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Article

Secondary Metabolite Profiles and Antibacterial Activity of *Mitragyna speciosa* Leaf Extracts According to Vein Color Variation

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Abstract

Empirically, the three distinct color variants of *M. speciosa* leaves (red, white, and green) vein are widely utilized and reported to possess varying therapeutic properties. This difference in efficacy is hypothesized to be a direct consequence of variances in their respective secondary metabolite profiles. Given the limited research comprehensively characterizing these metabolites and assessing the comparative antibacterial activity of the three variants, this study aimed to identify the secondary metabolite groups present in the red, white, and green vein leaves and determine their antibacterial activity against *Salmonella typhi* and *Staphylococcus aureus*. The methodology involved maceration extraction using 96% ethanol, followed by phytochemical screening and antibacterial testing via the agar well diffusion method across concentrations of 5%, 10%, 20%, and 40%. The negative control (96% ethanol) and positive control (chloramphenicol) were simultaneously utilized. The results confirmed that all three vein samples contained major secondary metabolite groups, including alkaloids, flavonoids, phenolics, saponins, and tannins. Differential antibacterial activity was noted: the red vein extract showed the highest efficacy against *S. typhi*, achieving an inhibition zone of 4.68mm at 10%, while the green vein extract was most effective against *S. aureus*, with an inhibition zone of 5.00 mm at 10%. These findings definitively demonstrate a difference in antibacterial potency across the *M. speciosa* vein variants, collectively affirming their significant potential for development as antibacterial active agents.

Keywords: Kratom; Phytochemical; Bactericidal

1. INTRODUCTION

Antibiotic resistance is a critical global public health crisis. This necessitates the urgent exploration of natural sources, especially plants, which are known reservoirs of antimicrobial compounds in traditional medicine. Scientists have successfully isolated active compounds from various botanicals like *Lamiaceae*, *Piperaceae*, and *Moringa oleifera*, confirming their antimicrobial promise [1-3]. However, the vast potential of indigenous flora, such as that in Southest Asia, remains largely unexplored. Many local plants lack extensive investigation, and existing studies often fail to provide in-depth chemical analysis crucial for new drug development.

Kratom (*Mitragyna speciosa* (Korth.) Havil.) is traditionally recognized across Southeast Asia, particularly Indonesia, for its medicinal properties, which stem from its high alkaloid concentration [4]. Mitragynine is the principal alkaloid, known for contributing to various therapeutic effects, including the regulation of blood sugar and lipid profiles [5]. Phytochemical screening of *M. speciosa* also confirms the presence of other key bioactive molecules, such as 7-hydroxymitragynine, quercetin, and rutin [6]. Crucially, recent evidence highlights mitragynine's potent antimicrobial activity. Research has established its inhibitory effects against several clinically relevant bacteria, including *Aeromonas hydrophila*, *Streptococcus pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus* [7-8]. Detailed investigations are already underway to fully characterize its mechanism of action. Given the urgent global need for new treatments against resistant pathogens, mitragynine emerges as a highly promising scaffold for novel antimicrobial drug development.

However, the local classification of *M. speciosa* into variants based on leaf vein color (red, green, white) is traditionally linked to perceived differences in pharmacological efficacy. This variation is hypothesized to stem from differences in the profile and concentration of secondary metabolites among the variants. Currently, adequate comparative data on the fundamental secondary metabolite and, subsequently, the antibacterial potential of extracts derived from these vein color variations remains limited. Therefore, this study aims to conduct a comparative analysis of the secondary metabolite profiles and to evaluate and contrast the resulting antibacterial activities of the different *M. speciosa* leaf vein color variants. The findings are expected to provide a robust scientific basis for optimizing the utilization of the most potent *M. speciosa* variant as a reliable source of novel antimicrobial agents.

