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In Vitro Assessment of Antimicrobial Activity and Synergistic Effects of Ethanolic Extracts from Six Medicinal Plants

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Abstract

This *in vitro* experimental study examines and evaluates the antimicrobial and synergistic effects of the ethanolic extract of six plants: *Biancaea sappan* (L.) Tod, *Bauhinia malabarica* Roxb, *Carthamus tinctorius* L., *Derris scandens* (Roxb.) Benth, *Hibiscus sabdariffa* L., and *Piper nigrum* L. against common microbial species representing gram-positive, gram-negative bacteria, and fungi, consisting of *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Candida albicans*. The plants were extracted using 90% ethanol. According to the standard method of agar diffusion assay, the micro-dilution method for minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. This study found that among the six plants, only *B. sappan* and *B. malabarica* exhibited moderate inhibitory effects against *S. aureus* and *S. epidermidis*. *B. sappan* had MIC values of 250 µg/ mL and 125 µg/ mL, respectively, and *B. malabarica* showed MIC values of 62.50 µg/ mL and 31.25 µg/ mL, respectively. The synergistic effects of a combination of *B. sappan* and *B. malabarica* extracts at a ratio of 25:75 were analyzed, and it was found that the combination inhibited *S. aureus* and *S. epidermidis* with MIC values of 250 µg/ mL and 125 µg/ mL, respectively. The fractional inhibitory concentration index (FICI) and the fractional bactericidal concentration index (FBCI) indicated antagonistic or synergistic effects of the combination, with FICI and FBCI values of 2.5–5.0 for both *B. sappan* and *B. malabarica* extracts in the 25:75 mixture. In conclusion, single plant ethanolic extracts of *B. sappan* and *B. malabarica* possess potent antimicrobial activity to varying degrees. However, the antimicrobial potency of the 25:75 ratio mixture of these extracts was shown to decrease against the same organisms, with *in vitro* antimicrobial activity and antagonistic effects observed only against the tested gram-positive bacteria.

Keywords**:** *antimicrobial activity; synergistic effect; antagonistic effect; Biancaea sappan (L.) Tod; Bauhinia malabarica Roxb*

1. Introduction

Infectious illnesses have been identified as one of the most significant threats to human health across the world. The majority of them are caused by microorganisms like bacteria and fungi. Unfortunately, the potential of synthetic or natural plant antibiotics has not been extensively researched, developed, or introduced into pharmaceutical care to mitigate the threat of microbial illnesses. Additionally, the abuse and misuse of synthetic antibiotics have gradually resulted in drug-resistant bacteria, posing a new worldwide therapeutic challenge to the public health

system known as antibiotic resistance. Presently, antimicrobial resistance (AMR) is one of the most significant health threats. It has reached high-risk levels concerning pathogens that do not respond to antimicrobial drugs. This makes infections more difficult to treat. (World Health Organization, 2019, 2022, 2023). The Centers for Disease Control and Prevention (U.S.) reported that Methicillin-resistant *Staphylococcus aureus* (MRSA), Multidrug-resistant *Staphylococcus epidermidis* (MDRSE), and *Candida* spp. were classified as serious threats (Centers for Disease Control and Prevention (U.S.), 2019; Murray et al., 2022; Siciliano et al., 2023). With an increase in bacterial resistance to antibiotics, natural antimicrobial plant products have gained attention in scientific research.

The use of natural products or therapeutic bioactive compounds from certain plant extracts has been ubiquitous for a long time. It is an important drug production source and raw material in the production of traditional, alternative, and modern medicines. Therefore, medicinal plants are considered important sources of new chemicals that may have therapeutic effects (Bulbul et al., 2011; Blumenthal et al., 2000; Li et al., 2024). Several plants have been employed for their antibacterial activity owing to active compounds, while others have been used by combining their common phytochemicals with antibiotics. Thus, the selection of six different plants (*B. sappan, B. malabarica, C. tinctorius, D. scandens, H. sabdariffa,* and *P. nigrum*) for the study was based on their traditional use and medicinal properties reported medicinal properties (Table 1).

For the extraction of most bioactive compounds, ethanol was used for extraction to compare biological activities, including antimicrobial activities. Ethanol was chosen as a widely used polar solvent for the preparation of plant extracts. It is a universal solvent capable of a higher safety profile and can dissolve a wide range of major polyphenol compounds (phenolic and flavonoid), alkaloids, saponins, tannins, steroids, and terpenoids found in plants (Abubakar, & Haque, 2020; Bashir et al., 2023; Ingle et al., 2017; Irfan et al., 2022; Pintać et al., 2018; Usman et al., 2022). It is a popular choice for preparing extracts available in the laboratory. Due to its ability to effectively extract compounds from the plant material, it can maintain the condition and value of the material along with reducing the risk of contamination before extraction (Abubakar, & Haque, 2020; Ćujić et al., 2016; Zhang et al., 2018). Numerous reports indicate that bioactive compounds from plant ethanolic extracts exhibit stronger antimicrobial properties compared to those extracted using methanol or water (Baluchamy et al., 2023; Acquavia et al., 2021; Chaudhry et al., 2022; Grozdanova et al., 2020; Hikmawanti et al., 2021; Valle et al., 2015; Valle et al., 2016).

