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Research article

Antibacterial Potential of the Ethyl Acetate Fraction of Ethanolic Extract of Kopasanda Leaves (Chromolaena odorata L.) Against Streptococcus Mutans

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ABSTRACT

Background: *Streptococcus mutans* is the primary bacterium responsible for dental caries. Continuous use of synthetic antibacterial agents may cause side effects; therefore, natural antibacterial alternatives are needed. Kopasanda leaves (Chromolaena odorata L.) are known to contain bioactive compounds with potential antibacterial activity. This study aimed to evaluate the antibacterial activity of the ethyl acetate fraction of the ethanolic extract of kopasanda leaves (*Chromolaena odorata* L.) against *Streptococcus mutans*.
Methods: An experimental laboratory study was conducted using the agar well diffusion method with ethyl acetate fractions at concentrations of 10%, 15%, and 20%. Gentamicin was used as the positive control and the solvent as the negative control. The inhibition zones were measured in millimeters and analyzed using One-Way ANOVA.
Results: The ethyl acetate fraction showed antibacterial activity against *Streptococcus mutans*, with mean inhibition zone diameters of 11.33 mm, 13.49 mm, and 15.98 mm at concentrations of 10%, 15%, and 20%, respectively. The 20% concentration exhibited the highest antibacterial activity, and statistical analysis indicated a significant difference among treatments ($p < 0.05$).
Conclusion: The ethyl acetate fraction of kopasanda leaf extract (*Chromolaena odorata* L.) exhibits antibacterial activity against *Streptococcus mutans*, with 20% being the most effective concentration.

I. Introduction

Oral diseases remain a significant global public health problem. According to the World Health Organization, oral diseases affect approximately 3.7 billion people worldwide, and untreated dental caries is the most common health condition globally based on the Global Burden of Disease 2021 data. Dental caries is a chronic disease caused by the activity of cariogenic microorganisms that metabolize carbohydrates into acids, leading to gradual demineralization of dental tissues. Microorganisms in the oral cavity form biofilm plaques and produce acids from carbohydrate metabolism, which subsequently trigger enamel demineralization. Globally, dental caries affects individuals across all age groups and contributes to reduced quality of life, impaired masticatory function, and increased economic burden on healthcare systems (Zhou et al., 2025).

One of the bacteria that plays a crucial role in the pathogenesis of dental caries is *Streptococcus mutans*. This Gram-positive bacterium is recognized as a primary microorganism contributing to dental plaque formation due to its ability to produce acid, tolerate acidic environments, and form strong biofilms on tooth surfaces. The ability of *S. mutans* to produce extracellular polysaccharides through the activity of the glucosyltransferase enzyme enables the bacteria to adhere and colonize the enamel surface effectively, thereby increasing the risk of dental caries. Recent studies also confirm that the presence of

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S. mutans is strongly associated with the incidence of dental caries in individuals with high caries risk (Fang et al., 2024). Furthermore, biofilm formation by *S. mutans* plays an important role in plaque development and caries progression because biofilms protect bacteria from host immune responses as well as antimicrobial agents (Kashi et al., 2025).

Control of cariogenic bacteria is generally achieved through the use of synthetic antibacterial agents such as chlorhexidine, fluoride, and certain antibiotics. However, long-term use of these agents may cause several side effects, including mucosal irritation, tooth discoloration, and disruption of the oral microbiota balance. In addition, the increasing resistance of microorganisms to antimicrobial agents has become a new challenge in controlling pathogenic bacteria. Therefore, the development of alternative antibacterial agents that are safer and more effective has become a major focus in dental and pharmaceutical research. Recent studies indicate that the exploration of bioactive compounds from natural sources, particularly medicinal plants, has great potential as a source of new antibacterial agents to inhibit the growth of *Streptococcus mutans* and the formation of cariogenic biofilms (Gao et al., 2024).

One of the plants with potential as a natural antibacterial source is kopasanda (*Chromolaena odorata* L.). This plant is known to contain various secondary metabolites, including flavonoids, tannins, alkaloids, and phenolic compounds, which have been reported to exhibit antibacterial activity against various pathogenic bacteria. Several studies have shown that leaf extracts of *Chromolaena odorata* possess inhibitory activity against both Gram-positive and Gram-negative bacteria, including bacteria associated with oral infections (Zainab et al., 2022a). Fractionation using solvents with specific polarity levels, such as ethyl acetate, is known to separate and concentrate semi-polar bioactive compounds that contribute to antibacterial activity, thereby potentially enhancing biological effectiveness compared with crude extracts.

