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Original Article

Impact of Sappan Wood Extract as Iron Chelator Adjuvant on Iron Concentration and Macrophage Polarization in Rat Spleen

Mohammad Ghozali[1], MD, PhD, MSc; Ratu Safitri[2], PhD; Erick Khristian[3], MSi; Amethyst Puspita Ainni[2], MSi; Yasmi Purnamasari Kuntana[2], MSi; Muhamad Hasan Bashari[1], MD, PhD, MKes; Jeri Nobia Purnama[3], MBiotek

[1]Department of Biomedical Sciences, Faculty of Medicine, Padjadjaran University, Jatinangor, West Java 45363, Indonesia;
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Jatinangor, West Java 45363, Indonesia;
[3]Department of Biotechnology, Faculty of Postgraduate, Padjadjaran University, Bandung, West Java 45363, Indonesia

Correspondence:
Ratu Safitri, PhD;
Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Jatinangor, West Java 45363, Indonesia
Tel: +87 719620318
Email: ratu.safitri@unpad.ac.id
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Abstract

Background: Iron accumulation in the spleen of thalassemia patients disrupts macrophage polarization, impairs immune function, and increases mortality. Sappan wood (*Biancaea sappan* L.) extract exhibits iron-chelating and immunomodulatory properties, making it a potential adjuvant with Deferiprone (DFP). This study aimed to assess the effects of Sappan wood extract (SWE) as a DFP adjuvant in rat models of iron overload.

Methods: This experimental study was conducted from January to March 2024 at Padjadjaran University, Indonesia. Thirty-five rats were divided into seven groups: normal, iron overload (IO) induced by iron dextran (ID) at a cumulative dose of 60 mg/Kg body weight (BW), positive control receiving DFP, and four groups receiving DFP+SWE at different doses of 50, 100, 150, and 200 mg/Kg BW. Iron levels and macrophage polarization (pro-inflammatory M1 and anti-inflammatory M2) in the spleen were assessed.

Results: SWE at 50 mg/Kg BW significantly reduced spleen iron levels to 60.77 ppm/mg and increased M2 intensity ($P<0.001$ compared to the IO group). At 100 mg/Kg BW, SWE effectively decreased M1 macrophage intensity ($P=0.001$ compared to the IO group).

Conclusion: SWE at 50 mg/Kg BW was comparable to DFP in reducing the spleen iron levels and M1 macrophage intensity, while 100 mg/Kg BW enhanced M2 macrophage polarization. These findings highlighted SWE's potential as a therapeutic adjuvant in IO conditions.

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Keywords ● Deferiprone ● Macrophage ● Iron overload ● Iron chelating agents

What's Known

- Iron overload disrupts immune system function by altering macrophage polarization, which plays a critical role in maintaining immune homeostasis.
- Dysregulated macrophage polarization is implicated in the pathogenesis of various diseases, including cancer, autoimmune diseases, and chronic inflammatory conditions.

What's New

- Sappan wood extract (*Biancaea sappan* L.) exhibits iron-chelating properties, effectively modulating iron levels in rat models.
- This extract demonstrates immunomodulatory potential by influencing macrophage polarization, suggesting its therapeutic relevance in iron-related immune dysregulation.

Introduction

Iron is an essential element involved in erythropoiesis and macrophage activation. About 90% of iron is utilized by splenic macrophages for recycling old erythrocytes.[1] However, iron overload (IO) poses a significant toxic threat, disrupting the polarization of proinflammatory (M1) and anti-inflammatory (M2) macrophages.[2,3] Corna and others showed that IO conditions are characterized by elevated cluster of differentiation 86 (CD86) and reduced cluster of differentiation 163 (CD163) expression in macrophages.[4] Koorella and others further established that CD86 expression is indicative of proinflammatory macrophages, as it is associated with production of proinflammatory cytokines (e.g., IL-2) through

binding to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).[5] Conversely, CD163, a receptor for the haptoglobin-hemoglobin (Hp-Hb) complex in the spleen, is closely related to anti-inflammatory activity through the production of interleukin-10 (IL-10).[5, 6] Elevated M1 expression signifies inflammation, while diminished M2 expression indicates impaired macrophage activity in erythrophagocytosis and tissue repair.[7] These findings underscore the pivotal role of iron in dictating macrophage polarization, suggesting its potential influence on the pathogenesis of macrophage-related diseases, such as nonalcoholic fatty liver disease, atherosclerosis, and cancer.[8]

IO increases susceptibility to pathogens and triggers inflammatory responses, necessitating effective strategies to reduce systemic iron levels.[9] For thalassemia patients, the primary therapeutic approach involves blood transfusion combined with iron chelation therapy.[10] Deferiprone (DFP), a clinically used iron chelator, is often limited by its potentially fatal side effects, such as agranulocytosis and neutropenia, as well as its high cost.[11] In addition, DFP has been shown to inadequately suppress ferritin (Ft) expression in M1 macrophages, resulting in relatively high levels of pro-inflammatory markers such as CD86.[4] Consequently, there is a pressing need for innovative and safer iron chelation strategies, particularly those leveraging natural compounds.