Recently, various studies have examined the effects of combining plant extracts in a different ratio mixture. These combinations produced various effects on microorganisms, exhibited antimicrobial activity, and altered interaction, resulting in synergistic/ antagonistic effects (Kongcharoensuntorn et al., 2024). These results may be due to interactions between the constituents of different parts (Adwan et al., 2010; Donkor et al., 2023; Saquib et al., 2021). However, the antimicrobial effects from different ratios of plant extract combinations with antimicrobial activity, tested within blended plant ethanolic extract preparations, have not been published. This study focuses on the first assessment of the natural antimicrobial activity of six different plant ethanolic extracts and then evaluates the interaction of those extracts that have antimicrobial activity in combination to test synergistic effects against several common microbial strains, representing groups of gram-positive, gram-negative bacteria, and fungi.

2. Objectives

This study assessed the natural antimicrobial activity of six plant ethanolic extracts and evaluated their synergistic effects against common gram-positive, gram-negative bacteria, and fungi.

3. Materials and Methods

3.1 Collection and Authentication of Plant Materials

Plant material of *B. sappan* (Heartwood), *B. malabarica* (Leaf), *C. tinctorius* (Leaf), *D. scandens* (Leaf), *H. sabdariffa* (Flower), and *P. nigrum* (Fruit) was sourced from various regions within Thailand, received in May 2022, and subsequently cultivated in Pathum Thani, Thailand. The chosen collection method was deemed most suitable for maintaining the plant's post-collection viability. These procedures strictly complied with the standardized collection guidelines of the Botanical Garden Organization (BGO) under the Ministry of Natural Resources and Environment (MNRE), Thailand. Assistant Professor Dr. Thanapat Songsak was responsible for the identification of the plant species. The collected plant materials have been meticulously preserved at the Herbarium in the Department of Pharmacognosy, College of Pharmacy, Rangsit University, Thailand. The herbarium's designated coding for the *B. sappan*, *B. malabarica*, *C. tinctorius*, *D. scandens*, *H. sabdariffa*, and

P. nigrum samples are B.sLT-HT-01, B.mR-L-02, C.tL-L-03, D.sRB-L-04, H.sL-FW-05, and P.nL.F-06, respectively.

3.2 Preparation of Plant Ethanolic Extracts

All components of the fresh plants in each part were chopped into small pieces after being thoroughly cleansed with tap water to remove any unnecessary contaminants, and they were dried at 50 °C using a laboratory hot air oven, at 50 °C until dry weight stability was observed. Then the dried plant material was crushed using an electric grinder. The plant powder from each dried plant part was sieved through a 400/65 mm sieve (mesh = 0.75 mm) (ISO 3310-1) for subsequent experiments. For each plant material powder (*B. sappan*, *B. malabarica*, *C. tinctorius*, *D. scandens*, *H. sabdariffa*, and *P. nigrum*), 90 grams of powder were macerated in 500 milliliters of 90% ethanol at room temperature (25–30 °C) for three days. The extract solutions obtained from maceration were filtered through Whatman No. 1 filter paper three times and then pooled together. After the filtering, the extract was subjected to rotary evaporation at reduced pressure and stored at -20 °C until use. The extract was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (50 mg/mL) stored at -20 °C until use. The working solution for each test was prepared from this concentrated stock solution.

3.3 Antimicrobial Activity Testing

3.3.1 Tested Microorganisms

This study assessed the following microbial strains: *Staphylococcus aureus* (*S. aureus*) (TISTR 1466), *Staphylococcus epidermidis* (*S. epidermidis*) (TISTR 518), *Escherichia coli* (*E. coli*) (ATCC 25922), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 27853), and *Candida albicans* (*C. albicans*) (ATCC 10231). They were assigned to three groups indicated by gram-positive bacteria, gram-negative bacteria and fungi, which were inoculated and isolated in culture medium for recovery and streak-plate subculture, then incubated overnight at 37°C in Mueller-Hinton agar/broth (MHA/MHB) and Sabouraud dextrose agar/broth (SDA/SDB) (HiMedia Laboratories LLC, USA), respectively, to be assessed for their *in vitro* antimicrobial activity and synergistic effect for all assay designs. The Drug and Herbal Product Research and Development Center, College of Pharmacy, Rangsit University, and Professor Emeritus Dr. Janenuj Wongthavatchai, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, assisted in obtaining and correctly identifying the bacteria used in this study.

