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Antibacterial activity of combined ethanol extracts of sappan wood (caesalpinia sappan l.) and mint leaves (mentha piperita l.) against escherichia coli

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ABSTRACT

Escherichia coli is an important bacterial pathogen associated with various infections, and the increasing prevalence of antimicrobial resistance highlights the need to investigate alternative antibacterial agents. This study evaluated the antibacterial activity of ethanol extracts of sappan wood (*Caesalpinia sappan* L.) and mint leaves (*Mentha piperita* L.), individually and in combination, against *E. coli* [insert the confirmed strain designation]. The extracts were prepared by maceration using 70% ethanol. Antibacterial activity was assessed using broth dilution and disk diffusion methods, while the interaction between extracts was evaluated qualitatively using the paper strip method. Disk diffusion tests were performed in triplicate, and inhibition zone data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with statistical significance set at $p < 0.05$. The minimum inhibitory concentration (MIC) could not be determined by visual observation because the extracts' color and turbidity interfered with the assessment of bacterial growth. No alternative quantitative MIC determination was performed; therefore, the MIC values remain undetermined. The minimum bactericidal concentration (MBC), determined by subculturing onto Endo Agar, was 12.5% for sappan wood extract and 25% for mint leaf extract. Among the tested combinations, the 2:1 ratio of sappan wood to mint leaf extract produced the largest inhibition zone (27.28 ± 0.33 mm; $n = 3$). One-way ANOVA indicated significant differences among treatment groups ($p < 0.001$). The paper strip test showed an inhibition pattern suggestive of a possible synergistic interaction; however, this finding was qualitative and was not confirmed using a combination index or fractional inhibitory concentration index. These findings indicate that the 2:1 combination exhibited the greatest antibacterial activity among the tested formulations under the experimental conditions, warranting further investigation using quantitative combination methods.

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INTRODUCTION

Escherichia coli is a Gram-negative bacterium commonly found in the human gastrointestinal tract, although certain pathogenic strains can cause infections, including urinary tract infections, diarrhea, and wound infections. The increasing prevalence of antimicrobial resistance has made bacterial infections more difficult to treat and has reduced the effectiveness of several commonly used antibiotics. The World Health Organization reported that approximately one in six laboratory-confirmed bacterial infections worldwide was resistant to antibiotic treatment in 2023, highlighting the continuing need to investigate potential antibacterial agents and alternative strategies for managing bacterial infections (World Health Organization, 2025). In this context, medicinal plants have attracted research interest because their extracts contain diverse chemical constituents that may exhibit antibacterial activity. However, their potential must be evaluated experimentally before their effectiveness can be established.

Sappan wood (*Caesalpinia sappan* L.) and mint leaves (*Mentha piperita* L.) are two medicinal plant materials that have been investigated for their potential antibacterial properties. Previous studies have reported antibacterial activity of sappan wood extracts against pathogenic bacteria, including *E. coli*, while research on *M. piperita* has also demonstrated antibacterial effects against several bacterial species (Sujana et al., 2013; Indrayudha, 2021; Pattananandecha et al., 2022; Chukiatsiri et al., 2024). Sappan wood has been reported to contain phenolic compounds, including brazilin-related constituents, whereas mint leaves are associated with volatile compounds such as menthol and menthone. Nevertheless, the presence and concentration of these individual compounds in the extracts used in the present study were not determined through instrumental chemical analysis. Therefore, their specific contributions to the observed antibacterial activity cannot be established from the present data. The preliminary phytochemical screening performed in this study provides information about broad classes of secondary metabolites but does not confirm the identity, concentration, or individual antibacterial contribution of specific compounds.