Herbal plants offer a promising alternative to iron chelation therapy. Sappan Wood (*Biancaea sappan* L.), rich in phenolic and flavonoid compounds, such as brazilin, exhibits iron-chelating, antioxidant, and anti-inflammatory properties.[12] Previous studies demonstrated that brazilin, isolated from sappan wood, reduced inflammatory indices and downregulated M1 macrophage markers, including proinflammatory cytokines such as TNF-α, IL-1β, and IL-6.[13] Given its antioxidant and anti-inflammatory capabilities, Sappan wood extract (SWE), may enhance the efficacy of DFP while mitigating its adverse effects. Adjuvant therapy, which involves the addition of supplementary substances to enhance the primary treatment, represents a promising approach. To date, no studies have explored the effect of SWE as an iron chelation adjuvant on spleen macrophage polarization. This study aimed to evaluate the role of SWE as an adjuvant to DFP in a rat model of IO. The research parameters included measuring spleen iron concentration and assessing the expression of M1 (CD86) and M2 (CD163) markers through immunohistochemical (IHC) staining.

Materials and Methods

The materials used in this study were Sappan wood (*Biancaea sappan* L.) and seven-week-old male Wistar rats (*Rattus norvegicus*) with an average body weight (BW) of 180-200 g, obtained from PT Bio Farma, Bandung, Indonesia. The inclusion criteria were based on the characteristics of healthy rats, which exhibited active behavior. The exclusion criteria included rats that were injured, deformed, or died before or during treatment. The study was conducted at the veterinary laboratory of Padjadjaran University, Indonesia, from January to March 2024.

Sappan Wood Extract Preparation

Sappan wood was obtained from the Forestry and Plantation Service, Yogyakarta Indonesia. The identification and voucher specimens (No. KSC001) were deposited at Padjadjaran University, Jatinangor, Indonesia. The wood was crushed into powder and macerated in 96% ethanol for four consecutive cycles of 24 hours each. The macerated extract was filtered using filter paper and then concentrated using a rotary evaporator at 40 °C to dryness.[14] The resulting thick extract was dried in an oven (Memmert UN110, Germany) at 70 °C until a dry extract was obtained. Then, the extract was ground using a grinder and filtered through an 80-mesh sieve (US Standard, No. 80, 180 µm). Afterward, the dried extract was dispersed in distilled water to prepare doses of 50, 100, 150, and 200 mg/Kg BW.

Ethical Considerations

All experimental procedures involving animals were conducted in accordance with ethical guidelines for animal research. The study protocol was reviewed, approved, and registered by the Institutional Research Ethics Committee of Padjadjaran University, Indonesia (Ethical Clearance No. 605/UN6.KEP/EC/2023). The study adhered to the 3R principles (Replacement, Reduction, and Refinement), by implementing humane treatment protocols, minimizing animal use, and incorporating methods to alleviate pain and distress.[15]

Animal Experiments

This experimental study was conducted over 28 days using a completely randomized design (CRD). Thirty-five male white Wistar rats (*Rattus norvegicus*) were divided into seven groups, with five animals per group. The rats were acclimatized for seven days and provided with CP-551 feed (Charoen Pokphand, Indonesia) containing 18.50%-20.50% protein and water *ad libitum*. The rats were housed in cages

(51×35×28 cm) under a 12-hour light/dark cycle, with bedding changed every 3 days.[16, 17]

The experimental animals were divided into seven groups. The first group served as the normal group, while the remaining six groups constituted the iron-overloaded rat model. The remaining six groups received intravenous injections of iron dextran (ID) (Sigma Aldrich, CAS Number: 9004-66-4, Singapore) at a cumulative dose of 60 mg/Kg BW administered as three separate 20 mg/Kg doses at 3-day intervals. Following iron loading, these groups were further subdivided into: (1) an IO control group, (2) a DFP control group, and (3) four adjuvant treatment groups receiving DFP plus SWE at doses of 50, 100, 150, or 200 mg/Kg BW. Three days after the final ID injection, oral treatments commenced. The normal group and the IO control group received distilled water, while the DFP control group was administered 1.35 mg/Kg BW DFP. The adjuvant treatment groups received 1.35 mg/Kg BW DFP followed by their respective SWE doses 2 hours later. All treatments were administered daily for 28 consecutive days, resulting in a total study duration of 40 days.

Organ Sample Collection

Rats were anesthetized with 10% ketamine HCl (200 mg/Kg, intraperitoneally; [CAS No. 1867-66-9, LGC GmbH, Luckenwalde, Germany]). After confirmation of death, spleen organs were collected, washed with physiological saline solution, and divided into two parts: one for iron concentration analysis and the other fixed in 10% Neutral Buffered Formalin (NBF; Sigma Aldrich, CAS No. 50-00-0, Singapore) at a ratio of 1:10.[15, 18, 19]

Spleen Iron Concentration Examination

Spleen iron concentration was measured using the Atomic Absorption Spectrophotometry (AAS) method (AAnalyst 400, Burladingen, Germany). Samples were weighed with a total weight of 0.2 g, placed in a 100 mL beaker glass (Corning Pyrex Beaker, Germany), and dissolved in 5 mL concentrated HNO_3 (Sigma Aldrich, CAS Number: 7697-37-2, Singapore) and 2 mL of 30% H_2O_2 (Sigma Aldrich, CAS Number: 7722-84-1, Singapore). The clear solution was diluted using aquabidest to obtain a total volume of 25 mL in a volumetric flask (Pyrex volumetric flask, Germany). The solution was then homogenized and measured using AAS with a wavelength of 248.3 nm. The results were expressed as ppm/mg of spleen tissue.