2. MATERIALS AND METHODS

2.1. Material

M. speciosa leaves, distinguished by their various vein colors (red, green, and white), were sourced from the Kota Bangun sub-district in the Kutai Kartanegara Regency, East Kalimantan Province. Initial authentication of the collected *M. speciosa* samples was carried out at the Laboratory of Ecology and Conservation of Tropical Forest Biodiversity, Faculty of Forestry, Mulawarman University. The verified samples were subsequently placed in storage at the Research and Development Laboratory of PT. Borneo Riseta Naturafram. Sample preparation began with sequential wet and dry sorting of all collected material. Following this, the *M. speciosa* samples were carefully arranged on drying racks and kept in a well-ventilated room where the temperature was maintained at 18 °C. The resulting dried leaves were then finely ground into a powder and stored in airtight containers awaiting the commencement of the extraction process. Meanwhile, the study utilized the following chemical reagents including ethanol (96% and 70%), distilled water (aquadest), and dimethyl sulfoxide (DMSO) were bought from Merck, Germany. For microbiological assays, the bacterial strains *Salmonella typhi* (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923) were cultured using Nutrient Agar (NA) medium and prepared in a 0.9% sodium chloride (NaCl) solution. Phytochemical screening involved the use of hydrochloric acid (HCl) solutions (concentrated and 2N), 1% ferric chloride ($FeCl_3$) solution, magnesium (Mg) powder, and the specific alkaloid reagents: Dragendorff's, Mayer's, Bouchardat's, and the Lieberman-Bouchard reagent were bought from Merck, Germany.

2.2. Instrument

The primary instruments and supporting laboratory equipment utilized in this research included: an autoclave, petri dishes, a desiccator, a freezer, glass beakers, measuring cylinders, a hot plate, an incubator, erlenmeyer flasks, a Laminar Air Flow (LAF) cabinet, micropipettes, a screw micrometer, an inoculating loop, measuring pipettes, a propipette (pipette filler), test tube racks, a rotary evaporator, test tubes, and an analytical balance.

2.3. Method

2.3.1. Extraction Processes

Extraction of *M. speciosa* was carried out according to previously published methods [9]. Extraction of the 150.0 g dried *M. speciosa* powder was performed using the maceration technique with 96% ethanol. The initial maceration spanned 24 hours. The resulting liquid filtrate was separated from the solid residue through filtration using Whatman filter paper No. 1 (GE Healthcare, UK). The residue was then subjected to three consecutive re-macerations. The collected filtrates were combined and concentrated to a thick consistency by drying under reduced pressure using a BUCHI R-210 series rotary evaporator, maintaining a temperature of approximately 50 °C.

2.3.2. Phytochemical screening

Phytochemical analysis was conducted to qualitatively screen the crude extracts for the presence of major secondary metabolites, including flavonoids, alkaloids, triterpenoids, tannins, steroids, saponins, and phenolics. All screening tests adhered to established standard qualitative procedures, as previously detailed by Syafrizal et al. [10], with the following specifics:

1 Alkaloid Test

The alkaloid test began by mixing the extract with 2mL of chloroform and 2mL of ammonia, and the resulting mixture was filtered. The filtrate was then acidified with 3–5 drops of concentrated sulfuric acid and shaken until two distinct layers were achieved, allowing for the collection of the chloroform fraction. This fraction was divided into two aliquots for confirmation using specific reagents. The presence of alkaloids was verified by two distinct reactions: the formation of a white or yellowish clumpy precipitate upon the addition of Mayer's reagent, and the appearance of a brick-red precipitate following the addition of Dragendorff's reagent.

2 Flavonoid Test

The extract was transferred via pipetting of 1 mL volume and subsequently deposited into a test tube. The extract was treated with magnesium powder and 2 to 4 droplets of concentrated hydrochloric acid, followed by agitation. The orange coloration observed signifies the presence of flavonoids.

3 Triterpenoid and Steroid Test (Lieberman-Bouchard Reagent)

The test for triterpenoids and steroids involved dissolving 1mL of the extract in acetone. To this solution, ten drops of anhydrous acetic acid and two drops of concentrated sulfuric acid (H_2SO_4) were added, and the mixture was briefly shaken. After sitting for several minutes, the reaction was assessed by color change: a red or purple color indicated the presence of triterpenoids, while green or blue indicated the presence of steroids.

4 Tannin Test

An aliquot of 1 milliliter of the extract was combined with 1 milliliter of a 1% ferric chloride solution.The appearance of a blue, black, or brownish-green coloration signifies the presence of tannins.