One plant with potential for development as a source of natural antibacterial agents is sappan wood (Caesalpinia sappan L.). Sappan wood contains various secondary metabolites, including brazilin, brazilein, flavonoids, tannins, lignans, steroids, and terpenoids. Brazilin is one of the characteristic compounds found abundantly in sappan wood and is known to contribute to various biological activities. The presence of phenolic compounds and other secondary metabolites provides a scientific basis for the use of C. sappan as a plant with potential antimicrobial activity (Leswara et al., 2024; Chukiatsiri et al., 2024; Nirmal et al., 2015; Pattananandecha et al., 2022).

In addition to sappan wood, mint leaves (Mentha piperita L.) are also plants with potential as a source of natural antibacterial agents. Mint leaves contain various bioactive compounds, particularly menthol, menthone, flavonoids, phenolic compounds, and other volatile components. These components can affect bacterial cell structure and function through interactions with cell membranes and disruption of metabolic processes. The volatile compounds in M. piperita may also contribute to antimicrobial activity because some of these components are lipophilic and can interact with bacterial membranes (Afrin et al., 2023; Sujana et al., 2013; Pulipati et al., 2016; Porwal et al., 2025).

The use of two antibacterial agents in combination may produce different activity compared with the use of each agent alone. Combinations containing bioactive compounds with different mechanisms of action may produce additive or synergistic effects. Sappan wood contains phenolic compounds such as brazilin and brazilein, whereas mint leaves contain components such as menthol, menthone, flavonoids, and phenolic compounds. The different characteristics of these compounds provide a basis for investigating whether mutually enhancing interactions occur when the two extracts are used together. However, the presence of a synergistic effect needs to be demonstrated experimentally because the response to a combination may be influenced by concentration, component ratio, and the characteristics of the bacteria used (Nirmal et al., 2015; Indrayudha, 2021).

Studies on antibacterial combinations have also shown that the use of plant extracts together with other antibacterial agents can produce different types of interactions.Indrayudha (2021) reported that a combination of peppermint leaf ethanol extract and amikacin exhibited antibacterial activity against Klebsiella pneumoniae, Staphylococcus aureus, and E. coli, and showed a synergistic interaction pattern under certain conditions. These findings provide a basis for the potential role of M. piperita extract in antibacterial combination systems. However, that study used an antibiotic as the combination partner, whereas the simultaneous use of sappan wood and mint leaf extracts against E. coli has been less extensively studied (Indrayudha, 2021).

In the present study, antibacterial activity was assessed using disk diffusion, while the interaction between the extracts was examined qualitatively using the paper strip method. These methods provide complementary information: disk diffusion allows comparison of inhibition-zone diameters, whereas the paper strip method permits observation of changes in inhibition-zone morphology where the two agents interact. However, qualitative changes in the inhibition zone cannot establish the magnitude of synergy or provide a quantitative interaction index. Moreover, the MIC of each extract could not be determined by visual observation because the color and turbidity of the extracts interfered with the assessment of bacterial growth. Although the minimum bactericidal concentration (MBC) was subsequently determined by subculture, MBC results cannot replace MIC measurements for calculating FICI. Therefore, the interaction observed in this study should be interpreted as a qualitative pattern suggestive of a possible synergistic interaction, rather than definitive quantitative evidence of synergy.

Despite previous reports of the antibacterial activity of sappan wood and mint leaf extracts, the interaction between these two extracts against *E. coli* requires further experimental clarification. Specifically, it remains uncertain whether their combination produces a genuinely synergistic effect and how different proportions of sappan wood and mint leaf extracts influence the observed antibacterial response. Evaluating the 1:1, 1:2, and 2:1 ratios can identify which tested formulation produces the largest inhibition zone, although quantitative confirmation of synergy would require additional MIC-based combination testing, such as a checkerboard assay with FICI calculation. Addressing these questions is important to distinguish enhanced antibacterial activity from a true synergistic interaction and to establish the appropriate interpretation of the experimental findings.