Immunohistochemical Examination

Spleens tissues fixed in 10% NBF were sliced into 0.5 cm sections, dehydrated in graded alcohol (Sigma Aldrich, CAS Number: 64-17-5, Singapore), cleared in xylene (Sigma Aldrich, CAS No. 1330-20-7, Singapore), and embedded in paraffin wax (Sigma Aldrich, CAS No. 64742-51-4, Singapore) at 58°C overnight. Sections (4 µm thick) were cut using a microtome and stained for IHC.[20]

Macrophage M1 was detected using CD86/B7-2 (BU63) Antibody (Novus Biologicals, Centennial, USA; Catalog #NBP2-25208) at a concentration of 4 µg/mL overnight at 4 °C. Macrophage M2 was stained using CD163 (EDHu-1)-BSA Free Antibody (Novus Biologicals, Centennial, USA; Catalog #NB110-40686). Tissues were stained using UltraTek HRP Anti-Polyvalent and DAB Staining Kit and counterstained with hematoxylin (blue) (ScyTek Laboratories, Inc., Utah, USA; Catalog #UCS015-IFU). Slides were observed under an Olympus CX23 microscope (Olympus Corporation, Japan) at ×400 magnification, and images were captured using an OptiLab Advance V2 microscope camera (Yogyakarta, Indonesia). Image analysis was performed using ImageJ software (Wayne Rasband, Maryland, USA), with density units calibrated to µm² using a microscope slide calibration (Berlin, Germany).

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) and Duncan's test in SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). A 95% confidence level ($P<0.05$) was used to determine significance. Graphs were generated using Microsoft Excel software (version 365; Microsoft Corporation, Washington, USA).

Results

Spleen Iron Concentration

The initial measurements focused on spleen iron concentration to evaluate the efficacy of SWE as an iron chelator. The lowest iron concentration was observed in the normal group (wild rats), with an average value of 19.36±1.27 ppm, while the highest iron concentration was found in the ID group, with a value of 104.44±12.67 ppm. In groups treated with SWE at a dose of 50, 100, 150, and 200 mg/Kg BW, the average spleen iron concentrations were 60.77±3.48 ppm, 81.73±10.01 ppm, 80.21±10.71 ppm, and 108.42±9.25 ppm, respectively. These findings indicated that the administration of SWE doses exceeding 50 mg/Kg BW might increase spleen iron content in the test animals. The results of Duncan's test for spleen iron concentration are shown in figure 1.

Duncan's test analysis revealed a statistically

significant reduction in spleen iron levels in all groups compared to the negative control group (IO), except for the 200 mg/Kg BW SWE group (figure 1). The 50 mg/Kg BW SWE group showed a decrease in spleen iron levels by 43.67 ppm (P<0.001), comparable to the reduction observed in the positive control group (DFP; 43.43 ppm, P>0.999). SWE doses of 100 and 150 mg/Kg BW also reduced spleen iron levels, though less markedly, with reduction of 22.71 ppm (P=0.004) and 24.23 ppm (P=0.002), respectively. Notably, 200 mg/Kg BW dose of SWE failed to reduce spleen iron levels and instead resulted in a quantitative increase. However, it was not statistically significant (P>0.999). Spleen iron levels in the normal group differed significantly from all other experimental groups (P<0.001), confirming pronounced disparities in iron accumulation.

Expression Level of Macrophages M1 and Macrophages M2

The expression levels of macrophages M1 and M2 were analyzed using ImageJ software. Microscopic images of CD86 staining for M1 macrophages are shown in figure 2.

Microscopic analysis revealed minimal M1 macrophage presence in the normal group, with near-undetectable levels. In contrast, the negative group (IO) exhibited the highest M1 macrophage intensity among all groups. Variable M1 expression patterns were observed in both the DFP-only and DFP+SWE combination groups. Quantitative analysis using ImageJ software measured the M1 macrophage area relative to the microscopic field of view (×400 magnification). The percentage intensities were as follows: normal group (0.01±0.01%), negative control (1.93±0.39%), DFP-only (0.43±0.07%), and DFP+SWE groups at 50 (0.48±0.06%), 100 (0.30±0.06%), 150 (0.49±0.12%), and 200 mg/Kg BW (0.71±0.13%). These results showed that the normal group showed negligible M1 presence, while the ID-treated negative control displayed the highest macrophage infiltration.

The results of Duncan's test for macrophage M1 are shown in figure 3.

Figure 3 demonstrates significant differences in all experimental groups compared to the normal group (P<0.001 for all comparisons). Similarly, the negative control group (IO) showed significant differences from all other groups (P<0.001).

The results of the *t* test showed that the SWE 100 mg/Kg BW treatment group achieved the greatest reduction in M1 macrophage percentage (0.30%), showing no significant difference from the normal group (P=0.222). While standard DFP therapy significantly reduced M1 percentage compared to the negative control (P<0.001), it remained significantly elevated relative to the normal group (P=0.01).

Dose-response analysis indicated that increasing SWE from 50 to 100 mg/Kg BW reduced M1 macrophage infiltration, while further dose escalation (150-200 mg/Kg BW) paradoxically increased M1 intensity in spleen tissue.