5 Saponin Test

The saponin test began by placing 1mL of the extract (dissolved in acetone) into a test tube and adding 10mL of hot water. After the solution cooled, it was shaken vigorously for 10 seconds. A positive result was indicated by the formation of persistent foam (1–10 cm high) for approximately 10 seconds, which did not dissipate upon the addition of one drop of 2N HCl

2.3.3. Minimum inhibitory concentration (MIC) assay

The antimicrobial activity of the crude extracts was evaluated using the disc diffusion method. Initially, single colonies of the test bacteria, *S.typhi* and *S.aureus*, were inoculated into Mueller-Hinton Broth (MHB) and incubated at 37 °C for 3–5 hours. The resulting bacterial cultures were then standardized to the McFarland 0.5 standard, yielding a final cell concentration of approximately 1.5×10^8 CFU/mL. For disc preparation, 6-mm diameter holes were punched onto Whatman No. 1 filter paper, which was subsequently sterilized by autoclaving at 121 °C for 20 minutes. Each crude extract was prepared by dissolving it in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL. Ten microliters (10µL) of this solution was applied to the center of each disc, which was then air-dried for approximately 24 hours. A sterile cotton swab was used to uniformly inoculate Mueller-Hinton Agar (MHA) plates with the standardized test pathogens. Six prepared discs were then placed onto the infected MHA plate using sterile forceps, ensuring a separation of 15–20 mm between disks and 15 mm from the plate edge. Plates were subsequently incubated at 37 °C for 24 hours. The diameter of the zone of inhibition was measured in triplicate using Vernier calipers. DMSO and tetracycline served as the negative and positive controls, respectively, prepared at the same concentration as the samples. The entire experiment was repeated three times, and the standard deviation was calculated.

The Minimum Inhibitory Concentration (MIC) was ascertained utilizing the standard microdilution method. The cultures of *S.typhi* and *S.aureus* were prepared. The crude extracts were serially diluted across a range of 0–10 mg/mL. Briefly, 100µL of the concentrated bacterial inoculum (1×10^6 CFU/mL) was introduced into each well containing Mueller-Hinton Broth (MHB), and the 96-well plate was incubated at 37 °C for 18–24 hours. The MIC value was defined as the lowest extract concentration preventing visible bacterial growth (lack of turbidity). Control wells comprising MHB only (sterility control) and MHB with the lowest extract concentration but lacking inoculation (negative control) were simultaneously assessed.

3. RESULT AND DISCUSSION

3.1 Phytochemical screening

In this work, phytochemical screening was performed on the three vein color variants of the *M. speciosa* leaf ethanol extract. The procedure involved dissolving the crude extracts in appropriate solvents, followed by the sequential addition of specific reagents into separate test tubes. The results of the screening were visually assessed based on changes in color, the formation of precipitate, or the production of stable foam upon the addition of each specific reagent. The observations revealed that all three *M. speciosa* leaf ethanol extract variants contained the secondary metabolite classes of alkaloids, flavonoids, triterpenoid, steroid, tannins, phenolics, and saponins, as summarized in Table 1.

Table 1. Phytochemical screening results of *M. speciosa* ethanol extract with various vein colors

Screening Phytochemical	Reagen	Ethanol extract of *M. speciosa*		
		Red vein	White vein	Green vein
Alkaloid	Mayer	+	+	+
	Dragendroff	+	+	+
	Bourchardat	+	+	+
Flavanoid	Mg powder and HCl concentrated	+	+	+
Triterpenoid and Steroid	Lieberman-Bouchard	–	–	–
Tannins	$FeCl_3$ 1%	+	+	+
Phenolics	$FeCl_3$ 10%	+	+	+
Saponins	HCl 2N	+	+	+

+ : identified, – : non identified

3.1.1 Identification of alkaloid

Confirmation of alkaloid compounds in the ethanol extract was conclusively achieved through consistent precipitation observed in the Mayer's, Bouchardat's, and Dragendorff's identification tests. The reaction of alkaloids with Mayer, Bouchardat, and Dragendorf reagents is illustrated in Figure 1. Specifically, the Mayer's test yields a positive result indicated by a yellow precipitate, hypothesized to be a potassium–alkaloid complex. The Mayer's reagent is formed in a sequence where mercury(II) chloride reacts with potassium iodide to yield mercury(II) iodide precipitate, which then complexes with excess potassium iodide to produce potassium tetraiodomercurate(II). Because alkaloids possess nitrogen atoms with lone pair electrons capable of forming coordinate covalent bonds with metal ions, the nitrogen in the alkaloid structure is thought to react with the potassium ion (K^+) from the tetraiodomercurate(II) complex, ultimately causing the desired precipitation.

