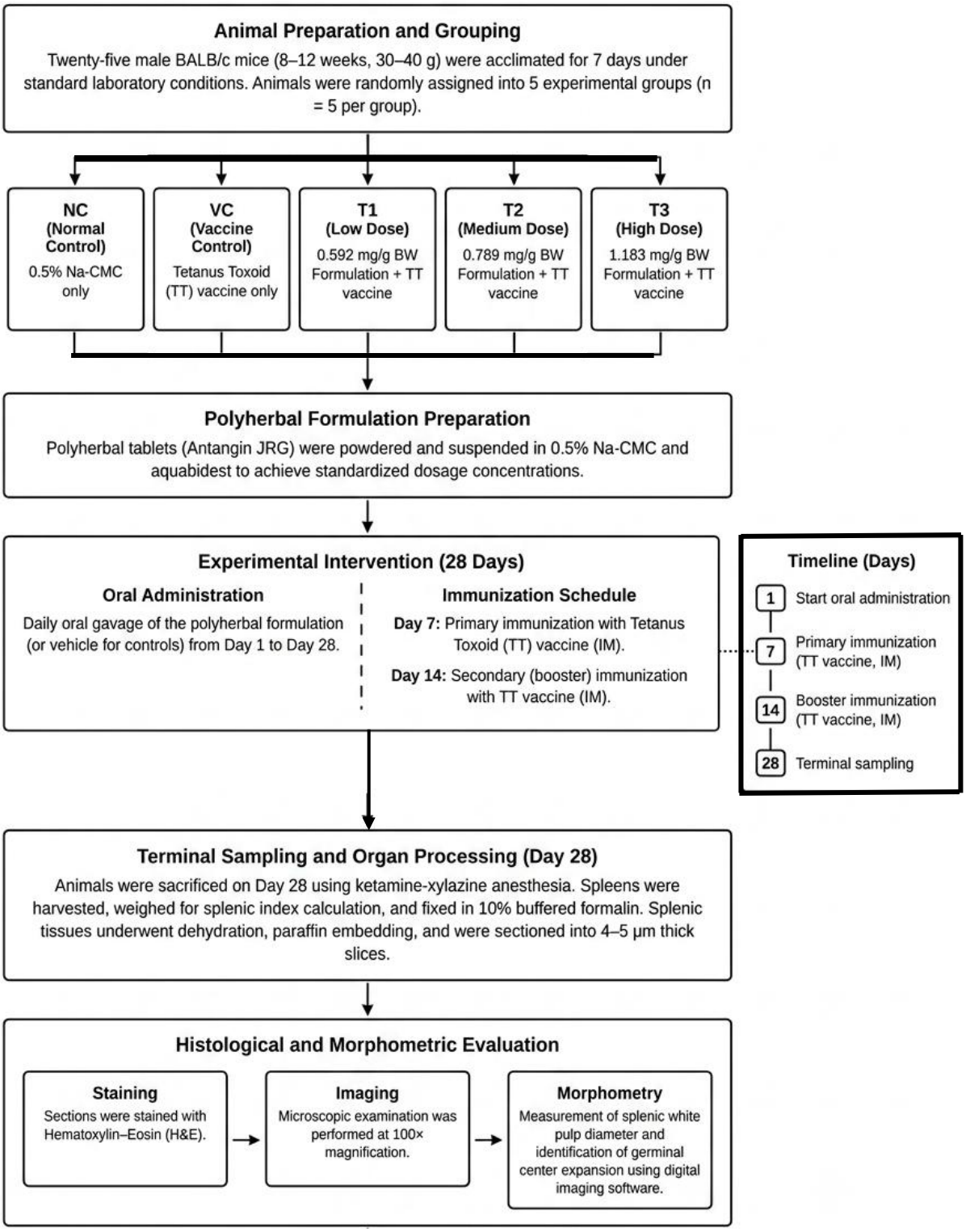

Figure 1. Research procedure

Polyherbal Formulation and Immunization Schedule

The polyherbal formulation (containing *Zingiber officinale*, *Panax ginseng*, and royal jelly) was administered orally via daily gavage for 28 consecutive days. Group T1, T2, and T3 received doses of 0.592 mg/20g BW, 0.798 mg/20g BW, and 1.183 mg/20g BW, respectively. To induce an immunogenic response,

mice in the VC and all treatment groups were injected subcutaneously with 20 µg of Tetanus Toxoid (TT) vaccine on days 7 and 14. The NC group received only a 0.5% Na-CMC placebo.