Figure 4 shows the distribution of $CD163^{+}$ M2 macrophages within the splenic white pulp. The normal group exhibited extensive M2 macrophage infiltration throughout the tissue, while the negative group (IO) demonstrated markedly reduced M2 presence compared to all other groups. Treatment groups receiving

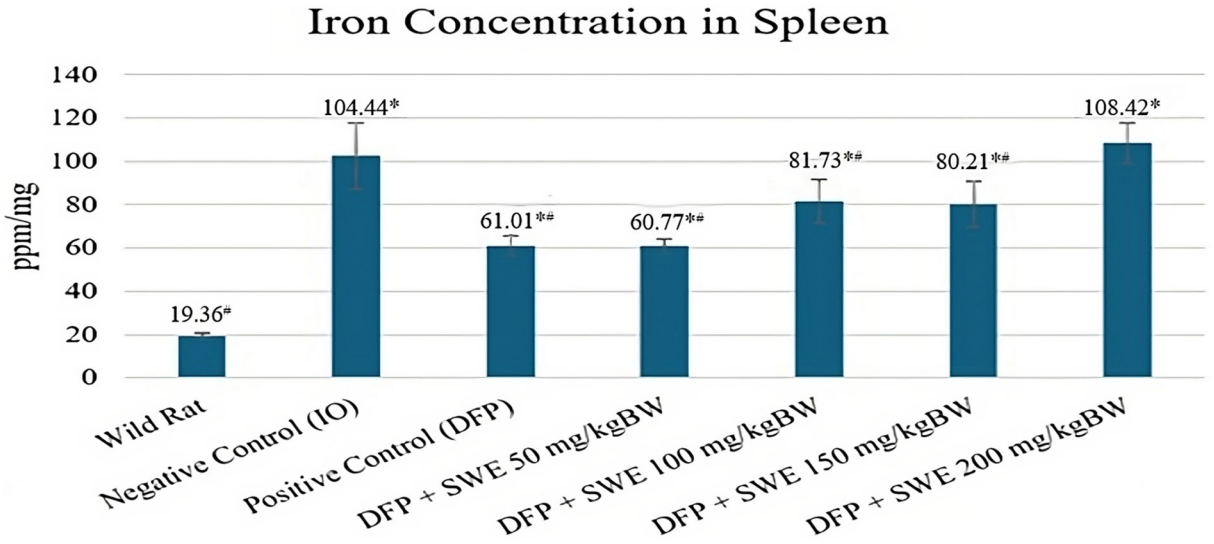

Figure 1: The graph illustrates differences in mean spleen iron concentration (ppm/mg) across experimental groups, with all treatment groups differing significantly from the normal group (wild-type rats; P<0.001). In the negative control group, only the highest dose of SWE shows no significant difference (P>0.999), while all other groups differ significantly (P<0.001). *Denotes significant differences compared to the normal group, and #indicates significant differences relative to the iron dextran (IO) group (P<0.05).

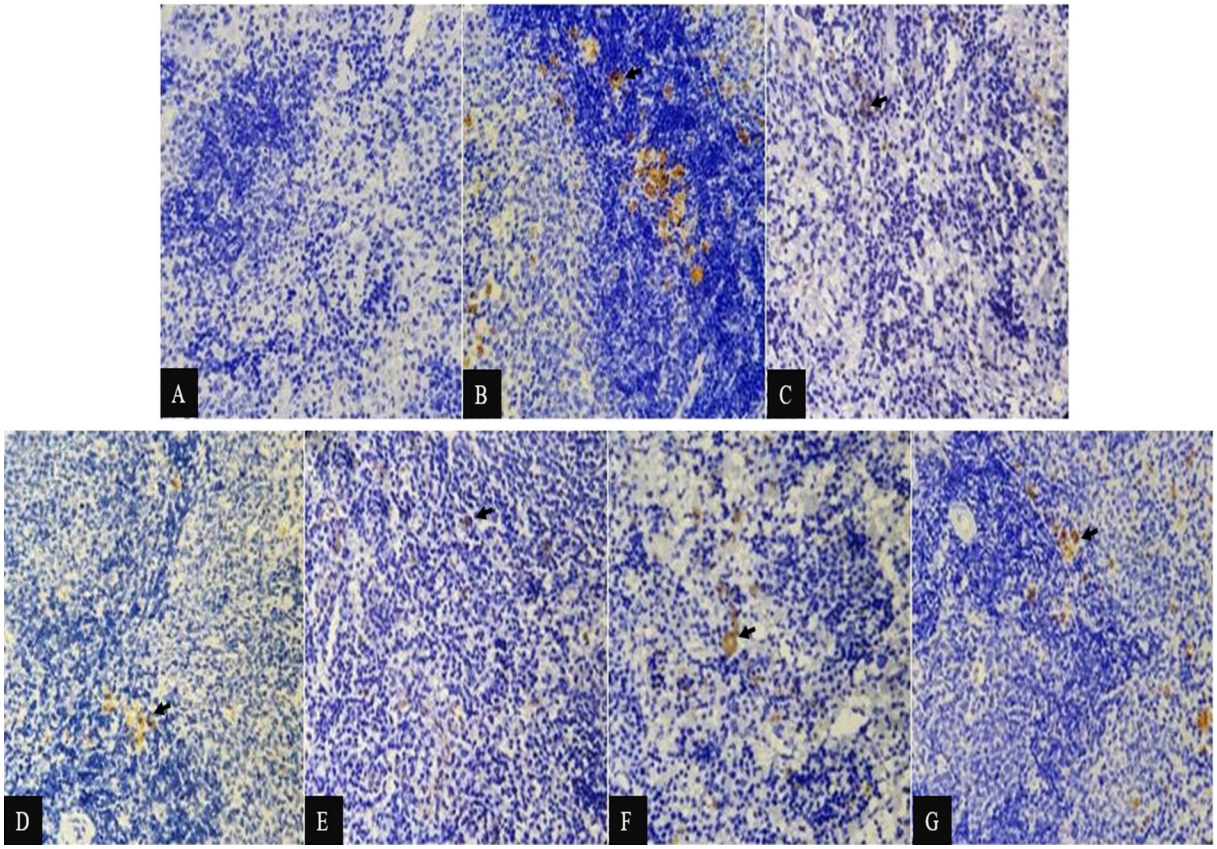

Figure 2: Spleen histology with immunohistochemical staining (×400 magnification) shows the distribution of M1 macrophages, characterized by positive CD86 staining in the marginal area. Black arrows indicate CD86-positive M1 macrophages. A) Normal group: No CD86 expression. B) Negative control group (IO): high CD86 intensity. C) Positive control group: single deferiprone (DFP) minimal CD86 expression. D-G) DFP+SWE groups: CD86 expression at doses of 50, 100, 150, and 200 mg/Kg BW, respectively, demonstrate dose-dependent CD86 expression patterns in marginal zones.