Accordingly, this study aimed to evaluate the antibacterial activity of ethanol extracts of sappan wood (*C. sappan* L.) and mint leaves (*M. piperita* L.), individually and in combination, against *E. coli*; determine the MBC of each extract when the MIC could not be reliably assessed; compare the antibacterial activity of combinations at ratios of 1:1, 1:2, and 2:1; and qualitatively examine the interaction pattern of the most active combination using the paper strip method. By distinguishing inhibition-zone activity from quantitative evidence of synergy, this study provides preliminary experimental information on the antibacterial potential of the two extracts and identifies the need for further MIC-based combination testing.

RESEARCH METHOD

This laboratory experimental study evaluated the antibacterial activity of ethanol extracts of sappan wood (*Caesalpinia sappan* L.) and mint leaves (*Mentha piperita* L.) against *Escherichia coli*. The study included extract preparation, phytochemical screening, bacterial identification, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), disk diffusion testing, and qualitative assessment of extract interactions using the paper strip method.

Sappan wood and mint leaves were dried, powdered, and extracted separately by maceration using 70% ethanol. For each plant material, 500 g of powder was used. The resulting extracts were concentrated by evaporation, yielding 45 g of sappan wood extract and 72 g of mint leaf extract, corresponding to extraction yields of 9.0% and 14.4%, respectively. The extracts were

subjected to preliminary phytochemical screening for flavonoids, alkaloids, tannins, saponins, and triterpenoids/steroids. The bacterial isolate was identified through macroscopic examination, microscopic examination using Gram staining, and biochemical testing. The specific strain designation and source of the *E. coli* isolate were not documented in the available method description and should be added if this information is available in the laboratory records.

The MIC and MBC were investigated using a dilution method with extract concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562%, 0.781%, 0.39%, and 0.195%. The preparations were incubated at 37°C for 24 h. MIC was intended to be determined as the lowest concentration that visibly inhibited bacterial growth. However, the intense color and turbidity of the extracts interfered with visual assessment; therefore, a reliable MIC endpoint could not be established. No alternative quantitative MIC method was performed. MBC was determined by subculturing samples onto Endo Agar and identifying the lowest concentration that showed no bacterial colony growth after incubation. The MBC values were 12.5% for sappan wood extract and 25% for mint leaf extract.

The antibacterial activity of the individual extracts and their combinations was evaluated using the disk diffusion method on Mueller–Hinton agar. The tested treatments comprised sappan wood extract, mint leaf extract, and combinations at sappan wood-to-mint ratios of 1:1, 1:2, and 2:1. Ciprofloxacin was used as the positive control, and dimethyl sulfoxide (DMSO) was used as the negative control. The plates were incubated at 37°C for 18–24 h, after which inhibition-zone diameters were measured in millimeters. Each treatment was tested in triplicate. The available method description does not specify the disk diameter, extract volume applied per disk, final concentration of each extract in the combinations, or the bacterial inoculum standardization procedure; these details should be added from the original laboratory records where available.

The 2:1 combination produced the largest inhibition zone among the tested extract treatments. However, the ratio represents the relative proportion of the extracts and does not, by itself, specify the final dose applied to each disk. The basis of the ratio and the final concentration and volume of each extract should therefore be clarified if recorded. The paper strip method was used to qualitatively examine the interaction pattern of the most active combination. Because this method provides a visual assessment and no MIC-based fractional inhibitory concentration index (FICI) was calculated, the observed pattern was interpreted as suggestive of a possible interaction rather than definitive quantitative evidence of synergy.

Inhibition-zone data were analyzed using SPSS version 25. Data normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was assessed using Levene's test. Data meeting the assumptions for parametric analysis were evaluated using one-way analysis of variance (ANOVA), whereas the Kruskal–Wallis test was used when the relevant assumptions were not met. Post hoc comparisons were conducted as appropriate, including Tukey's test following a significant ANOVA result. Statistical significance was set at $p < 0.05$. The original results reported significant differences among treatment groups ($p < 0.001$). The three replicates reported for each treatment must be clarified as independent experimental replicates or technical replicates. The ANOVA F statistic, degrees of freedom, effect size, and confidence intervals should be reported using the original statistical output and raw data.