Table 1 A list of plants reported to have traditional uses and pharmacological properties

Scientific names	Family names	Bioactive compounds	Traditional and Pharmacological properties	References
Biancaea sappan (L.) Tod.	Fabaceae or Leguminosae	Phenolics like brazilin, Xanthone, Coumarin, Chalcones, Flavones, Diterpenes, Homo-Isoflavonoids.	Antibacterial, Antifungal, Antiviral, Antioxidant, Anthelmintic, Anticonvulsant Immunomodulatory, Insecticidal, Analgesic, Anti-inflammatory	(POWO, 2024b; Rajput et al., 2022; Prashith et al., 2021)
Bauhinia malabarica Roxb.	Fabaceae or Leguminosae	Flavonoids like quercetin, Isoquercitrin, Glycoside, Polyphenolic, Hyperoside, kaempferol, Afzelin, 6,8-di-C-methylkaempferol-3-methyl ether	Anthelmintic, Antiperiodic, Antioxidant, Analgesic, Antibacterial, Antifungal, Anti-inflammatory, Nephroprotective, Hypolipidemic, Antiatherogenic	(Igwe, & Okeke, 2017; POWO, 2024a; Thetsana, 2019; Thetsana et al., 2019)
Carthamus tinctorius L.	Asteraceae	Flavonoids, Alkaloids, Organic acids, Glycosides, Polyacetylenes, Steroids, Coumarins, Fatty acids, Phenylethanoid	Anticoagulant, Antihypertensive, Cardioprotective, Antioxidant, Neuroprotective, Antitumor, Anti-melanogenic, Antidiabetic, Immunostimulants, Anti-obesity, Anti-arthritic, Anti-inflammatory	(Asgarpanah, & Kazemivash, 2013; Lamichhane et al., 2022; POWO, 2024c; Zhang et al., 2016)

Table 1 A list of plants reported to have traditional uses and pharmacological properties (Cont.)

Scientific names	Family names	Bioactive compounds	Traditional and Pharmacological properties	References
Derris scandens (Roxb.) Benth.	Fabaceae	Flavonoids, Isoflavones, Coumarins	Antibacterial, Antioxidant, Anticancer, Anti-inflammatory, Musculoskeletal pain, Immunostimulants	(Hussain et al., 2015; Madhiri, & Panda, 2018; POWO, 2024d; Puttarak et al., 2016)
Hibiscus sabdariffa L.	Malvaceae	Natural acids, Phenolic acids, Organic acids, Anthocyanins	Antibacterial, Antioxidant, Nephron/Hepatoprotective, Diuretic, Anti-cholesterol, Anti-diabetic, Anti-hypertensive	(Da-Costa-Rocha et al., 2014; POWO, 2024e; Riaz, & Chopra, 2018)
Piper nigrum L.	Piperaceae	Alkaloid like piperine, Phenolics, Flavonoids, Terpenes, Tannin, Carotenoids, Sterols	Antibacterial, Anti-inflammatory, Antipyretic, Antioxidant, Antitumor	(Ashokkumar et al., 2021; POWO, 2024f; Shityakov et al., 2019)

3.3.2 Screening of Antimicrobial Activity of Plant Ethanolic Extracts by Agar Diffusion Assay

The agar diffusion assay, using the agar well diffusion technique, involved placing the antimicrobial agent into an agar well made with a sterile disposable pasteur pipette (inside diameter of agar well: approximately 6–7 mm) under aseptic conditions. It was widely used as a first part of research methods for screening plant extracts for antibacterial activity (Balouiri et al., 2016). The microbial preparation begins with picking the pure single colonies of each microorganism from their culture plate, which should be prepared in sterile saline solution (0.85% NaCl by autoclaving) to help disperse and dilute the bacteria, then after checking an adjusted McFarland turbidity scale of 0.5 ($1.5x10^8$ CFU/mL), sterile cotton swabs was employed to inoculate the agar plate surface with each bacterium, distributing a volume of microbial inoculum evenly and thoroughly throughout the surface. 100 µL of the targeted antimicrobial agent, which was 1,000 µg/mL of each plant ethanolic extracts, was transferred into the agar wells. This was performed in triplicate for each strain, and the test plates were incubated under appropriate conditions at 37°C for approximately 24 hours, depending on the test microorganism. The antimicrobial agent diffused from the agar well and spread into the gel of the agar plate. A clear zone was observed to determine the inhibitory activity of each microbial strain tested. The sizes of the inhibition clear zones surrounding the wells were measured to the nearest whole on a millimeter scale with vernier calipers. The sensitivities of each isolated bacterial species were assessed against numerous antibiotics: Positive controls (PC) were ampicillin 10 µg/disc, gentamicin 10 µg/disk, and ketoconazole 50 µg/disk was used agar disc diffusion technique (Kirby-Bauer) according to Clinical and Laboratory Standards Institute (CLSI) recommendations (Bubonja-Šonje et al., 2020; Clinical and Laboratory Standards Institute, 2024; Ii et al., 2022; The American Society for Microbiology, 2009), and a diluent of extract (5% DMSO) was employed as a negative control (NC) in this investigation, then ensuring that antimicrobial activity is evaluated accurately and objectively. For each antimicrobial assay, the results were provided as the average of the least-triplicate trials (n = 3).