Spleen Harvesting and Histological Preparation

On day 28, animals were euthanized using ketamine-xylazine anesthesia. Spleens were harvested, weighed to calculate the splenic index, and fixed in 10% buffered formalin. For histological analysis, tissues were dehydrated in graded alcohol, cleared in toluene, and embedded in paraffin at 60°C. Paraffin blocks were sectioned at 4 µm using a microtome and mounted on glass slides.

Staining and Histomorphometry Evaluation

Tissue sections were deparaffinized with xylene and rehydrated through a series of decreasing alcohol concentrations. Sections were stained with Hematoxylin-Eosin (HE) to differentiate between red and white pulp structures. To ensure objectivity, all samples were anonymized and coded before being examined independently by a certified anatomical pathologist. The diameter of the splenic white pulp was measured at 100× magnification using digital imaging software. Three observers performed independent measurements to ensure consistency and accuracy.

Ethical considerations

This study received ethical approval from the Health Research Ethics Committee, Faculty of Medicine, under approval number KE/FK/1372/EC/2024 on September 5, 2024. The approval was subsequently amended and re-approved on December 12, 2024, under the same approval number.

Data analysis

Data were analyzed using statistical methods, and results were presented as mean ± SD. To assess normality, the Shapiro-Wilk test ($p>0.05$) was performed, followed by Levene's test for homogeneity ($p>0.05$). If the data were both normally distributed and homogeneous, a one-way ANOVA ($p<0.05$) was used to assess differences among groups. When significant differences were detected, post hoc analysis was performed using the Least Significant Difference (LSD) test. Statistical significance was set at $p<0.05$ with a 95% confidence interval.

RESULTS AND DISCUSSION

Splenic index

The splenic index is the ratio of spleen weight (mg) to body weight (g) in immunogenicity model animals on day 28. This parameter serves as an indirect indicator of immunological status, as changes in the spleen-to-body weight ratio often reflect variations in immune activity, lymphoid proliferation, or tissue perfusion (25,26). The index was calculated using the following formula (1).

$$Splenic\ Index = \frac{Spleen\ Weight\ (mg)}{Body\ Weight\ on\ Day\ 28\ (g)} \quad (1)$$

To evaluate the systemic physiological impact and ensure the safety profile of the polyherbal formulation, the splenic index—a quantitative marker of immune activity and organ-level stress—was calculated for all experimental groups. Individual splenic index values and statistical analyses are presented in Table 1.

Table 1. Splenic Index of Immunogenicity Model Animals

Variable	Group	Animal 1	Animal 2	Animal 3	Animal 4	Mean ± SD	Shapiro-Wilk p-value
Splenic Index (mg/g)	NC	61.62	59.19	59.18	60.15	60.04±1.15	0.225
	VC	60.86	58.62	60.54	60.36	60.10±1.00	0.128
	T1	61.94	61.07	60.95	61.63	61.40±0.47	0.495
	T2	62.35	62.19	60.82	60.43	61.45±0.97	0.249
	T3	61.57	60.04	62.00	60.45	61.02±0.92	0.552

Note. Data are expressed as mean ± SD (n=5). All diameter measurements were performed on day 28. Statistical significance compared to the vaccine control (VC) group is indicated by * ($p<0.05$) and ** ($p<0.001$). NC: normal control (0.5% Na-CMC); VC: vaccine control (tetanus toxoid); T1, T2, T3: treatment groups receiving low, medium, and high doses of the polyherbal formulation plus tetanus toxoid vaccine, respectively

The Shapiro–Wilk test (Table 1) indicated that the data were normally distributed across all groups ($p > 0.05$), and Levene's test confirmed homogeneity of variance ($p = 0.470$). Therefore, the data met the assumptions for one-way ANOVA. The ANOVA results showed no statistically significant difference in splenic index among groups ($p = 0.119$). LSD post hoc analysis also showed no significant difference between the vaccine control group and the treatment groups ($p > 0.05$).

Statistical analysis showed no significant difference in splenic index among the experimental groups ($p = 0.119$). These findings indicate that administration of the polyherbal formulation at the tested doses did not cause marked changes in spleen weight relative to body weight over the 28-day experimental period. Therefore, although the formulation was evaluated for its immunostimulatory potential, the unchanged splenic index suggests that the tested doses did not induce apparent splenic enlargement or excessive immune-related organ burden under the present experimental conditions [27,28,29].