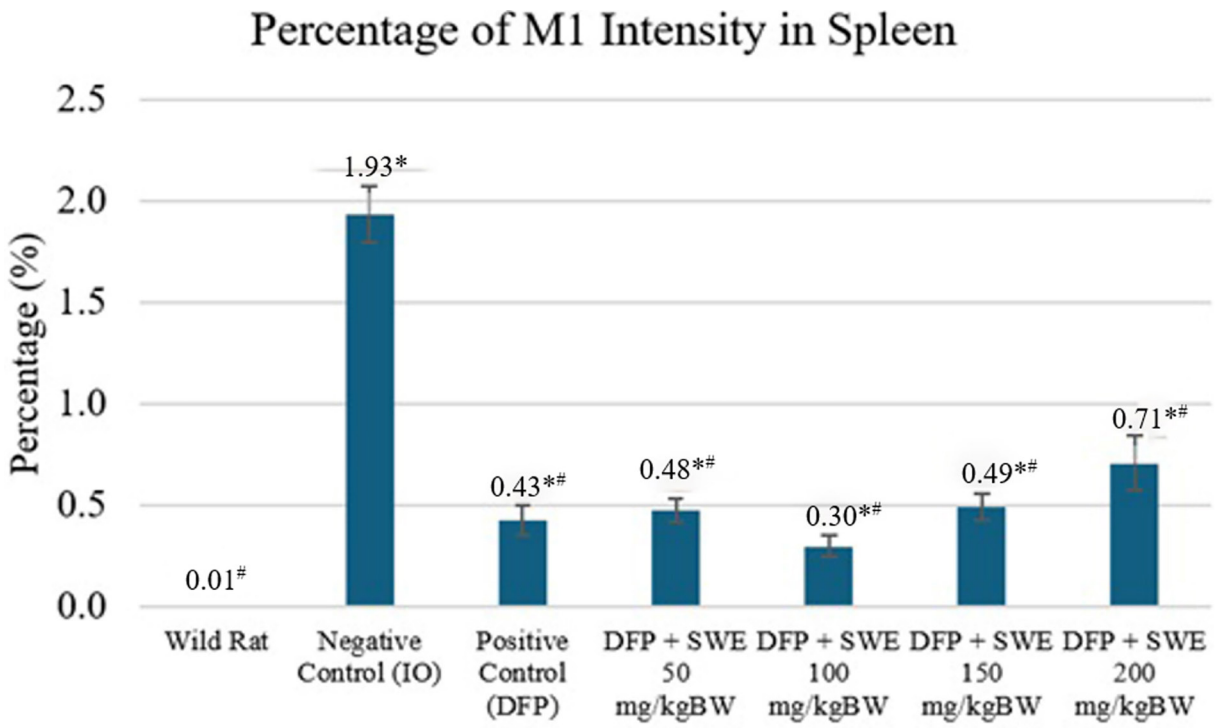

Figure 3: Percentage value shows CD86 (M1) macrophage expression intensity in the spleen tissue as measured by immunohistochemical staining and analyzed using ImageJ software. *Indicates significant differences against the normal group (wild-type rats; P<0.05). #Indicates significant differences against the iron dextran (IO) control group (P<0.05).

either DFP alone or DFP combined with SWE showed variable M2 macrophage intensity, with the lowest SWE dose displaying the most pronounced infiltration. These qualitative observations were subsequently quantified through digital image analysis using ImageJ software to measure $CD163^+$ M2 macrophage distribution intensity.

Quantitative analysis revealed significant differences in M2 macrophage intensity across groups (mean±SD): normal group (24.46±2.08%), negative control (IO, 6.25±2.08%), DFP treatment (17.86±0.68%), and SWE combination groups at 50 (17.45±0.80%), 100 (12.86±0.64%), 150 (11.65±0.51%), and 200 mg/Kg BW (10.46±0.18%).

The results demonstrated that DFP administration significantly enhanced M2 macrophage levels compared to the negative control group (IO). The 50 mg/Kg BW SWE group showed comparable M2 enhancement to