$$\% \text{ RIZD} = \frac{(\text{IZD sample - IZD negative control})}{(\text{IZD antimicrobial standard})} \text{ x } 100$$

The expression used to quantify antibacterial activity was the Relative Inhibition Zone Diameter (RIZD), calculated using the following equation: % RIZD = (IZD of sample – IZD of negative control)/(IZD of an antimicrobial standard of each strain) that reflects the percentage of the relative diameter of the inhibitory zone, measured in millimeters. The calculated findings reveal that the percentages equal 100, indicating that specific extracts are as effective in inhibiting bacterial growth as the antibiotics used in the study. The common interpretation of the breakpoint values is as follows: 0% = no effect, > 0 – 100% = moderate efficacy, > 100% = good efficacy, and > 200% = high efficacy (Patthamasopasakul et al., 2024; Costa et al., 2023; Gouvinhas et al., 2018; Leal et al., 2020; Rojas et al., 2006).

3.3.3 Determination of Minimal Inhibitory Concentration (MIC) of Plant Ethanolic Extracts by the Resazurin-based 96-well Plate Method

Determination of the MIC of the plant ethanolic extracts against each microbial strain by micro-broth dilution assays using MHB and SDB. The concentrations of the plant ethanolic extracts ranged from 1,000 μg/mL to 1.95 μg/mL prepared by two-fold serial dilutions. Consequently, 100 μL culture of inoculum for each strain at 1.5×10^5 CFU/mL was transferred into the wells of polystyrene sterile flat-bottom 96-well plates. Each concentration from the 2-fold dilution of the plant ethanolic extract (100 μL) was loaded in triplicate wells for each strain. 100 μL of 2% DMSO were loaded in triplicate wells and considered negative control. After incubating the plate for 24 hours at 37°C, the MIC value was determined as the first well with the lowest concentration of compounds showing no visible bacterial growth or turbidity (Kowalska-Krochmal, & Dudek-Wicher, 2021; Swebocki et al., 2023). To confirm microbial growth by the colorimetric assay (Elshikh et al., 2016; Han et al., 2024), 30 μL of 0.015 % resazurin solution were added to all test wells and incubated for 2–4 hours at 37°C. Wells that exhibited microbial growth showed a color change, as the microorganism reduced resazurin (blue dye) to resofurin (pink).

3.3.4 Determination of Minimum Bactericidal Concentration (MBC) of Plant Ethanolic Extracts from the Micro-dilution Method by Drop Plate Method

Determination of the MBC of the plant ethanolic extracts against each microbial strain was done by pipetting 50 μL of the culture from each well of the micro-broth assay onto each MHA or SDA culture plate, performed in triplicate for each strain. They were then incubated for 24 hours at 37°C. The lowest concentration of extracts that showed no bacterial growth was considered the point of MBC value (Swebocki et al., 2023; Thongdonphum et al., 2023).

3.3.5 Synergistic Antimicrobial Activity Testing of Plant Ethanolic Extracts by Fractional Inhibitory Concentration Index (FICI) and Fractional Bactericidal Concentration Index (FBCI)

$$\Sigma FIC=[FIC(A) + FIC(B)]= \left[\frac{MIC(A,B)}{MIC(A)}+\frac{MIC(B,A)}{MIC(B)}\right]$$

As usual, the results of the determination of the MIC or MBC values of the plant ethanolic extracts against each microbial strain by micro-broth dilution assays. The values of the MIC or MBC were used in the analysis of the synergistic effects of plant ethanolic extracts in combination using the fractional inhibitory concentration (FICI) and fractional bactericidal concentration (FBCI) index. Following the formula that determines the index: ΣFIC = FIC(A) + FIC(B) = (MIC(A, B)/MICA) + (MIC(B, A)/MICB), where MIC(A) and MIC(B) were the MIC or MBC of the plant ethanolic extracts A and B alone, respectively. The MIC (A, B) and MIC (B, A) were the values of the MIC or MBC of the plant ethanolic extracts in combination, respectively, with the minimum concentration of effective combinations included. The correlation between the FIC or FBC values and the effect of the antibacterial agent combinations was calculated using the FIC or FBC index formula. The common interpretation of the breakpoint values was as follows: Synergistic effect (FICI or FBCI $\leq$ 0.5), additive effect (0.5 $<$ FICI or FBCI $\leq$ 1), indifferent effect (1 $<$ FICI or FBCI $\leq$ 4), and antagonistic effect (FICI or FBCI $>$ 4) (Vuuren, & Viljoen, 2011).