Splenic index

Unlike the splenic index, the mean diameter of the splenic white pulp increased in a clear, dose-dependent manner across the treatment groups. Quantitative measurements showed mean diameters of 231.6 ± 14 µm in the normal control (NC), 280.8 ± 11 µm in the vaccine control (VC), 354.2 ± 15 µm in the low-dose group (T1), 436.4 ± 21 µm in the medium-dose group (T2), and 518 ± 21 µm in the high-dose group (T3). These results are summarized in Table 2, which presents the mean white pulp diameters and standard deviations for all experimental groups. One-way ANOVA revealed a highly significant difference among groups ($p < 0.001$). LSD post hoc analysis confirmed significant pairwise differences ($p < 0.05$), demonstrating a consistent dose–response pattern following administration of the polyherbal formulation.

Histological examination corroborated the morphometric data and revealed that the spleen in all groups exhibited normal histoarchitecture, with clearly differentiated white and red pulps. In the NC and VC groups, the white pulp appeared smaller, with less compact lymphoid tissue surrounding the central arterioles [30,31]. Conversely, in the treated groups (T1–T3), the white pulp regions were progressively enlarged, displaying increased cellular density and more prominent periarteriolar lymphoid sheaths (PALS). Furthermore, representative photomicrographs of splenic sections are shown in Figure 2, illustrating the comparative morphology of the white pulp among groups. Collectively, these images demonstrate a clear dose-dependent expansion of lymphoid follicles in animals receiving the polyherbal treatment compared with the controls.

Table 2. Splenic Index of Immunogenicity Model Animals

Variable	Group				
	NC	VC	T1 (0.592 mg/gWB)	T2 (0.789 mg/gWB)	T3 (1.183 mg/gWB)
Mean White Pulp Diameter (µm)	231.6±14	280.8±11	354.2±15**	436.4±21**	518±21**

Note. Data are expressed as mean ± SD (n=5). All diameter measurements were performed on day 28. Statistical significance compared to the vaccine control (VC) group is indicated by * ($p<0.05$) and ** ($p<0.001$). NC: normal control (0.5% Na-CMC); VC: vaccine control (tetanus toxoid); T1, T2, T3: treatment groups receiving low, medium, and high doses of the polyherbal formulation plus tetanus toxoid vaccine, respectively

No signs of necrosis, congestion, or architectural disruption were observed in any group. The splenic capsule, central arteries, and the boundary between the red and white pulps remained intact. These findings indicate that the observed enlargement of the white pulp occurred under physiological conditions. Overall, administration of the polyherbal preparation resulted in a significant, dose-dependent increase in white pulp diameter without altering splenic architecture, suggesting normal tissue integrity throughout the treatment period [32,33].

This study aimed to evaluate the immunostimulatory activity of a polyherbal formulation by assessing histological changes in the diameter of the splenic white pulp, a key indicator of humoral immune response, in an immunogenicity model. The formulation consisted of natural ingredients *Zingiber officinale* var. amarum (white ginger), *Zingiber officinale* var. rubrum (red ginger), *Blumea balsamifera* leaves, *Mentha arvensis* leaves, *Alstonia scholaris* bark, *Myristica fragrans seed*, Panax ginseng, and Royal jelly. The synergistic interaction among these components positively affected immune responsiveness, as reflected in splenic architecture.

The spleen is a secondary lymphoid organ that plays crucial roles in filtering blood, processing antigens, and facilitating lymphocyte differentiation. It is anatomically divided into two distinct regions: the red pulp, which removes senescent erythrocytes and recycles iron, and the white pulp, which contains lymphoid tissue where immune cells, particularly B and T lymphocytes, interact with antigens [19,34]. Because of its role in both innate and adaptive immunity, the spleen is a sensitive indicator of immunomodulatory effects, especially in histological assessments that reflect lymphoid activation and cellular proliferation.