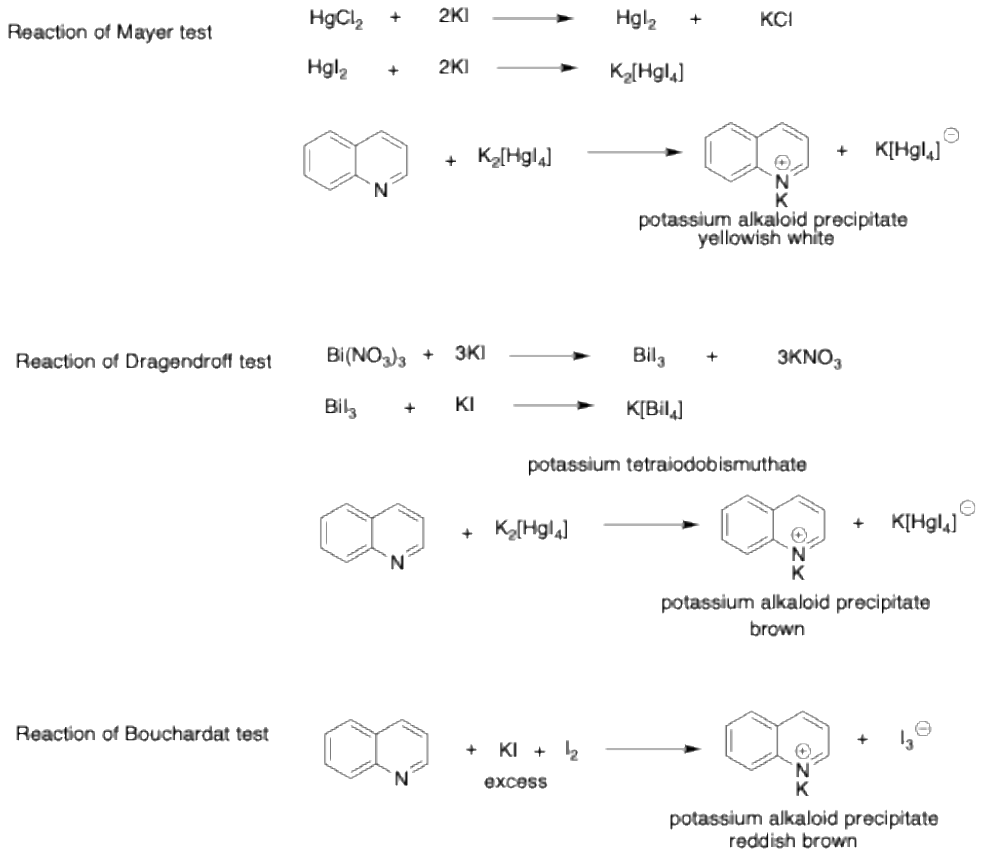

Figure 1. Proposed reactions of the Mayer test, Dragendorff test, and Bourchat test

Meanwhile, a positive identification of alkaloids via the Dragendorff's test was established by the formation of a reddish-brown or yellowish precipitate. This color change signifies the precipitation of a complex formed between the alkaloid and potassium. The effective formulation of Dragendorff's reagent requires dissolving bismuth nitrate in hydrochloric acid (HCl). This critical step prevents the hydrolysis of bismuth salts, which readily form the BiO^+ ion, thereby maintaining the concentration of the active Bi^{3+} ion in the solution. Furthermore, the Bi^{3+} ion reacts with potassium iodide to first produce bismuth(III) iodide (BiI_3), which subsequently dissolves in excess potassium iodide (KI) to form the reactive agent, potassium tetraiodobismuthate (K[BiI4]). The next step, the nitrogen atom with its lone pair electrons in the alkaloid structure—forms a coordinate covalent bond with the potassium ion (K^+) from the tetraiodobismuthate complex, thus leading to the observed alkaloid–potassium complex precipitation [11].

Furthermore, Bouchardat's reagent is a qualitative chemical assay used to detect alkaloids, sharing a common iodine–potassium iodide base with Wagner's reagent. Although both reagents are used for the same diagnostic purpose, Bouchardat's solution contains a higher concentration of potassium iodide (KI) [11]. This difference in concentration results in a more pronounced, darker precipitate when alkaloids are present, making Bouchardat's reagent often favored for yielding clearer and more intense results.The underlying mechanism involves a straightforward chemical interaction: alkaloid molecules react with the potassium–iodide complex within the reagent, leading to the precipitation of a potassium–alkaloid complex. The formation of this characteristic precipitate, ranging from light brown to yellow, confirms the presence of alkaloids in the tested sample.