3.4 Statistical Analysis

The measurements for all experiments were presented as the average of triplicate run (n = 3). For each result, the data were summarized as the mean ± standard error of the mean (SEM). The significance of the differences between two related groups of the single tested plant ethanolic extracts was determined using an independent t-test and a one-way analysis of variance (ANOVA), followed by a Tukey HSD multiple comparison test to interpret the significance of the differences between the three combination related groups of ratio mixtures of the tested plant ethanolic extracts. A probability ($P \leq 0.05$) was considered statistically significant.

4. Results and Discussion

4.1 Antimicrobial Activity of Plant Ethanolic Extracts by Agar Diffusion Assay

The findings from this study reveal significant insights into the antibacterial properties of six various plant ethanolic extracts using the agar diffusion technique. The diameter of the inhibition zone was measured to correlate with the antimicrobial properties of the plant ethanolic extracts and the strains of organisms (Table 2). The single plant ethanolic extracts of *C. tinctorius*, *D. scandens*, *H.*

sabdariffa, and P. nigrum did not have the ability to inhibit the growth of *S. aureus, E. coli, S. epidermidis, P. aeruginosa,* and *C. albicans*. These results were inconsistent with previous studies reporting on the antimicrobial activity of ethanol extracts from these plants (Balali et al., 2023; Haleem et al., 2023; Sri Chaithanya, & Seedevi, 2023; Zarai et al., 2013). It may be troublesome since the composition of plant extracts varies according to local climate, environmental factors, differences in the cultivation sources, harvest season, and quality control processes of these plant materials before extraction. This may result in reduced amounts of active compounds. Thus, these absences of activity demonstrate that these the plant extracts lacked sufficient antimicrobial properties were insufficient to provide an observed effect against these specific microbes (Balekundri, & Mannur, 2020; Radulović et al., 2013; Vaou et al., 2021). Conversely, the single plant ethanolic extracts of *B. sappan* and *B. malabarica* presented a notable antibacterial with moderate effect against two bacteria in the genus of *Staphylococcus*, *S. aureus* and *S. epidermidis*, which included important opportunistic pathogens colonized on the skin that cause commensal infectiousness and the most common nosocomial infections (Ahmed, 2011; Atunnisa et al., 2023; Chessa et al., 2015, 2016; Otto, 2009; Siciliano et al., 2023).

Previous studies have shown that alcoholic crude extracts of sappan heartwood are potent in inhibiting *S. aureus* (Hemthanon, & Ungcharoenwiwat, 2022) and *S. epidermidis* (Atunnisa et al., 2023). According to research on sappan wood, using ethanol as an extraction solvent has the highest wood extraction yield. There were included many different biological actions and several structurally unique phenolic components such as brazilin, xanthone, coumarin, chalcones, flavones, and homo-isoflavonoids. They have antimicrobial activity against a variety of pathogenic bacteria, although slightly less potent than methanol extraction (Nirmal et al., 2015; Nirmal & Panichayupakaranant, 2015; Niu et al., 2020; Rajput et al., 2022; Srinivasan et al., 2012; Vij et al., 2023). Additionally, ethanol had been used as a solvent in previous work to extract a series of flavonoid derivatives, including the essential ingredient in *B. malabarica* leaf ethanolic extract. It was identified several distinctive bioactive substances, including hyperoside, kaempferol, afzelin, 6,8-di-C-methyl kaempferol-3-methyl ether, quercetin, isoquercitrin, and a glycoside molecule, all of which demonstrated antibacterial effects against a broad range of bacterial strains. These compounds can be used to prevent and treat various bacterial infections, although they exhibit slightly less potency when extracted with ethanol compared to methanol. (Nguyen, & Bhattacharya, 2022; Thetsana, 2019; Thetsana et al., 2019; Yang et al., 2020). This evidence suggests that plants contain bioactive chemical compounds, with most previous studies reporting that the major active ingredients belong to phenolic and flavonoid compound groups (Aliyu et al., 2009; Fernandes et al., 2012; Kaewamatawong et al., 2008; Manso et al., 2021; Sasmal, n.d.; Sharma et al., 2014). This finding was consistent with research on the activity that may be able to inhibit these specific strains of gram-positive bacteria. It may be related to variances in cell wall structures and resistance mechanisms among bacteria and species. Due to the gram-positive bacteria have a more accessible peptidoglycan layer, antibacterial substances can penetrate more effectively. In contrast, the outer membrane of gram-negative bacteria or fungi operates as a barrier to a wide range of substances through distinct mechanisms. (Reygaert, 2018; Uddin et al., 2021). Therefore, the extracts tested in this study were ineffective against gram-negative bacteria, including *E. coli* and *P. aeruginosa*, as well as fungi like *C. albicans*. This may be a result of gram-negative bacteria having cell walls with a thin lipopolysaccharide outer membrane that acts as a permeability barrier. This membrane may have successfully reduced the quantity of plant extract that the bacteria's efflux pump mechanisms could release. Therefore, in some cases, it has been shown that gram-negative bacteria were more resistant than gram-positive bacteria to antimicrobials generated from plant extract that they may even show no impact at all (Biswas et al., 2013; Saxena et al., 2023). Furthermore, the complex structural and biological differences between eukaryotic fungi compared with the prokaryotic pathogens of bacteria. Their integrative resistance mechanisms of fungi may result in higher antimicrobial resistance (Fisher et al., 2022; Lee et al., 2023).