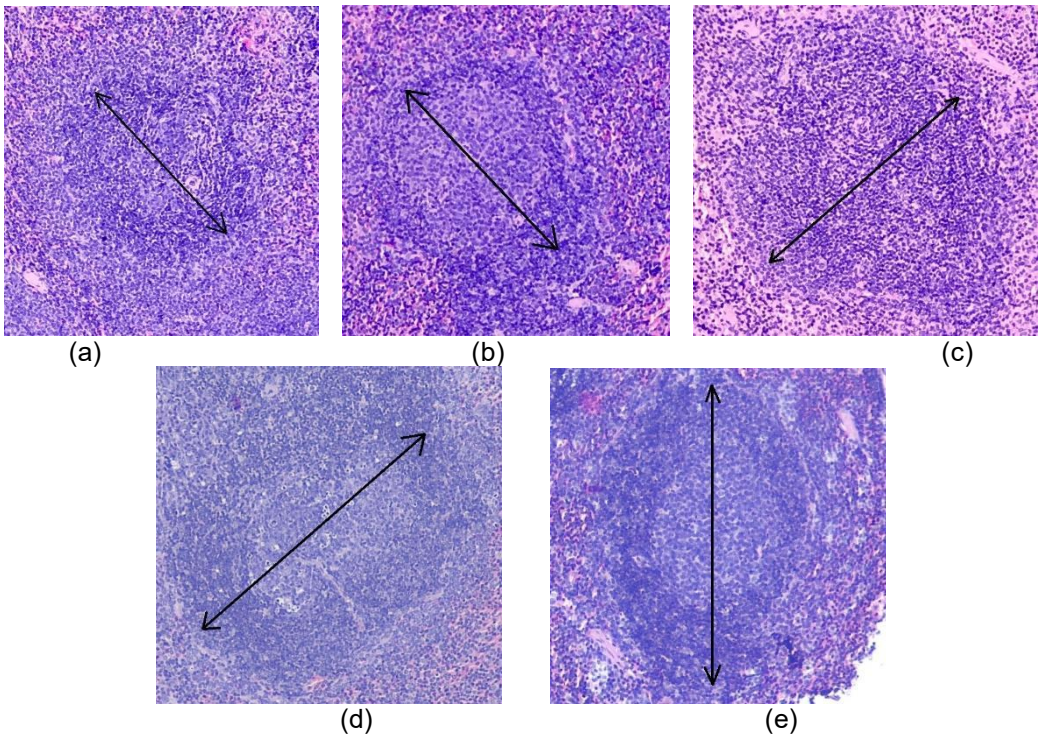

Figure 2. Histological appearance of splenic tissue in immunogenicity model animals across all groups (Hematoxylin-Eosin staining, 100x magnification). Visual Indicators: Black lines demarcate the boundaries of the white pulp; white lines identify the germinal centers

The photomicrographs (Figure 2) illustrate the comparative morphology and dose-dependent white pulp expansion observed on day 28. (a) Normal Control (NC): Group receiving standard feed/water and 0.5% Na-CMC only. (b) Vaccine Control (VC): Group induced with Tetanus Toxoid (TT) vaccine without herbal treatment (mean white pulp diameter: 287.3 µm). (c) T1 (Low Dose): Group treated with 0.592 mg/g BW polyherbal formulation and TT vaccine (mean diameter: 359.8 µm). (d) T2 (Medium Dose): Group treated with 0.789 mg/g BW polyherbal formulation and TT vaccine (mean diameter: 445.5 µm). (e) T3 (High Dose): Group treated with 1.183 mg/g BW polyherbal formulation and TT vaccine, showing maximum lymphoid expansion (mean diameter: 527.3 µm).

Although enlargement of the splenic white pulp suggests increased lymphoid activity and may reflect activation of adaptive immune responses, this finding should be interpreted cautiously. The present study did not measure serum IgM or IgG levels, nor did it assess antigen-specific antibody titers against tetanus toxoid. Therefore, the observed increase in white pulp diameter cannot be taken as direct evidence of increased antibody production or enhanced tetanus-specific humoral protection. Measuring total serum IgM and IgG, anti-tetanus toxoid antibody titers, and other functional immune parameters is needed to determine whether the histological changes observed in the spleen reflect a stronger humoral immune response [35,36].

The primary histological parameter analyzed in this study was the white pulp diameter. White pulp enlargement is generally associated with heightened immunological activity, particularly during antigenic stimulation or after exposure to immunostimulants that promote lymphocyte proliferation and germinal center formation [27,36]. Thus, morphological changes in splenic architecture provide crucial evidence of systemic immune activation in vivo.

The splenic index, defined as the ratio of spleen weight to total body weight, is commonly used as a quantitative indicator of immune status and splenic physiological load [28]. In this study, ANOVA and LSD tests showed no significant differences in splenic index values among experimental groups. The absence of statistically significant changes suggests that administration of the polyherbal formulation did not induce splenomegaly or pathological hypertrophy. This finding implies that the formulation neither imposed immunological stress nor elicited systemic toxicity [29,37].