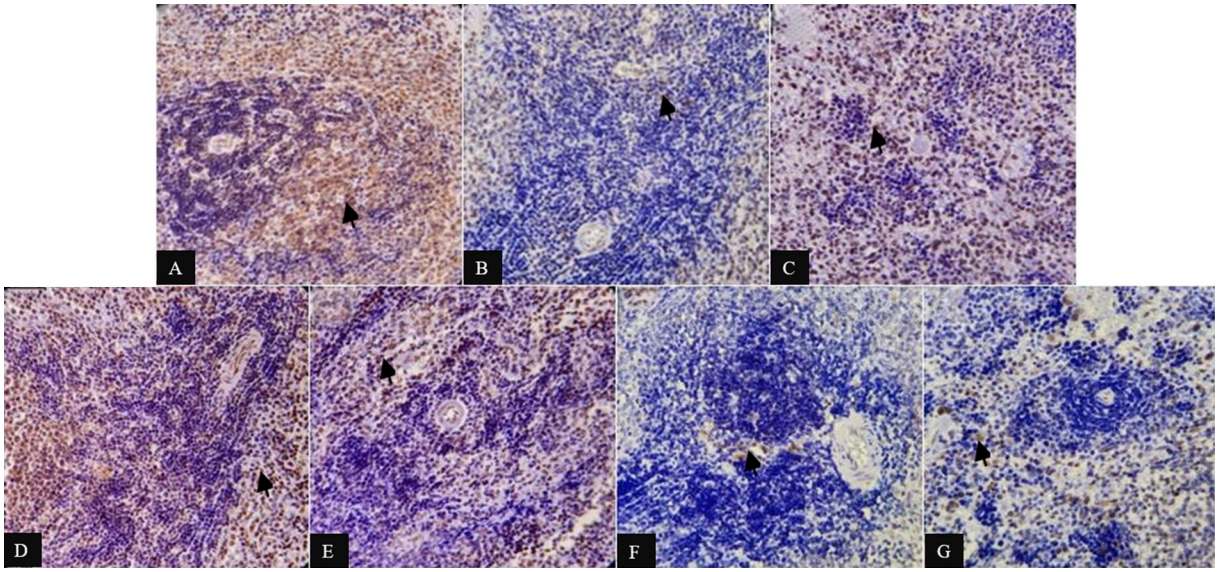

Figure 4: Spleen histology with immunohistochemical staining (×400 magnification) shows the distribution of M2 macrophages, characterized by positive CD163 staining in the white pulp area. Black arrows indicate $CD163^+$ M2 macrophages. A) Normal group: Highest CD163 intensity. B) Negative control group (IO): Lowest CD163 intensity. C) Positive control group, single deferiprone (DFP) administration: higher CD163 expression intensity than SWE-treated groups. D-G) DFP+SWE groups: CD163 expression, at doses of 50, 100, 150, and 200 mg/Kg BW, respectively, shows a decreasing trend with increasing SWE dosage.

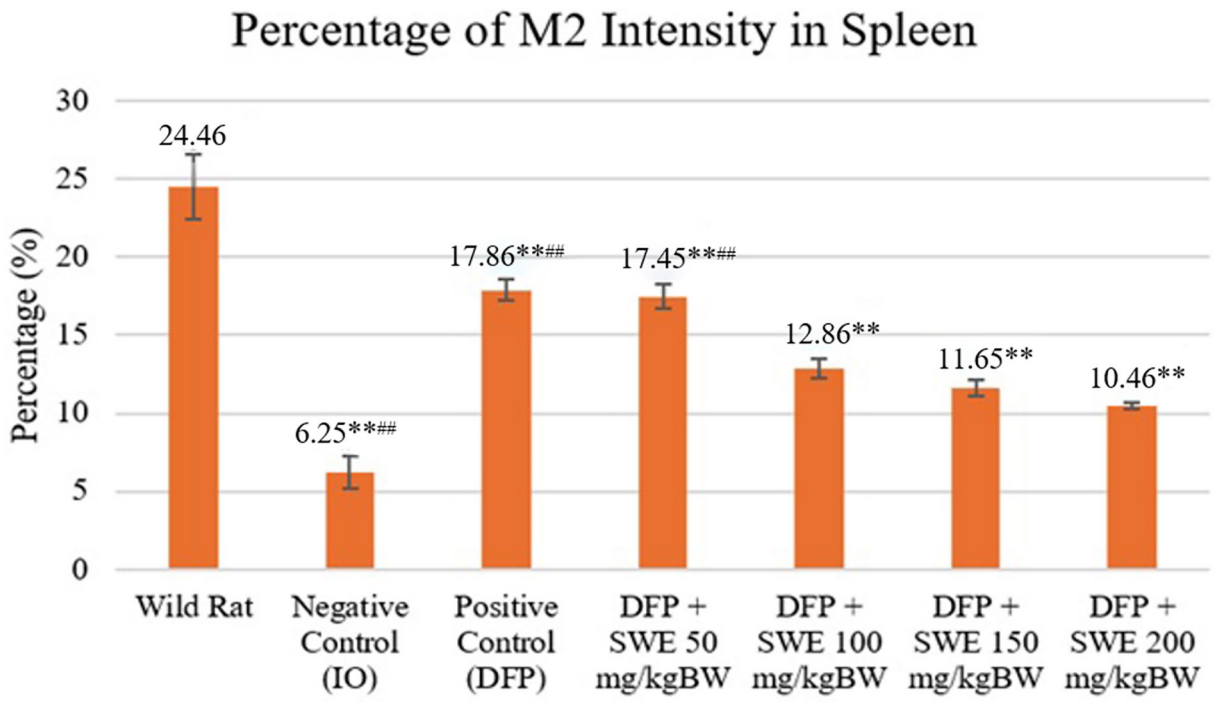

Figure 5: Percentage value of CD163 (M2) expression intensity in the spleen, analyzed using ImageJ software. *Indicates a significant difference compared to the normal group (wild rat), and #indicates a significant difference compared to the (IO) administration group ($P<0.05$).

DFP monotherapy. However, higher SWE doses (100-200 mg/Kg BW) produced progressively weaker M2 responses, establishing an inverse dose-response relationship in splenic tissue. Statistical comparisons between groups (Duncan's test) are presented in figure 5.