The antimicrobial properties of single and combined plant ethanolic extracts in different ratios (25:75, 50:50, and 75:25) between *B. sappan* and *B. malabarica* were evaluated. They revealed a consistent antibacterial effect against *S. aureus* and *S. epidermidis*, while the combinations did not inhibit *E. coli*, *P. aeruginosa*, or *C. albicans*. The results demonstrated that the combined extracts show moderate antimicrobial efficacy, both individually and in combination, when mixed in varying ratios of *B. sappan* and *B. malabarica*, as shown in Table 3. For

the single plant ethanolic extracts, the %RIZD values against *S. aureus* were 23.93 ± 0.39 and 31.56 ± 0.90, while the %RIZD values against *S. epidermidis* were 26.50 ± 0.94 and 35.40 ± 1.34 for *B. sappan* and *B. malabarica*, respectively, at the same ratio of plant ethanolic extracts. Furthermore, when the ethanolic extracts of *B. sappan* and *B. malabarica* were combined in varying ratios (25:75, 50:50, and 75:25), the mixtures preserved antibacterial potency against a moderate level for *S. aureus*, with %RIZD values of 31.30 ± 0.37, 30.27 ± 1.38, and 23.50 ± 1.12. Like *S. epidermidis* that was shown with %RIZD values of 25.66 ± 0.67, 23.50 ± 2.85, and 18.98 ± 0.21, respectively, but still had not inhibited the growth of *E. coli*, *P. aeruginosa*, or *C. albicans*

These results indicate that while there is a noticeable antibacterial effect, the efficacy does not significantly improve with the combination, and in some ratios, it appears to be slightly reduced. This might be due to the specialized interactions between the bioactive compounds found in these plants, which may not improve effectiveness against the resistant outer membrane of gram-negative bacteria or the different cellular structures of fungi. This aligns with the first screening and previous studies, indicating that while individual plant extracts can exhibit specific antibacterial activities, their combinations do not always result in synergism or a broader spectrum of action. For instance, the bioactive compounds in *B. sappan* and *B. malabarica* are known for their effectiveness against gram-positive bacteria and this may potentially be due to small molecules or mechanisms involving cell wall disruption or inhibition of essential bacterial enzymes. The mechanisms by which these extracts exert their antibacterial effects could involve disruption of cell wall synthesis, inhibition of protein synthesis, or interference with other critical bacterial processes. However, this did not give any indication regarding more specific mechanisms of action (Oulahal, & Degraeve, 2022; Sullivan et al., 2020; Vaou et al., 2022).

Table 2 Results of the antimicrobial activity testing of the investigated single plant ethanolic extracts in an agar diffusion assay

Antimicrobial agent	**Inhibition zone (mm)[IZ] against tested microbial**				
	S. aureus	***S. epidermidis***	***E. coli***	***P. aeruginosa***	***C. albicans***
Single-plant ethanolic extracts					
- 1,000 µg/mL of *B. sappan* L.Tod (Heartwood)	9.30 ± 0.15	12.70 ± 0.45	-	-	-
- 1,000 µg/mL of *B. malabarica* Roxb (Leaf)	12.27 ± 0.39	16.97 ± 0.64	-	-	-
- 1,000 µg/mL of *C. tinctorius* L. (Leaf)	-	-	-	-	-
- 1,000 µg/mL of *D. scandens* (Roxb) Benth (Leaf)	-	-	-	-	-
- 1,000 µg/mL of *H. sabdariffa* L. (Flower)	-	-	-	-	-
- 1,000 µg/mL of *P. nigrum* L. (Fruit)	-	-	-	-	-
Ratio mixture of *B. sappan* L.Tod : *B. malabarica* Roxb					
- 25:75	12.17 ± 0.15	12.30 ± 0.32	-	-	-
- 50:50	11.77 ± 0.54	11.27 ± 1.37	-	-	-
- 75:25	9.13 ± 0.44	9.10 ± 0.10	-	-	-
Positive control					
- 10 µg/disk of ampicillin	38.87 ± 1.32	47.93 ± 0.60	NA	NA	NA
- 10 µg/disk of gentamicin	NA	NA	18.00 ± 0.25	29.13 ± 0.64	NA
- 50 µg/disk of ketoconazole	NA	NA	NA	NA	47.40 ± 1.15
Negative control					
- 2% DMSO	-	-	-	-	-