This observation is critical from a pharmacological safety standpoint: an effective immunostimulant should promote immune activation without compromising physiological balance or triggering uncontrolled

immune proliferation. Therefore, the stable splenic index across treatment groups underscores the safety profile of the tested formulation and supports its suitability for long-term use as a natural immunomodulator.

In contrast to the splenic index, the white pulp diameter differed significantly among treatment groups. The most pronounced effect was observed in the high-dose treatment group (T3), with an average white pulp diameter of 527.3 µm (Figure 2e). Morphologically, this enlargement signifies intense lymphoid activation and proliferation, particularly of B lymphocytes within the germinal centers. B-cell proliferation in the spleen indicates an adaptive immune response involving clonal expansion and antibody synthesis, likely triggered by antigenic or bioactive stimulation from the polyherbal components.

The increased white pulp diameter, therefore, provides compelling histological evidence that the polyherbal formulation functions not merely as a biochemical immunostimulant but also as an agent that enhances the structural and functional maturation of secondary lymphoid tissues. This dual effect supports the hypothesis that the formulation promotes both the qualitative and quantitative aspects of immune responsiveness.

The observed enhancement of splenic white pulp structure aligns with previous reports indicating that polyphenolic and flavonoid compounds can stimulate lymphocyte proliferation and expand lymphoid tissue. Ethanol extracts rich in flavonoids have been shown to significantly increase both the diameter and area of the splenic white pulp in Wistar rats, reflecting enhanced humoral immune responses. The immunostimulatory effects of flavonoids are mediated through multiple mechanisms, including upregulation of interleukin-2 (IL-2) production, modulation of B cell receptor signaling, and activation of transcription factors such as NF-κB and STAT3, which regulate lymphocyte proliferation [30].

Among the herbal constituents, *Zingiber officinale* (ginger) is particularly noteworthy for its broad spectrum of immunopharmacological activity. The bioactive compounds gingerol and shogaol possess strong antioxidant and immunomodulatory properties [7,38]. These compounds not only scavenge reactive oxygen species (ROS), protecting immune cells from oxidative damage, but also directly enhance humoral responses by stimulating antibody production and lymphocyte proliferation.

In vitro studies have shown that gingerol and shogaol promote human B cell proliferation at concentrations as low as 50 µg/mL, even under oxidative stress induced by paraquat exposure. [8] This finding suggests that components of ginger may strengthen immune function by both reducing oxidative burden and upregulating immune signaling pathways. Additionally, the coexistence of white and red ginger varieties in the formulation may produce complementary effects: red ginger tends to have higher concentrations of gingerol and shogaol, whereas white ginger contributes sesquiterpenes and diarylheptanoids that further support antioxidant defense.

In addition to ginger, *Blumea balsamifera* (sembung) substantially contributes to the formulation's immunostimulatory potential. Its quercetin content has been shown to enhance peripheral B lymphocyte populations and modulate cytokine profiles, particularly by elevating IL-4 and IL-10 levels [9, 10]. These cytokines play pivotal roles in promoting humoral immunity and antibody class switching from IgM to IgG, a key indicator of long-term immune memory.

Panax ginseng, another major constituent, contains ginsenosides that activate macrophages and dendritic cells, increase expression of MHC class II molecules, and enhance antigen presentation efficiency. Furthermore, ginsenosides have been reported to upregulate CD19 and CD40 signaling in B cells, thereby promoting their differentiation into antibody-secreting plasma cells. The synergistic inclusion of Alstonia scholaris bark, Myristica fragrans seed, and Mentha arvensis leaves further amplifies this immune response by providing alkaloids, essential oils, and terpenoids that modulate both innate and adaptive immune pathways [15,16].

Royal jelly, a non-plant component, has been extensively studied for its ability to stimulate the proliferation of both B and T lymphocytes and to increase natural killer (NK) cell activity [17]. It contains peptides and fatty acids, including 10-hydroxy-2-decenoic acid (10-HDA), which have been shown to enhance B cell differentiation into immunoglobulin-producing plasma cells. Thus, the inclusion of Royal jelly in the formulation not only strengthens the humoral arm of the immune response but also supports cytotoxic and regulatory aspects of adaptive immunity.