Based on the above description, exploring the potential of the ethyl acetate fraction derived from the ethanol extract of kopasanda (*Chromolaena odorata* L.) leaves as an antibacterial agent against *Streptococcus mutans* is important to be conducted. This study is expected to provide scientific information regarding the potential of medicinal plants as sources of natural antibacterial agents and to support the development of alternative active compounds for the prevention and control of bacteria responsible for dental caries. Therefore, this study aimed to evaluate the antibacterial activity of the ethyl acetate fraction of the ethanol extract of kopasanda (*Chromolaena odorata* L.) leaves at concentrations of 10%, 15%, and 20% against the growth of *Streptococcus mutans*.

This study aimed to evaluate the antibacterial activity of the ethyl acetate fraction of the ethanolic extract of kopasanda leaves (*Chromolaena odorata* L.) against *Streptococcus mutans*.

METHODS

This study was conducted at the Laboratory of the Undergraduate Pharmacy Study Program, Universitas Strada Indonesia. The research design employed an experimental laboratory method to evaluate the antibacterial activity of the ethyl acetate fraction derived from the ethanol extract of kopasanda (*Chromolaena odorata* L.) leaves against *Streptococcus mutans*. The independent variable in this study was the concentration of the ethyl acetate fraction of kopasanda leaf extract (10%, 15%, and 20%). The dependent variable was the antibacterial activity indicated by the inhibition of *Streptococcus mutans* growth. The controlled variables included the bacterial strain (*Streptococcus mutans*), culture media (Nutrient Agar), incubation temperature, and incubation duration used during the experimental procedures. The materials used in this study included kopasanda (*Chromolaena odorata* L.) leaves, 70% ethanol, ethyl acetate, acetic acid, concentrated sulfuric acid, 2N HCl, Dragendorff reagent, Mayer reagent, Wagner reagent, $FeCl_3$ 1%, NaCl 0.9%, bacterial suspension, and Nutrient Agar (NA) media. The equipment used included test tubes, Erlenmeyer flasks, and a Bunsen burner.

Research Stages:

1. **Plant Identification**: Plant identification was conducted to ensure that the kopasanda leaves used in this study corresponded to the correct plant species. The plant material used in this research was kopasanda leaves (*Chromolaena odorata* L.).
2. **Preparation of Simplicia and Extract Preparation**: Fresh kopasanda leaves weighing 7 kg were washed thoroughly under running water and drained, then cut into smaller pieces. The cleaned leaves were dried in an oven at 50°C in a drying cabinet for two days. After drying, dry sorting was performed to remove unsuitable materials. The dried leaves were then ground into powder and sieved to obtain a fine powder, which was subsequently used for extraction. The

extraction process was carried out using the maceration method with 70% ethanol as the solvent for 3 × 24 hours.