3.1.2 Identification of flavanoid

The characteristic red coloration observed in the flavonoid test is a result of the reduction of flavonoid compounds, a reaction initiated by the addition of magnesium (Mg) powder and hydrochloric acid (HCl) to the extract as depicted in Figure 2. The positive finding in this assay is unequivocally confirmed by the distinct color change to a reddish solution in all *M. speciosa* sample.

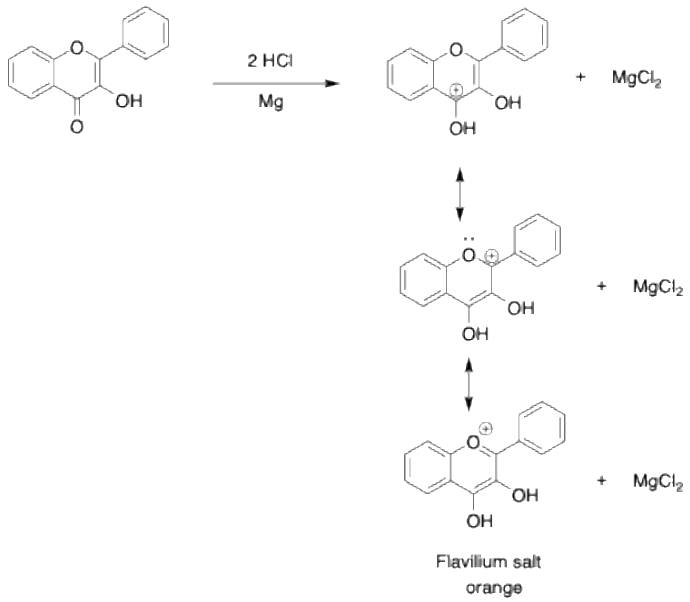

Figure 2. Flavonoid identification reaction proposed

3.1.3 Identification of triterpenoid and steroid

The screening for steroids and triterpenoids relies on the ability of these compounds to generate characteristic colors when treated with concentrated sulfuric acid (H_2SO_4) in an acetic anhydride solvent medium [12]. The colors expected are typically red, orange, or purple for triterpenoids, and blue for steroids. The ethanol extract of the *M. speciosa* leaves yielded a positive result, evidenced by a marked reddish–purple color change, which unequivocally confirms the presence of triterpenoid compounds.

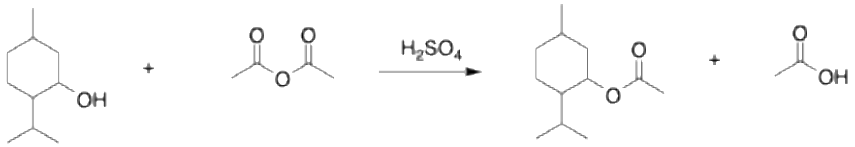

Figure 3. triterpenoid or steroid identification reaction proposed

3.1.5 Identification of tanine

The positive qualitative finding for tannins across all *M. speciosa* ethanol extracts was visually confirmed by the solution turning a dark color following the addition of $FeCl_3$ (Figure 4). This reaction occurs because the phenolic hydroxyl groups present in tannin structures readily chelate with the ferric ion (Fe^{3+}), thereby forming a characteristic colored complex.