(-) = No activity of antimicrobial property, (NA) = Not analysis, [IZ] Inhibition zones including the diameter of the paper disc/agar well (6 mm), The results are represented as mean ± SEM values of 3 independent tests (n = 3)

Table 3 Results of percentage of inhibition zone diameter (%RIZD) in relation to the tested antibiotic

Antimicrobial agent / Concentration (µg/mL)	% RIZD against tested microbial (Antibiotic drug : Ampicillin 10 µg/disc)	
	S. aureus	***S. epidermidis***
Single-plant ethanolic extracts		
- 1,000 µg/mL of *B. sappan* L.Tod	23.93 ± 0.39	26.50 ± 0.94
- 1,000 µg/mL of *B. malabarica* Roxb	31.56 ± 0.90	35.40 ± 1.34
Ratio mixture of *B. sappan* L.Tod : *B. malabarica* Roxb		
- 25:75	31.30 ± 0.37	25.66 ± 0.67
- 50:50	30.27 ± 1.38	23.50 ± 2.85
- 75:25	23.50 ± 1.12	18.98 ± 0.21

0% = No effect, > 0 - 100% = Moderate efficacy, > 100% = Good efficacy, > 200% = High efficacy, The results are represented as mean ± SEM values of 3 independent tests (n = 3)

Table 4 Results of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the antimicrobial activity testing of single plant ethanolic extracts

Antimicrobial agent	Antimicrobial activity against tested microbial			
	S. aureus		***S. epidermidis***	
	MIC	**MBC**	**MIC**	**MBC**
Single-plant ethanolic extracts				
- *B. sappan* L.Tod	250.00	> 500.00	125.00	125.00
- *B. malabarica* Roxb	62.50	125.00	31.25	31.25
Ratio mixture of *B. sappan* L.Tod : *B. malabarica* Roxb				
- 25:75	250.00	> 250.00	125.00	125.00

The statistical analysis of the % RIZD among the different mixture ratios revealed insightful patterns regarding their antibacterial efficacy. Specifically, the ratios of 25:75 and 50:50 did not exhibit statistically significant differences in their % RIZD values ($P > 0.05$). This indicates that the antibacterial activities of these mixtures are relatively similar. However, a significant difference was observed with the 75:25 ratio, suggesting a variation in antimicrobial effectiveness with moderate effect with this combination. (Gouvinhas et al., 2018; Leal et al., 2020). This result was achieved with the 25:75 mixture. This finding underscores the potential of the 25:75 ratio to maximize antibacterial efficacy from calculated when compared with their combination in this study. Therefore, focusing on this specific ratio for further testing is warranted. Subsequently, the 25:75 mixture ratio was chosen based on these preliminary %RIZD results. It may provide a deeper understanding of the antimicrobial potency and effectiveness or some interaction of the extract ratios in combination, further validating its antimicrobial agent in this study. Although the potency of all ratios in the mixture was shown to be moderate efficacy used for the experiment to determine the MIC and MBC for use in the next interpretation of other parameters of synergistic antimicrobial agents.

4.2 Determination of MIC and MBC of Plant Ethanolic Extracts by the Resazurin-based 96-well Plate Micro-dilution and Drop Plate Methods

The determination of MIC and MBC for the ethanolic extracts of *B. sappan* and *B. malabarica* against *S. aureus* and *S. epidermidis* provides a deeper understanding of their antibacterial potency. The MIC and MBC values, which were tested over a concentration range of 1,000 to 1.95 µg/mL, reveal significant antibacterial activity for both extracts. Table 4 highlights the efficacy of these extracts, both single and the mixtures, in different ratios. For *S. aureus*, the MIC values were 250 µg/mL for *B. sappan* and 62.5 µg/mL for *B. malabarica*, while the MBC values were > 500 µg/mL and 125 µg/mL, respectively. These results indicate that *B. malabarica* was more potent against *S. aureus* compared to *B. sappan*, requiring lower concentrations to inhibit and kill the bacteria. Previous research has demonstrated that

alcoholic crude extracts of *B.sappan* heartwood, including lead compounds such as brazilin (Muangrat, & Thipsuwan, 2023; Nirmal et al., 2015), or lead bioactive flavonoid of quercetin from *B.malabarica* extract (Nguyen, & Bhattacharya, 2022) were highly effective in inhibiting *S. aureus* with low MIC and MBC values (Hemthanon, & Ungcharoenwiwat, 2022). Consistent with these findings. Similarly, regarding antimicrobial activity against *S. epidermidis*, *B. sappan* showed MIC and MBC values of 125 μg/mL, whereas *B. malabarica* exhibited even lower MIC and MBC values of 31.25 μg/mL, revealing significant antibacterial activity for both extracts. This again underscores the greater efficacy of *B. malabarica* 's comparative effectiveness, with consistently lower MIC and MBC values than *B. sappan*, implying that it contains stronger antibacterial agents or may have higher levels of main bioactive components. The MIC and MBC values were accurate and precise for measuring antimicrobial potency; nevertheless, it cannot identify specific bioactivity or interaction (Dafale et al., 2016). When the extracts were combined at a 25:75 ratio (*B. sappan* : *B. malabarica*), the results align with previous findings for the single extracts, where the effect against *S. aureus* showed MIC and MBC values of 250 and >250 μg/mL, respectively, and exhibited *S. epidermidis* showed both MIC and MBC values of 125 μg/mL. These findings suggest that combining the extracts did not significantly enhance the antibacterial activity compared to the stronger individual extracts (Cacace et al., 2023). Therefore, the moderate antibacterial potency of *B. malabarica* and *B. sappan* highlights their properties as therapeutic agents against only gram-positive bacteria such as *S. aureus* and *S. epidermidis*. However, the lack of enhanced activity in combinations suggests that further research will be needed to optimize formulations and potentially discover synergistic interactions with other antibacterial compounds or extracts.