3. **Phytochemical Screening**: Phytochemical screening was performed on the ethanol extract of kopasanda (*Chromolaena odorata* L.) leaves prior to the fractionation process to qualitatively identify the secondary metabolites present in the extract. The screening was conducted using the test tube method with specific reagents for each class of compounds. The tested compound groups included alkaloids, flavonoids, saponins, tannins, and terpenoids/steroids.
4. **Fractionation:** The ethanol extract of kopasanda leaves obtained from the extraction process was concentrated using a rotary evaporator under controlled temperature to obtain a viscous extract. The extract was then subjected to fractionation using ethyl acetate through a liquid–liquid partition method. The concentrated extract was first dissolved in a suitable solvent and transferred into a separatory funnel, followed by the addition of ethyl acetate. The mixture was gently shaken until two distinct phases were formed. The ethyl acetate layer was separated from the other phase. The partition process was repeated several times to obtain the ethyl acetate fraction. The ethyl acetate fraction obtained was then evaporated using a rotary evaporator to produce a concentrated ethyl acetate fraction, which was subsequently used for antibacterial activity testing against *Streptococcus mutans*.
5. **Solvent-Free Test:** A solvent-free test was performed to ensure that the obtained fraction did not contain residual solvents, particularly ethanol, which may influence the antibacterial activity results since ethanol is known to possess antibacterial and antifungal properties.
6. **Phytochemical Analysis of Ethyl Acetate Fraction:** Phytochemical analysis of the ethyl acetate fraction was carried out using Thin Layer Chromatography (TLC). Silica Gel 60 F_{254} plates were used as the stationary phase, while the mobile phase consisted of a mixture of n-butanol: acetic acid: distilled water (4:1:5). The ethyl acetate fraction sample was spotted onto the TLC plate and eluted in a chamber previously saturated with the mobile phase until reaching the development limit. The plate was then dried and observed under ultraviolet light at a wavelength of 366 nm. The appearance of yellowish-green fluorescence indicated the presence of flavonoid compounds in the ethyl acetate fraction (Urbain & Simões-Pires, 2020).
7. **Preparation of Culture Media and Bacterial Suspension:** The culture medium was prepared by weighing 14 g of Nutrient Agar (NA) and dissolving it in 500 mL of distilled water in an Erlenmeyer flask. The solution was heated until boiling for approximately 1 minute while being stirred until homogeneous. The medium was sterilized using an autoclave at 121°C for 15 minutes and then cooled to 40–45°C. For bacterial rejuvenation, approximately 5 mL of NA medium was poured into sterile test tubes and closed with sterile cotton plugs. The tubes were placed at an angle of approximately 30° until the medium solidified to form slant agar. *Streptococcus mutans* from a pure culture was inoculated onto the slant agar using a sterile inoculating loop and incubated at 37°C for 24 hours to obtain an active culture. A bacterial suspension was prepared by adding 10 mL of physiological NaCl solution (0.9%) into a sterile test tube. Bacterial colonies from the rejuvenated culture were suspended in the NaCl solution and homogenized until the turbidity matched the 0.5 McFarland standard, corresponding to approximately 1×10^8 CFU/mL.
8. Antibacterial Activity Assay: The antibacterial activity was evaluated using the well diffusion method. Approximately 15 mL of sterile Nutrient Agar (NA) medium was poured into Petri dishes and allowed to solidify. The *Streptococcus mutans* suspension was evenly spread on the surface of the agar using a sterile spreader and allowed to stand for 5–10 minutes. Five wells with a diameter of approximately 5 mm were then made in the agar medium. Each well was filled with the ethyl acetate fraction of kopasanda leaf extract according to the treatment concentrations (10%, 15%, and 20%). A 0.2% chlorhexidine solution was used as the positive control, while sterile distilled water served as the negative control. All treatments were performed in triplicate and incubated at 37°C for 24 hours. After incubation, antibacterial activity was determined by measuring the diameter of the inhibition zone formed around the wells using a caliper in millimeters (mm). The results were expressed as the mean inhibition zone diameter from three replicates.

RESULTS

1. **Plant Identification Results:** Based on the morphological identification conducted by the Technical Implementation Unit (UPT) of the Herbal Material Medica Laboratory, Batu, Malang, the kopasanda plant was confirmed to belong to the Asteraceae family, which is one of the largest and most widely distributed families of flowering plants. The plant species was identified as *Chromolaena odorata* (L.) R.M. King & H. Rob., which is the officially accepted scientific name according to the International Code of Botanical Nomenclature.
2. **Simplicia Preparation and Extract Preparation Results:** Based on the preparation of kopasanda (*Chromolaena odorata* L.) leaf simplicia powder, a total yield of 2.5 kg of simplicia powder was obtained.

Table 1. Results of Kopasanda Leaf Simplicia Preparation

Sample	Weight
Fresh leaves	7 kg
Dried leaves	4,5 kg
Simplicia powder	2,5 kg

The moisture content analysis of the kopasanda (*Chromolaena odorata* L.) leaf simplicia powder showed an average moisture content of 5.63% from three replicates. This value meets the quality requirements for simplicia, where the moisture content generally should not exceed 10% to prevent microbial growth and to maintain the stability of the material during storage (Kemenkes RI, 2017; WHO, 2018).

The extraction of kopasanda (*Chromolaena odorata* L.) leaf simplicia powder resulted in 936.98 g of concentrated extract.

3. **Extraction Results:** The powdered simplicia was extracted using methanol, producing **5 grams** of concentrated extract. The extract yield obtained was **23.07%**.
4. **Phytochemical Screening Results:** Based on the identification of chemical constituents in the ethanol extract of kopasanda (*Chromolaena odorata* L.) leaves, several secondary metabolites were detected. The results of the phytochemical screening are presented in Table 2.

Table 2. Phytochemical Screening Results of the Ethanol Extract of Kopasanda Leaves

Metabolite Compounds	Reagents	Indicators	Observation Results	Kesimpulan
Flavonoid	Serbuk mg HCI 5ml	Larutan warna merah tua, jingga atau kuning	Warna merah tua	(+)
Alkaloid	HCI 2N dragen drof, wagner	Endapan merah/ jingga putih, endapan coklat	Endapan merah dan coklat	(+)
Saponin	HCI 2N	Terbentuk buih tinggi 1-10 cm	Berbusa dengan ketingian 2 cm	(+)

Note: (+) Positive

5. **Fractionation Results:** The ethanol extract of kopasanda (*Chromolaena odorata* L.) leaves obtained from the extraction process was concentrated using a rotary evaporator under controlled temperature to obtain a viscous extract. The extract was subsequently subjected to fractionation using ethyl acetate as the solvent through a liquid–liquid partition method.