The administration of ID negative control group (60 mg/Kg BW) showed statistically significant differences from all treatment groups ($P<0.001$), including ID, DFP, and SWE-treated groups across the 50-200 mg/Kg BW dose range. Quantitative analysis of M2 macrophage intensity revealed that the normal group displayed the highest intensity values, while the untreated IO group exhibited the lowest levels. Both the DFP monotherapy group and the group receiving 50 mg/Kg BW SWE demonstrated M2 intensity values most similar to the normal group (though still significantly below normal levels; $P<0.001$). Statistical comparisons showed significant differences between the normal group versus both the negative control group and the 50 mg/Kg BW SWE group ($P<0.001$ for both), while no significant difference was observed between the negative control and 50 mg/Kg BW SWE groups ($P>0.999$). These findings indicated that DFP administration alone and in combination with 50 mg/Kg BW SWE produced equivalent therapeutic effects on M2 macrophage polarization.

Discussion

The results demonstrated that SWE at varying doses significantly reduced spleen iron levels and M1 macrophage intensity while increasing M2 macrophage intensity. The optimal dose for reducing spleen iron levels was observed in the group treated with DFP alongside SWE (50 mg/Kg BW) as an adjuvant. In contrast, the most pronounced reduction in M1 macrophage intensity occurred at a dose of 100 mg/Kg BW, whereas the highest increase in M2 macrophage intensity was achieved with SWE at 50 mg/Kg BW. The finding was comparable to a single DFP administration. Notably, increasing the SWE dose did not consistently enhance its efficacy in lowering spleen iron levels, suppressing M1 macrophages, or promoting M2 macrophage polarization. These findings suggested a non-linear dose-response relationship, where higher doses did not necessarily yield maximal therapeutic effects. The spleen, composed predominantly of macrophage-rich tissue, plays a pivotal role in erythrocyte recycling and immune modulation.[21] Labile iron, also known as non-transferrin-bound iron (NTBI), enters the spleen macrophage cells through divalent metal transporter 1 (DMT1). This iron is initially stored in the labile iron pool[22] before accumulating in the storage pool.[23] The resulting IO creates oxidative stress conditions that are toxic to cellular components, causing damage to DNA, proteins, and lipids (ferroptosis). In addition, IO stimulates erythrophagocytosis and increases iron recycling from both ferritin and the labile iron pools.[24] According to Çetinçakmak and others, elevated iron levels in the spleen could lead to hypersplenism, a condition that might ultimately require splenectomy.[25]

Brazilin, a flavonoid compound in SWE, contains two phenolic hydroxyl groups capable of forming excretable iron-flavonoid complexes.[26] Interestingly, while SWE promotes iron excretion, the observed increase in splenic iron levels with higher SWE doses may result from iron redistribution from other organs. This apparent paradox can be explained through multiple mechanisms. As a polyphenolic compound, brazilin exhibits dual functionality as both an antioxidant and metal chelator. In iron metabolism, it binds Fe^{2+} and Fe^{3+} ions, potentially enhancing iron bioavailability in systemic circulation.[14, 27] In addition, brazilin appears to modulate splenic macrophage activity, enhancing their capacity for iron processing through phagocytosis and iron elimination pathways.[28, 29] The consequent increase in macrophage-mediated iron uptake and processing may explain the dose-dependent accumulation of iron within the spleen following SWE administration.

Deferiprone effectively reduces splenic iron levels through its action as a bidentate chelator, forming a 3:1 ligand-iron complex that readily crosses cell membranes and binds circulating iron, facilitating excess iron removal.[30] When administered as adjuvant therapy with DFP, SWE at 50 mg/Kg BW demonstrated enhanced efficacy in reducing splenic iron levels compared to the single administration of DFP. However, this study revealed a paradoxical dose-response relationship: increasing SWE doses beyond 50 mg/Kg in adjuvant therapy resulted in rising iron levels, ultimately approaching those observed in untreated controls. These findings aligned with research by Safitri and others who reported that SWE doses between 50-200 mg/Kg BW functioned as iron chelators in iron-overloaded rats, evidenced by increased total iron-binding capacity (TIBC) and reduced transferrin saturation.[14] The biphasic response observed in the present study, where lower SWE doses potentiate DFP's effects while higher doses diminish efficacy, suggested complex dose-dependent interactions in iron metabolism regulation.

The IO significantly disrupts the equilibrium between M1 and M2 macrophage populations. Research showed that excess iron promoted M1 macrophage activation, which sustained chronic inflammatory states while simultaneously impairing the transition to the M2 phenotype required for tissue repair.[28] This polarization into proinflammatory M1 and anti-inflammatory M2 macrophages represents a crucial immunoregulatory mechanism, particularly in IO conditions.

Elevated iron levels preferentially drive macrophage polarization toward the M1 phenotype, characterized by enhanced production of inflammatory cytokines, including TNF-α and IL-1β. This process is mediated through iron-dependent activation of inflammatory pathways such as NF-κB. However, prolonged IO can also lead to immune dysfunction, as excessive iron can interfere with the effective activation of M1 macrophages.[8]

In contrast, M2 macrophages, which typically mediate tissue repair and anti-inflammatory responses, demonstrate significant suppression under IO conditions. Inhibitory effect occurs primarily through downregulation of characteristic M2 markers, particularly interleukin-10 (IL-10). Experimental evidence from animal models confirmed that iron supplementation actively suppressed M2 polarization, consequently impaired anti-inflammatory responses.[31]

The observed increase in M1 macrophage polarization under IO conditions occurs through multiple mechanisms. Intracellular labile iron interacts with reactive oxygen species (ROS) via the Fenton reaction, generating oxidative stress that contributes to cellular and tissue damage.[32] Furthermore, IO downregulates ferroportin (FPN) expression, thereby promoting M1 polarization as evidenced by elevated CD86 expression and increased production of proinflammatory cytokines, such as IL-6 and TNF-α.[33] This shift toward the M1 phenotype reflects enhanced inflammatory responses and bactericidal activity.