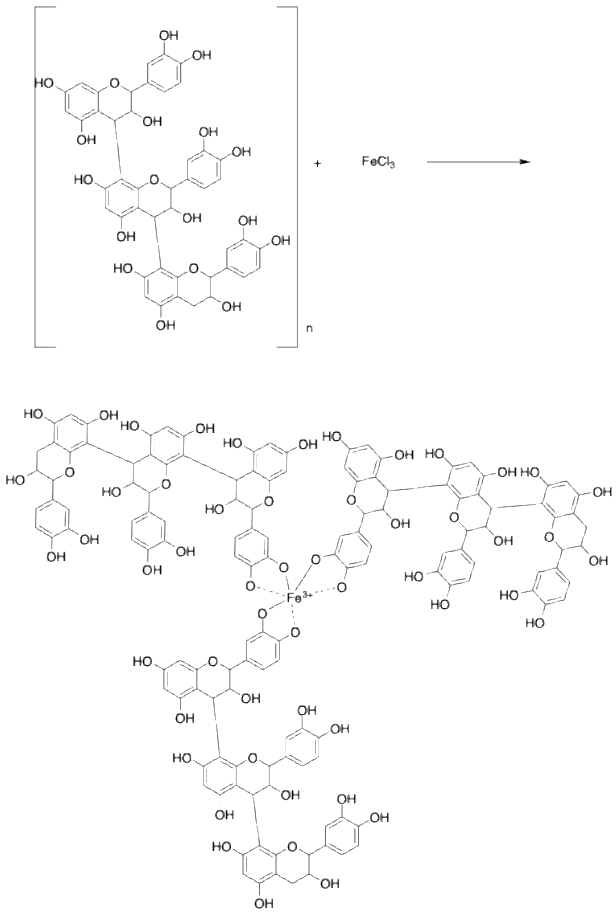

Figure 4. Taninn identification reaction proposed

3.1.5 Identification of saponin

The formation of stable foam in the saponin test is conclusive proof of these glycosides, confirming their surface-active nature and ability to hydrolyze in water to yield foam, glucose, and other compounds [13] (as demonstrated in Figure 5). Saponins act as surfactants because their structure contains both polar (glycosyl) and nonpolar (steroid/triterpenoid) groups. When agitated in water, they form micelles—structures where the polar groups face the exterior and the nonpolar groups are internalized—a phenomenon that creates the persistent foam. In this study, however, minimal foam production was observed, suggesting a relatively low concentration of saponins within the *M. speciosa* leaf extract.

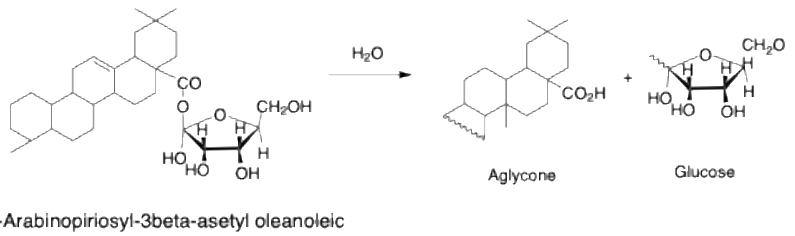

Figure 5. The hydrolysis reaction of saponins in aqueous solutions

3.2 Antibacterial activity

The antibacterial activity assessment demonstrated varying efficacy among the three test samples. Specifically, the red vein extract exhibited the highest activity against the Gram-negative bacterium, *S. typhi*. Conversely, the green vein extract showed the best activity against the Gram-positive bacterium, *S. aureus*, while the white vein extract displayed moderate activity against both bacterial strains. These results are summarized in Figure 6. The negative control, 96% ethanol, showed no antibacterial activity, confirming that the observed inhibition was derived solely from the active compounds within the *M. speciosa* leaf extract.