RESULTS AND DISCUSSIONS

Extract Characteristics

Extraction of 500 g of sappan wood powder and 500 g of mint leaf powder using 70% ethanol yielded 45 g and 72 g of concentrated extracts, respectively. The corresponding extraction yields were 9.0% for sappan wood and 14.4% for mint leaves (Table 1). Extraction yield was calculated as the mass of concentrated extract divided by the initial mass of dried plant powder, multiplied by 100.

Table 1. Yield of sappan wood and mint leaf extracts

Sample	Powder weight (g)	Extract weight (g)	Yield (%)
Sappan wood	500	45	9.0
Mint leaves	500	72	14.4

The mint leaf extract had a higher yield than the sappan wood extract. This difference indicates that a greater mass of concentrated extract was recovered from the mint leaf material under the extraction conditions used. However, extraction yield alone does not establish antibacterial potency or the concentration of individual active compounds. The difference may be associated with the composition of each plant material and the compounds extracted by 70% ethanol, although these factors were not separately evaluated in this study.

The measured moisture content was 5.99 ± 0% for sappan wood extract and 8.99 ± 0% for mint leaf extract. Both values were below 10%. Any claim that these values meet a formal quality standard should be supported by a specific, applicable reference that defines the standard for the type of extract examined. The yield calculation described above is a gravimetric calculation; it should not be presented as compliance with a particular pharmacopoeial or regulatory yield standard unless that standard was actually used.

Phytochemical Screening

Preliminary phytochemical screening identified several classes of secondary metabolites in both extracts. The results are presented in Table 2.

Table 2. Phytochemical screening results of the extracts

Compound class	Sappan wood	Mint leaves
Flavonoids	+	+
Alkaloids	+*	+*
Tannins	+	+
Saponins	+	+
Triterpenoids/steroids	+	+

The screening results indicate that the extracts contained detectable members of the tested phytochemical classes. Nevertheless, these qualitative tests do not establish the identity or concentration of individual compounds. Although previous studies have reported brazilin-related constituents in sappan wood and volatile constituents such as menthol and menthone in peppermint, these compounds were not individually identified or quantified in the extracts used in the present study. Therefore, the phytochemical findings provide preliminary information about the chemical classes detected but do not demonstrate which compounds were responsible for the observed antibacterial effects. Claims about specific compounds or their mechanisms should be treated as hypotheses informed by previous literature rather than as direct findings of this experiment. Chromatographic analysis, such as HPLC or GC–MS where appropriate, would be required to characterize relevant constituents more specifically.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The dilution test was performed at concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.5625%, 0.7812%, 0.3906%, and 0.1953%. Following incubation, all tubes containing sappan wood and mint leaf extracts remained visually turbid at the tested concentrations. Consequently, the MIC could not be reliably determined because the color and turbidity of the extracts interfered with the visual assessment of bacterial growth. The individual observations are shown in Table 3.

Table 3. MIC test results

Concentration (%)	Sappan wood R1	R2	R3	Mint leaves R1	R2	R3
100	+	+	+	+	+	+
50	+	+	+	+	+	+

Concentration (%)	Sappan wood R1	R2	R3	Mint leaves R1	R2	R3
25	+	+	+	+	+	+
12.5	+	+	+	+	+	+
6.25	+	+	+	+	+	+
3.125	+	+	+	+	+	+
1.5625	+	+	+	+	+	+
0.7812	+	+	+	+	+	+
0.3906	+	+	+	+	+	+
0.1953	+	+	+	+	+	+

Note: (+) bacterial growth present/turbid observation

The results in Table 3 do not establish an MIC endpoint. No alternative quantitative MIC method was performed in this study; therefore, MIC values should not be inferred from the turbidity observations. This limitation is particularly important when interpreting the combination experiments because an MIC-based interaction index cannot be calculated without reliable MIC values for the individual extracts and their combinations.