Deferiprone administration demonstrates significant efficacy in reversing these effects. Treatment normalizes M1 macrophage ($CD86^+$) populations to levels comparable with healthy controls (N) through its iron-chelating capacity, which directly reduces Fenton reaction-mediated oxidative damage. Additionally, DFP downregulates key genes involved in oxidative stress response, iron homeostasis, and proinflammatory cytokine production (TNF-α, IL-7, IL-8).[34] These findings were in agreement with a previous study by Vinchi and others, demonstrated that the iron chelator deferasirox similarly reduced hepatic and splenic iron content while modulating macrophage polarization - decreasing M1 markers and increasing M2 markers.[35] Collectively, these studies suggest that iron chelation not only normalizes iron homeostasis but also restores macrophage functional responses to pathogenic challenges.

Administration of SWE at varying doses as adjuvant therapy demonstrated significant efficacy in reducing M1 macrophage polarization. The active component brazilin mediated this effect through suppression of proinflammatory cytokine secretion, particularly IL-6 and TNF-α.[36] Further supporting these findings, brazilin administration was shown to reduce systemic inflammatory markers, including decreased serum levels of TNF-α, IL-1β, and IL-6.[13] These results collectively demonstrated that combination therapy using SWE (50 to 200 mg/Kg BW) with DFP (1.35 mg/Kg BW) effectively reduced inflammation in the spleen. The therapeutic effect was shown to be mediated through dual mechanisms: iron chelation by DFP and anti-inflammatory modulation by SWE-derived brazilin.

Under IO conditions, the observed reduction in M2 macrophages suggested iron-induced cell death mediated by excessive free radicals, leading to either necrosis or lipid peroxidation (ferroptosis).[28, 37] This depletion of M2 macrophages impairs critical physiological functions, particularly erythrocyte recycling and hemoglobin clearance (Hb:Hp complex), which may subsequently promote tissue damage through hydroxyl radical production.[22]

Our findings demonstrated that DFP administration significantly increased M2 macrophage populations. As reported by Maras and others, this effect likely occurs through DFP's ability to reduce TNF-α levels, chelate-free iron, and mitigate Fenton reaction-mediated oxidative stress.[34] Deferiprone effectively reduces oxidative stress and inflammatory reactions and increases the expression of anti-inflammatory M2 macrophages. Administration of SWE at doses of 50, 100, 150, and 200 mg/Kg BW and DFP 1.35 mg/Kg BW enhanced M2 macrophage numbers, with the 50 mg/kg SWE dose showing optimal efficacy-achieving levels comparable to positive controls.

The abundance of M2 macrophage expression is related to its role in tissue repair and angiogenesis.[32] Chemical compound components found in SWE such as brazilin, flavonoids, and sappanols have anti-inflammatory and immunomodulatory properties.[38] Previous research showed that ethanol extract of Sappan wood at a dose of 25 mg/Kg BW had immunomodulatory activity on peritoneal macrophages of albino mice, which showed a significant increase in macrophage

phagocytosis activity.[39] Collectively, these results showed that SWE not only reduced iron concentration and M1 macrophage polarization but also actively promoted macrophage differentiation in the spleen.

The findings of the study showed that higher doses of SWE did not have additional therapeutic benefits and paradoxically increased splenic iron concentration. This study also showed that serological iron profiles from previous studies require correlation with tissue-level iron measurements, as systemic iron reduction may not preclude tissue-specific accumulation.

One of the limitations of this study was the absence of interleukin profiling (both pro-inflammatory and anti-inflammatory cytokines), which precluded definitive conclusions about the relationship between macrophage polarization (M1/M2) and humoral immunity.

Conclusion

The administration of SWE as an adjuvant effectively reduced iron levels in the spleen decreased the average number of M1 macrophages, and increased the average number of M2 macrophages in iron-overloaded rat models. This study highlighted the potential of SWE as an adjuvant therapy to DFP in promoting M2 macrophage polarization and exerting anti-inflammatory effects. The optimal effective dose for reducing spleen iron levels, decreasing M1 macrophage intensity, and increasing M2 macrophage (CD163) expression was found to be 50 mg/Kg BW of SWE combined with 1.35 mg/Kg BW of DFP. However, careful dose adjustment is necessary, as higher doses of SWE may lead to increased iron trapping in activated macrophages, potentially diminishing its therapeutic efficacy.

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Authors' Contribution

E.K: Involved in developing research concept, collecting data, and conducting statistical analysis and interpretation of results; A.P.A: Conduction experiment on animals, involved in collecting related literature and drafting the initial version of the article; M.G: Involved in developing the concept and substance of the research, conducting a critical review of the final version of the article, and ensuring the accuracy of the research results; Y.P.K: Validation of the histopathological results and participate in providing feedback on interpretation of data for the discussion section; M.H.B: Handling the animals and validator of laboratory examination results, especially spleen iron, participate in providing input on interpretation of data for the discussion section, especially in the medical field; J.N.P: Involved in technical research and data collection; R.S: Principal investigator and funding manager for the research project. Although her direct contribution to the experimental work may be limited, her role in securing funding and overseeing the project was critical; Participated in the revision of the article based on reviewers' feedback. All authors have reviewed the final version critically for important intellectual content and have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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