The concentrated extract was first dissolved in an appropriate solvent and transferred into a separatory funnel, followed by the addition of ethyl acetate. The mixture was gently shaken until two distinct phases were formed. The ethyl acetate layer was then separated from the other phase. The partition process was repeated several times to obtain the ethyl acetate fraction optimally. The obtained ethyl acetate fraction was subsequently evaporated again using a rotary

evaporator to produce 25.57 g of concentrated ethyl acetate fraction, which was then used for antibacterial activity testing against *Streptococcus mutans*.

6. **Solvent-Free Test Results:** The results of the solvent-free test indicated negative results for residual ethanol solvent.

Table 2. Results of the Solvent-Free Test

Sample	Results
Ethanol extract	The extract did not exhibit an ester odor (Azwanida NN, 2015)
Ethyl acetate fraction	No characteristic solvent odor was detected (Abubakar & Haque, 2020)

7. **Phytochemical Screening Results:** Phytochemical analysis using Thin Layer Chromatography (TLC) showed that the methanol extract contained flavonoids, saponins, and alkaloids.

Table 1. Phytochemical Screening Results of the Ethyl Acetate Fraction

Constituent	Mobile Phase	UV 254 nm	UV 366 nm	Reference Standard	Rf Value	Result
Flavonoid	Chloroform : Ethyl acetate (6:4)	Yellow–green	Yellow–green	Quercetin	0,65	Positive
Saponin	Chloroform : Methanol: Water (64:50:1)	Blue–brick red	Blue–brick red	-	0,86	Positive
Alkaloid	Toluene:Ethyl acetate:Diethylamine (7:2:1)	Brown–orange	Brown–orange	-	0,70	Positive

8. **Antibacterial Activity Test Results:** The antibacterial activity test of the ethyl acetate fraction of the ethanol extract of kopasanda (*Chromolaena odorata* L.) leaves against *Streptococcus mutans* showed the presence of inhibition zones around the wells at various treatment concentrations. The concentrations of the ethyl acetate fraction used in this study were 10%, 15%, and 20%. The inhibition zones were indicated by the formation of clear areas surrounding the wells, which reflected the inhibition of bacterial growth. The diameters of the inhibition zones were measured using a caliper in millimeters (mm) and expressed as the average of three replicates. In general, increasing the concentration of the ethyl acetate fraction showed a tendency to increase the diameter of the inhibition zone, indicating that higher concentrations of the fraction exhibited greater antibacterial activity against *Streptococcus mutans*.

Table 5. Inhibition Zone Diameter of the Ethyl Acetate Fraction

Test Sample	Inhibition Zone Diameter (mm)			Mean	Standard Deviation	Category
	RI	RII	RIII			
Fraksi 10%	13,51	10,68	9,79	11,33	± 1,94	Very strong
Fraksi 15%	15,32	12,76	12,38	13,49	± 1,60	Very strong
Fraksi 20%	11,65	12,70	23,58	15,98	± 6,61	Very strong
Kontrol Positif	23,45	24,10	22,80	23,45	±0,65	Strongest
Kontrol Negatif	0	0	0,1	0	0	Weak

Notes:

RI : Replication I
RII : Replication I
RIII : Replication I

Inhibition Zone Criteria (Valgas et al., 2007):

≥ 20 mm **:** Strongest
10–19,9 mm **:** Very strong
5–9,9 mm **:** Moderate
< 5 mm **:** Weak

DISCUSSION

Plant identification was conducted to ensure that the sample used in this study was correctly identified as kopasanda (*Chromolaena odorata* L.). The identification process is essential to avoid species misidentification that may affect the composition of active compounds and the research outcomes. *Chromolaena odorata* belongs to the Asteraceae family, which is known to contain various secondary metabolites such as flavonoids, phenolics, tannins, alkaloids, and terpenoids with potential antibacterial properties. Several studies have reported that this plant is widely used in traditional medicine due to its biological activities, including antibacterial activity against various pathogenic bacteria (Zainab et al., 2022b).