4.3 Analysis of the antimicrobial synergistic effects in the combination of plant ethanolic extracts assays by FICI and FBCI

The analysis and investigation into the antimicrobial synergistic effects of the combination of *B. sappan* and *B. malabarica* ethanolic extracts at a 25:75 ratio provide important insights into their potential interactions by FICI and FBCI to assess synergy. We found varying results indicating the nature of the interactions between the extracts, as shown in Table 5. For both *S. aureus* and *S. epidermidis*, FICI and FBCI values were calculated as 5, which indicates an antagonistic interaction since FICI and FBCI values greater than 4 are typically interpreted as antagonistic.

This suggests that the ratio of 25:75 in combination with *B. sappan* and *B. malabarica* reduced their overall efficacy against these bacteria. Although the FBCI value for *S. aureus* was 2.5, which was lower than *S. epidermidis*, this slight difference suggests a somewhat antagonistic interaction of both plant ethanolic extracts against *S. aureus* compared to *S. epidermidis* (Vuuren & Viljoen, 2011). The implications of antagonistic interactions observed in the combination of *B. sappan* and *B. malabarica* extracts at a 25:75 ratio imply that the compounds within each extract may compete for the same bacterial targets or interfere with each other's mechanisms of action (Álvarez-Martínez et al., 2021; Sullivan et al., 2020; Vaou et al., 2022). These findings highlight the need for careful consideration when combining plant extracts, as interactions between bioactive compounds may reduce antibacterial efficacy. Further research is needed to optimize the beneficial use of plant extracts as ingredients in effective antibacterial prescription drugs (Caesar, & Cech, 2019).

Table 5 Result of analyzed the synergistic effects of plant ethanolic extracts in combination by the fractional inhibitory concentration (FICI) and the fractional bactericidal concentration (FBCI) index

Antimicrobial agent	**Synergistic effect of antimicrobial activity against tested microbial**			
	S. aureus		***S. epidermidis***	
	FICI	**FBCI**	**FICI**	**FBCI**
Ratio mixture of *B. sappan* L.Tod : *B. malabarica* Roxb				
- 25:75	5.00	2.50	5.00	5.00

Synergistic effect (FICI or FBCI $\leq$ 0.5), Additive effect (0.5 < FICI or FBCI $\leq$ 1), Indifferent effect (1 < FICI or FBCI $\leq$ 4), Antagonistic effect (FICI or FBCI > 4)

5. Conclusion

These data demonstrate that different plant extracts produce variable results against each microorganism. Among the six ethanolic plant extracts tested, only *B. sappan* and *B. malabarica* demonstrated antibacterial activity against *S. aureus* and *S. epidermidis*, both of which are *Staphylococcus* species known to cause common nosocomial infections. These findings highlight the moderate potential of these extracts as sources of new antibacterial agents, specifically targeting gram-positive bacteria. However, the extracts were ineffective against gram-negative bacteria and fungi, likely due to the structural differences in their cell walls and other resistance mechanisms. The ethanolic extracts of *B. sappan* may contain phenolic components of brazilin and *B. malabarica* rich in quercetin flavonoid, exhibiting promising antibacterial properties based on the previous research. However, they show less potency than extracts obtained with other solvents. The observed MIC and MBC values provide a quantitative measure of the potency of the major bioactive compound, and the antimicrobial activity of *B. malabarica* extract is stronger than that of *B. sappan* extract. Although the combination of both extracts was explored for potential synergistic or antagonistic effects, it did not significantly enhance antibacterial activity. The results indicated that the antagonistic effects of the combination of *B. sappan* and *B. malabarica* were limited to gram-positive antibacterial activity. Future research should focus on isolating and characterizing the active compounds within these extracts, determining their specific mechanisms of action, and exploring their effectiveness. These compounds may not interact synergistically with other compounds that target bacteria or fungi, necessitating further investigation into the specific phytochemical interactions to elucidate these pathways and fully understand their potential and limitations as antibacterial agents.

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7. References

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