From a mechanistic perspective, the dose-related enlargement of the splenic white pulp observed in this study may reflect increased lymphocyte activation and proliferation within the spleen's immune-active region. The mean white pulp diameter increased from 359.8 ± 13 µm in the low-dose group to 445.5 ± 13 µm in the medium-dose group and 527.3 ± 23 µm in the high-dose group, indicating that higher doses of the polyherbal preparation produced greater expansion of the splenic white pulp. Similar findings have been reported in previous studies, in which herbal extracts containing bioactive compounds such as flavonoids increased the diameter and area of splenic white pulp and germinal centers, supporting their role as natural

immunomodulators [23]. In addition, an ethanol extract of Dioscorea alata was reported to significantly increase splenic white pulp diameter at 50 and 250 mg/kg. However, a higher dose showed a lower response, suggesting that the immunomodulatory effect of plant extracts may be dose-dependent or dose-optimal [24]. Therefore, the increased white pulp diameter in this study likely reflects enhanced proliferative and differentiation events associated with humoral immune activation [20,39].

Additionally, bioactive constituents of the polyherbal formulation may enhance intercellular communication within the spleen by upregulating cytokines such as IL-6, TNF-α, and interferon-γ. These cytokines are known to facilitate B cell proliferation and antibody production, and to promote helper T cell activation, thereby providing co-stimulatory signals through CD40L-CD40 interactions [25].

The structural maturation of the white pulp also implies improved immunological architecture, including well-developed germinal centers and mantle zones. This morphological organization supports efficient antigen trapping, lymphocyte migration, and antibody secretion functions essential for robust humoral defense.

The absence of significant changes in the splenic index, coupled with pronounced histological enhancement of the white pulp, indicates that the immune response induced by the polyherbal formulation is physiological rather than pathological. In other words, the spleen's functional enhancement occurs without signs of splenic congestion, necrosis, or inflammatory infiltration, which are often associated with excessive immune activation. These findings have therapeutic implications. The formulation could serve as a safe, natural immunostimulant suitable for preventive and adjunctive applications, particularly in conditions characterized by immunosuppression, such as chronic infection, aging, or stress-induced immunodeficiency. Moreover, structural evidence of enhanced humoral activity supports its potential role as an adjuvant in vaccination strategies, where improved B-cell activation and antibody production are desired outcomes.

CONCLUSION AND SUGGESTION

In conclusion, this study successfully provided histological evidence of enhanced humoral immunity by a polyherbal formulation in an animal immunogenicity model. The research achieved its objective of demonstrating that the formulation significantly improves the structure of the splenic white pulp by dose-dependently expanding lymphoid follicles and germinal centers. Importantly, this enhancement occurred without altering the splenic index or causing tissue damage, confirming that the formulation promotes immune activation while maintaining physiological safety and avoiding systemic immune stress.

Based on these findings, the polyherbal formulation is suggested for application as a supportive natural immunostimulant or a vaccine adjuvant to optimize adaptive immune responses, particularly in preventive healthcare. To further validate its clinical utility, future studies should incorporate functional immunological markers, such as antigen-specific antibody titers (IgG and IgM) and specific cytokine profiling, to fully elucidate the molecular mechanisms underlying its synergistic effects in human translational models.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Ai S, Li Y, Zheng H, Zhang M, Tao J, Liu W, et al. Collision of herbal medicine and nanotechnology: a bibliometric analysis of herbal nanoparticles from 2004 to 2023. J Nanobiotechnology. 2024; 22:140. doi: https://doi.org/10.1186/s12951-024-02426-3
2. Mahboubi M. *Mentha piperita* and its main constituents: a review of their immunomodulatory and anti-inflammatory effects. Phytother Res. 2020; 34:2098-108. doi: https://doi.org/10.1002/ptr.6701
3. Atanasov AG, Zotchev SB, Dirsch VM, Supuran CT. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov. 2021; 20:200-16. doi: https://doi.org/10.1038/s41573-020-00114-z
4. Al-Nahdi AM, John A, Raza H. Ginger extract and its main bioactive component, 6-gingerol, as immunomodulators. Molecules. 2021; 26:4374. doi: https://doi.org/10.3390/molecules26144374
5. Bhalani DV, Nutan B, Kumar A, Singh Chandel AK. Bioavailability enhancement techniques for poorly aqueous soluble drugs and therapeutics. Biomedicines. 2022; 10:2055. doi: https://doi.org/10.3390/biomedicines10092055