The MBC test was subsequently performed by subculturing the tested preparations onto Endo Agar. The results are presented in Table 4.

Table 4. MBC test results

Concentration (%)	Sappan wood R1	R2	R3	Mint leaves R1	R2	R3
100	−	−	−	−	−	−
50	−	−	−	−	−	−
25	−	−	−	−	−	−
12.5	−	−	−	+	+	+
6.25	+	+	+	+	+	+
3.125	+	+	+	+	+	+
1.5625	+	+	+	+	+	+
0.7812	+	+	+	+	+	+
0.3906	+	+	+	+	+	+
0.1953	+	+	+	+	+	+

Note: (+) bacterial growth present; (−) no bacterial growth

The MBC was 12.5% for sappan wood extract and 25% for mint leaf extract. Under the conditions of this experiment, the sappan wood extract therefore reached the no-growth endpoint at a lower tested concentration than the mint leaf extract. This finding is consistent with the larger inhibition zone produced by the individual sappan wood extract in the disk diffusion assay. However, the results do not identify the specific chemical constituents responsible for this difference. Any explanation involving brazilin, flavonoids, tannins, or other compounds should be presented as a possible explanation based on previous literature, not as a mechanism confirmed by the current experiment (Nirmal et al., 2015; Pattananandecha et al., 2022).

Antibacterial Activity Using the Disk Diffusion Method

Antibacterial activity was subsequently determined based on inhibition zone diameter. The positive control, ciprofloxacin 5 µg/mL, produced a mean inhibition zone of 39.58 ± 1.02 mm, whereas the negative control, 1% DMSO, produced no inhibition zone. These results indicate that the test method was able to distinguish the antibacterial activity of the test materials and that 1% DMSO did not produce an inhibitory effect under the study conditions.

Table 5. Antibacterial activity of extracts and combinations against E. coli

Treatment	Replication I (mm)	Replication II (mm)	Replication III (mm)	Mean ± SD (mm)	Category
Ciprofloxacin 5 µg/mL	40.55	39.70	38.50	39.58 ± 1.02	Very strong
DMSO 1%	0	0	0	0	No inhibition zone

Treatment	Replication I (mm)	Replication II (mm)	Replication III (mm)	Mean ± SD (mm)	Category
Sappan wood extract	14.10	13.60	15.20	14.30 ± 0.81	Strong
Mint leaf extract	10.10	9.60	9.10	9.60 ± 0.50	Moderate
EKS 1 : EDM 1	13,10	14.90	14,30	14,10 ± 0,91	Strong
EKS 1 : EDM 2	15.80	15.87	16.08	15.92 ± 0.22	Strong
EKS 2 : EDM 1	27.25	27.05	27.54	27.28 ± 0.33	Very strong

Note: EKS = sappan wood extract; EDM = mint leaf extract

The results showed that all individual extracts and combinations were able to inhibit the growth of E. coli. Sappan wood extract produced an inhibition zone of 14.30 ± 0.81 mm, which was larger than that produced by mint leaf extract, 9.60 ± 0.50 mm. This result is consistent with the MBC findings, in which sappan wood had a lower bactericidal concentration. This activity was likely associated with the flavonoid, tannin, saponin, and phenolic compounds in sappan wood, which can disrupt bacterial cell structure and function (Batubara et al., 2010; Nirmal et al., 2015).

The findings demonstrate that the measured antibacterial response differed among the tested formulations. However, disk diffusion results can be affected by both antibacterial activity and the ability of extract constituents to diffuse through agar. Furthermore, the manuscript does not provide the final concentration and volume of each extract applied to the disks. The ratio alone is insufficient to establish the actual dose of each component. Consequently, the larger inhibition zone of the 2:1 combination should be interpreted as enhanced inhibition under the specific disk diffusion conditions, rather than definitive evidence of synergy.