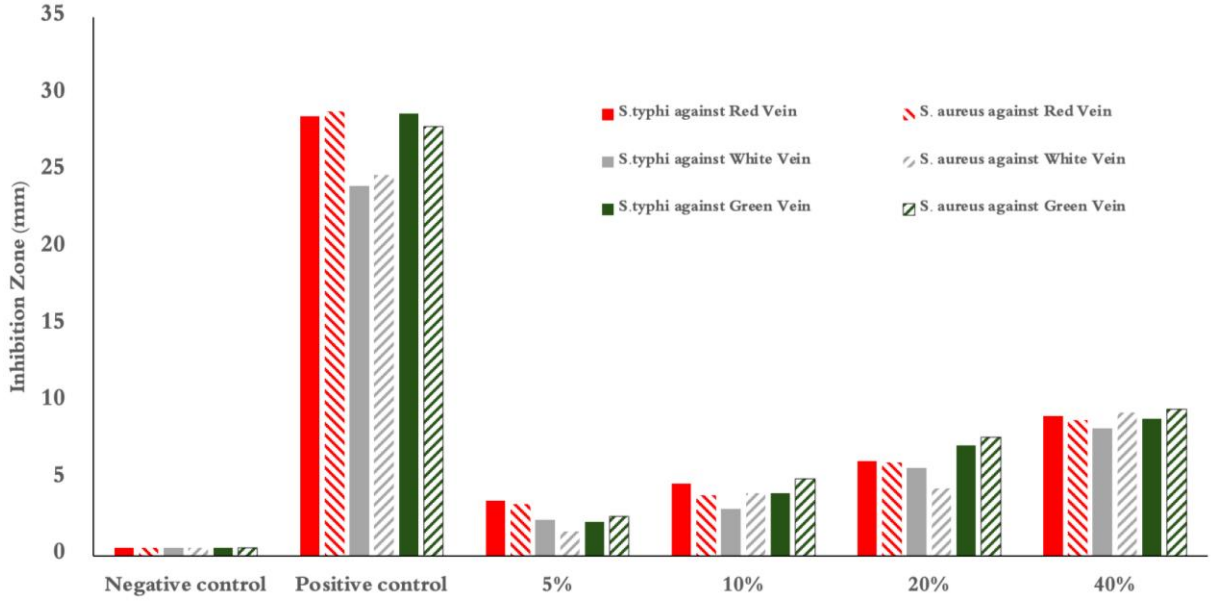

Figure 6. Antibacterial inhibition efficacy of M. speciosa ethanol extracts from different vein color variants against *Salmonella typhi* and *Staphylococcus aureus*.

Testing the three vein extracts across various concentrations revealed a clear dose-response relationship, where the diameter of the inhibition zone increased proportionally with the concentration of the red, white, and green extracts. However, all *M. speciosa* samples displayed generally weak inhibitory capabilities at the 5% concentration and reached only a moderate category at the 20% concentration.

Overall, the data indicate that the *M. speciosa* extracts possess significant antibacterial activity against both *S. typhi* and *S. aureus*, confirming the presence of bioactive compounds readily extracted by ethanol. Phytochemical screening results support this finding, as all samples were confirmed to contain active compound classes known for antimicrobial properties, including alkaloids, flavonoids, tannins, and saponins. The observed activity can be attributed to several synergistic mechanisms: Alkaloids (lipophilic or amphipathic) disrupt the bacterial cell membrane by interacting with lipids and proteins. This action compromises membrane integrity, leading to the leakage of essential cellular components (e.g., K^+, Na^+, and ATP) and ultimately causing cell death [14]. Flavonoids (possessing free hydroxyl groups) similarly disrupt the cytoplasmic membrane's lipid layer. This structural breakdown enhances cell permeability, resulting in the efflux of critical intracellular components (e.g., ATP, ions, proteins, and nucleic acids), which leads to lysis [15]. Tannins (rich in phenolic OH groups) utilize strong hydrogen and hydrophobic bonding to bind to proteins on the bacterial surface and within the cell. This binding can inactivate essential cytoplasmic enzymes crucial for energy production and replication (such as DNA polymerase or ATP-ase) [16]. In addition, saponins exhibit antibacterial activity through the disruption of bacterial cell membranes. This process results in the leakage of essential proteins and enzymes, potentially forming pores that ultimately lead to cellular mortality. This mechanism is effective against both Gram-positive and Gram-negative bacteria [17]. The

verified presence of alkaloids, flavonoids, tannins, and saponins across the different vein color extracts therefore reinforces the potential of *M. speciosa* as a natural source for developing novel antimicrobial agents.

4. CONCLUSION

The phytochemical screening of *M. speciosa* ethanol extracts from the red, white, and green vein variants confirmed the presence of multiple bioactive secondary metabolites, including alkaloids, flavonoids, phenolics, saponins, and tannins. The subsequent antibacterial testing established a clear dose dependent inhibitory effect against *S. typhi* and *S. aureus*, with activity stemming solely from the extract and not the ethanol solvent. Differential efficacy was observed: the red vein extract demonstrated the highest inhibitory activity against the Gram-negative bacterium, *S. typhi*, while the green vein extract was most potent against the Gram-positive bacterium, *S. aureus*. This observed antibacterial effect is mechanically supported by the synergistic action of the detected compounds: alkaloids and flavonoids primarily disrupt bacterial cell membrane integrity, leading to leakage; while tannins inactivate essential enzymes by binding to proteins and chelating metal ions. These findings collectively affirm the significant potential of the various *M. speciosa* vein extracts as promising natural sources for the development of new antimicrobial agents.

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