6. Chyou S, Benoit-Lizon I, Grigorova I. The spleen in immune responses. Curr Opin Immunol. 2020; 65:1-8. doi: https://doi.org/10.1016/j.coi.2020.01.003
7. Chaachouay N, Zidane L. Plant-derived natural products: a source for drug discovery and development. Drugs Drug Candidates. 2024; 3:184-207. doi: https://doi.org/10.3390/ddc3010011
8. Che CT, Wang ZJ, Chow MSS, Lam CWK. Herb-herb combination for therapeutic enhancement and advancement: theory, practice, and future perspectives. Molecules. 2013; 18:5125-41. doi: https://doi.org/10.3390/molecules18055125
9. Choi JH, Choi SH, Yun KW. Risk factors for severe COVID-19 in children: a systematic review and meta-analysis. J Korean Med Sci. 2022;37: e35. doi: https://doi.org/10.3346/jkms.2022.37.e35
10. Darmawan E, Ahmad H, Perwitasari DA, Kusumawardani N. Pharmacist intervention can reduce the potential use of inappropriate drug medications in Indonesian geriatric patients. J Appl Pharm Sci. 2020; 10:1-7. doi: https://doi.org/10.7324/JAPS.2020.101012
11. Huang S, He T, Liu X, Wu Y, Li W. Quercetin and its derivatives: immunomodulatory effects and potential therapeutic applications. Front Immunol. 2021; 12:689044. doi: https://doi.org/10.3389/fimmu.2021.689044
12. Desjarlais M, Wirth M, Lahaie I, Ruknudin P, Hardy P, Rivard A, Chemtob S. Nutraceutical targeting of inflammation-modulating microRNAs in severe forms of COVID-19: a novel approach to prevent the cytokine storm. Front Pharmacol. 2020; 11:602999. doi: https://doi.org/10.3389/fphar.2020.602999
13. Dewi MK, Chaerunisaa AY, Muhaimin M, Joni IM. Improved activity of herbal medicines through nanotechnology. Nanomaterials (Basel). 2022; 12:4073. doi: https://doi.org/10.3390/nano12224073
14. Mallick S, Dutta A, Dey S, Majumdar S, Pal S. Immunomodulatory effects of edible mushrooms: a review. Int J Biol Macromol. 2022; 208:708-21. doi: https://doi.org/10.1016/j.ijbiomac.2022.03.158
15. El-Guendouz S, Lyoussi B, Miguel MG. Insight into the immunomodulatory effects of royal jelly. J Food Biochem. 2021;45: e13812. doi: https://doi.org/10.1111/jfbc.13812
16. Gu X, Ma Z, Fang J, Cai D, Zuo Z, Liang S, et al. Obesity enhances antioxidant capacity and reduces cytokine levels of the spleen in mice to resist splenic injury challenged by *Escherichia coli*. J Immunol Res. 2020; 2020:5948256. doi: https://doi.org/10.1155/2020/5948256
17. Harun NH, Mohamad MF. The immunomodulatory effects of *Zingiber officinale* (ginger): a systematic review. Res J Pharm Technol. 2022;15:3776-81. doi: https://doi.org/10.52711/0974-360X.2022.00634
18. Jayanti ASD. Uji aktivitas imunostimulan sediaan poliherbal yang mengandung *Zingiber officinale* var. *amarum*, *Zingiber officinale* var. *rubrum*, *Blumea balsamifera*, *Mentha arvensis*, *Alstonia scholaris*, *Myristica fragrans*, *Panax ginseng*, dan royal jelly pada hewan model imunogenisitas [Tesis]. Yogyakarta: Universitas Gadjah Mada; 2024. https://etd.repository.ugm.ac.id/penelitian/detail/248728
19. Jirakitticharoen S, Wisuitiprot W, Jitareerat P, Wongs-Aree C. Phenolics, antioxidant and antibacterial activities of immature and mature *Blumea balsamifera* leaf extracts eluted with different solvents. J Trop Med. 2022; 2022:7794227. doi: https://doi.org/10.1155/2022/7794227
20. Karole S, Shrivastava S, Thomas S, Soni B, Khan S, Dubey J, et al. Polyherbal formulation concept for synergic action: a review. J Drug Deliv Ther. 2019; 9:453-66. doi: https://doi.org/10.22270/jddt.v9i1-s.2339
21. Lei L, Li Y, Li M, Xin H, Tian X, Zhang Y, et al. Pathological changes in the spleen of mice subjected to different time courses of restraint stress. Sci Rep. 2024; 14:13543. doi: https://doi.org/10.1038/s41598-024-64428-3
22. den Haan JMM, Martinez-Pomares L. Stromal cells of the spleen: organization, development and immunological functions. Int Rev Cell Mol Biol. 2021; 362:189-228. doi: https://doi.org/10.1016/bs.ircmb.2021.02.001
23. Kim J, Byun J, Lee S, Kim H, Lee J, Kim J, et al. Immune-enhancing effects of red ginseng extract in cyclophosphamide-induced immunosuppressed mice. J Ethnopharmacol. 2022; 283:114724. doi: https://doi.org/10.1016/j.jep.2021.114724
24. Jiang W, Pan Y, Wang Q. Spleen: a central regulator of immune responses and systemic metabolism. Immunology. 2022; 165:130-44. doi: https://doi.org/10.1111/imm.13424
25. Onisei T, Tihăuan BM, Dolete G, Axinie M, Răscol M, Isvoranu G. In vivo acute toxicity and immunomodulation assessment of a novel nutraceutical in mice. Pharmaceutics. 2023; 15:1292. doi: https://doi.org/10.3390/pharmaceutics15041292
26. Parham P. The Immune System. 5th int. student ed. New York: W.W. Norton & Company, 2021.
27. Neely JL, Khamina K, Pillai S. Splenic marginal zone B cells: guardians of the blood. J Immunol. 2021; 206:1765-74. doi: https://doi.org/10.4049/jimmunol.2100014
28. Rastogi S, Pandey MM, Rawat AKS. Traditional herbs and polyherbal formulations: a review of their immunomodulatory potential. J Ethnopharmacol. 2020; 263:113205. doi: https://doi.org/10.1016/j.jep.2020.113205