The original analysis reported that the data met the assumptions of normality and homogeneity and were analyzed using one-way ANOVA, followed by Tukey's post hoc test. The overall significance value reported as 0.000 should be written as $p < 0.001$. The complete ANOVA table, including the F statistic and degrees of freedom, as well as the exact post hoc comparisons, effect size, and confidence intervals, should be added using the original statistical output. These values cannot be reconstructed reliably from the summary means and standard deviations alone, particularly without confirmation of the replication structure.

Interaction Pattern of the Combination Using the Paper Strip Method

The 2:1 combination of sappan wood and mint leaf extracts was selected for qualitative assessment using the paper strip method because it produced the largest inhibition zone in the disk diffusion assay. The original observation showed a change in the inhibition-zone morphology at the intersection of the strips. The original figure documenting this observation should be retained as Figure 1.

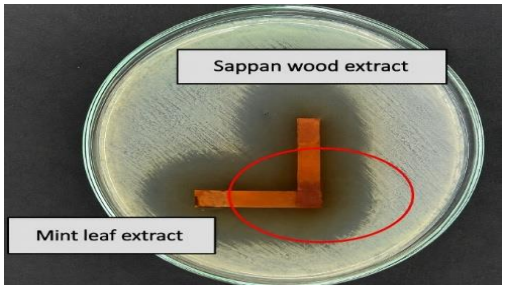


Figure 1. Antibacterial activity test of the combination of sappan wood and mint leaf extracts using the paper strip method

The observed inhibition-zone pattern suggests a possible interaction between the two extracts under the test conditions. Nevertheless, the paper strip method provides a qualitative observation and does not quantify the magnitude of interaction. A larger inhibition zone from a

combination cannot by itself distinguish synergistic activity from the combined contribution of the individual extracts, differences in component concentrations, or differences in agar diffusion.

Quantitative approaches such as a checkerboard assay with calculation of the fractional inhibitory concentration index (FICI) can be used to evaluate extract interactions when reliable MIC values are available. Time-kill experiments can provide complementary evidence by examining bacterial survival over time. Other interaction models, including Bliss independence, Loewe additivity, or ZIP, may also be considered when the experimental design and data meet the requirements of those analyses. None of these quantitative analyses was performed in the present study (Hanifa et al., 2022; Novitri & Kurniati, 2021).

Accordingly, the paper strip findings should be described as a qualitative pattern suggestive of a possible interaction, rather than proof of synergism. The present results support further evaluation of the 2:1 combination but do not establish the type or magnitude of the interaction. In addition, because the individual compounds were not identified or quantified, the observed response cannot be attributed directly to brazilin, menthol, menthone, or any other specific compound. Overall, the study found that the 2:1 combination produced the largest inhibition zone among the tested extract formulations, while sappan wood extract had a lower MBC than mint leaf extract. These are the principal experimental findings. Further studies using reliable MIC determination, quantitative combination testing, and chemical characterization would be needed to confirm whether the observed interaction is synergistic and to investigate the compounds potentially contributing to the antibacterial response.

CONCLUSION

The ethanol extracts of sappan wood (*Caesalpinia sappan* L.) and mint leaves (*Mentha piperita* L.) exhibited antibacterial activity against *Escherichia coli* under the experimental conditions. Among the tested formulations, the combination at a 2:1 ratio of sappan wood to mint leaf extract produced the largest inhibition zone, measuring 27.28 ± 0.33 mm. The MBC values were 12.5% for sappan wood extract and 25% for mint leaf extract. The paper strip test revealed an interaction pattern suggesting potential synergy between the two extracts. However, because the MIC could not be reliably determined and no quantitative combination assay or fractional inhibitory concentration index (FICI) was obtained, synergism cannot be conclusively established. Further studies should employ a checkerboard assay with FICI calculation and, where appropriate, complementary methods such as time-kill analysis to quantitatively characterize the interaction between the extracts.

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