29. Sapra L, Bhardwaj A, Azam Z, Madhry D, Verma B, Rathore S, Srivastava RK. Phytotherapy for treatment of cytokine storm in COVID-19. Front Biosci (Landmark Ed). 2021; 26:51-75. doi: https://doi.org/10.52586/4927
30. Agustina R, Permatasari R, Tjandrawinata RR. Immunomodulatory effect of *Carica papaya* L. leaf extract in cyclophosphamide-induced Wistar rats. J Ethnopharmacol. 2022; 291:115150. doi: https://doi.org/10.1016/j.jep.2022.115150
31. Kemp R, Glynn D, Woolley P, Smith A, Brown M. Nonproliferative and proliferative lesions of the rat and mouse spleen. J Toxicol Pathol. 2021;34(Suppl):1S-56S. doi: https://doi.org/10.1293/tox.34.1S
32. Srinivasan K. Ginger (*Zingiber officinale*) and its bioactive components: a comprehensive review of immunomodulatory effects. Phytother Res. 2022; 36:2680-708. doi: https://doi.org/10.1002/ptr.7463
33. Tangkiatkumjai M, Boardman H, Walker DM, Pradubpongse S, Srisurapanont M. Motivations and barriers to using herbal medicines: a systematic review. Complement Ther Med. 2020; 53:102534. doi: https://doi.org/10.1016/j.ctim.2020.102534
34. World Health Organization. Global traditional medicine strategy 2025–2034. Geneva: World Health Organization; 2025. Available from: https://www.who.int/publications/i/item/9789240102965
35. World Health Organization. WHO global report on traditional and complementary medicine 2019. Geneva: World Health Organization; 2019. Available from: https://apps.who.int/iris/handle/10665/312342
36. Yuandani, Jantan I, Haque MA, Rohani AS, Nugraha SE, Salim E, et al. Immunomodulatory effects and mechanisms of the extracts and secondary compounds of *Zingiber* and *Alpinia* species: a review. Front Pharmacol. 2023; 14:1222195. doi: https://doi.org/10.3389/fphar.2023.1222195
37. Zebeaman M, Tadesse MG, Bachheti RK, Bachheti A, Gebeyhu R, Chaubey KK. Plants and plant-derived molecules as natural immunomodulators. Biomed Res Int. 2023; 2023:7711297. doi: https://doi.org/10.1155/2023/7711297
38. Li T, Liang K, Gao Y, Chen J, Liang W. Herbal medicine and gut microbiota: a review of their interactions and implications for health. Medicine (Baltimore). 2021;100: e27600. doi: https://doi.org/10.1097/MD.0000000000027600
39. Zhu H, Guo L, Yu D, Du X. New insights into immunomodulatory properties of lactic acid bacteria fermented herbal medicines. Front Microbiol. 2022; 13:1073922. doi: https://doi.org/10.3389/fmicb.2022.1073922