The preparation of simplicia involved washing, drying, and grinding the kopasanda leaves into simplicia powder. This process aims to increase the surface area of the material so that the extraction of active compounds can occur more efficiently. In this study, the moisture content of the simplicia was 5.63%, which meets the quality requirements for herbal simplicia. A low moisture content can inhibit microbial growth and improve the stability of the material during storage. The extraction process was carried out using ethanol as the solvent because it can dissolve various secondary metabolites with medium to high polarity, such as flavonoids, tannins, and alkaloids. Plant extraction using the maceration method with ethanol solvent is also widely applied in phytochemical studies because it can produce extracts with high levels of bioactive compounds (Fitriana et al., 2025a).

Phytochemical screening was conducted to identify the secondary metabolites present in the kopasanda leaf extract. The preliminary screening results indicated that the extract contained several classes of secondary metabolites such as flavonoids, tannins, alkaloids, saponins, and terpenoids. These compounds are known to possess various biological activities, including antibacterial activity. Flavonoids and phenolic compounds can damage bacterial cell membranes and disrupt cellular metabolic processes, while tannins can cause protein denaturation in bacterial cells, thereby inhibiting microbial growth. Several studies have also reported that *Chromolaena odorata* leaf extract qualitatively contains flavonoids, tannins, phenols, alkaloids, and saponins that contribute to its antibacterial activity (Muharraran et al., 2025).

Fractionation was performed to separate active compounds based on their polarity in order to obtain a more specific fraction. In this study, ethyl acetate was used as the solvent to obtain a semi-polar fraction suspected to contain antibacterial active compounds. Ethyl acetate is commonly used in fractionation processes because it can extract compounds such as flavonoid aglycones, phenolics, and certain terpenoids that possess biological activity. This fractionation process is important because the resulting fraction often exhibits stronger biological activity than crude extracts due to the higher concentration of active compound (Fitriana et al., 2025b).

A solvent-free test was conducted to ensure that the obtained fraction no longer contained residual extraction solvents that could influence the results of antibacterial activity testing. This step is important because some organic solvents, such as ethanol, have antimicrobial activity that may cause bias in the research results. Therefore, fractions that have been evaporated using a rotary evaporator must be confirmed to be free of solvent residues before further testing. The removal of residual solvents is also essential to obtain extracts or fractions that are stable and safe for biological assays (Fitriana et al., 2025b).

Thin Layer Chromatography (TLC) analysis was performed to identify the active compounds present in the ethyl acetate fraction. The results showed the presence of yellowish-green fluorescence under UV light at a wavelength of 366 nm, indicating the presence of flavonoid compounds. Flavonoids are among the secondary metabolites commonly found in medicinal plants and possess various biological activities, including antibacterial activity. Flavonoids are known to inhibit bacterial growth through several mechanisms, such as damaging bacterial cell membranes, inhibiting nucleic acid synthesis, and interfering with bacterial energy metabolism (Phetburom et al., 2025) (Bunkaew et al., 2025).

The results of this study showed that the ethyl acetate fraction of kopasanda leaf extract was able to inhibit the growth of *Streptococcus mutans*, as indicated by the formation of inhibition zones around the wells. The diameter of the inhibition zones increased with increasing concentrations of the fraction, indicating that the antibacterial activity is dose-dependent. This phenomenon is commonly observed in studies involving antibacterial activity of plant extracts, where higher extract concentrations increase the amount of bioactive compounds responsible for inhibiting bacterial growth (Yadav et al., 2015).

The ethyl acetate fraction demonstrated relatively strong antibacterial activity with inhibition zone diameters ranging from 11.33 to 15.98 mm. This activity is likely associated with the presence of secondary metabolites detected during phytochemical screening, particularly flavonoids and phenolic compounds. Flavonoids are known to disrupt bacterial cell membranes, inhibit nucleic acid synthesis, and interfere with bacterial cellular energy metabolism, thereby inhibiting microbial growth.

Furthermore, the results also showed that the positive control gentamicin produced a larger inhibition zone compared with the ethyl acetate fraction. This result is expected because gentamicin is a broad-spectrum antibiotic that specifically inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit. In contrast, plant extracts generally contain a mixture of various secondary metabolites with more complex mechanisms of action.

Statistical analysis was conducted to determine whether there were differences in antibacterial activity among the treatment groups, namely the ethyl acetate fraction of kopasanda leaf extract at concentrations of 10%, 15%, and 20%, as well as the positive and negative control groups. Normality testing was performed using the Shapiro–Wilk method. The results showed that all treatment groups had significance values of $p > 0.05$, indicating that the inhibition zone diameter data in each group were normally distributed. Variance homogeneity was tested using Levene's Test, which showed a significance value of $p > 0.05$, indicating that the data distribution among the treatment groups was relatively homogeneous and fulfilled the assumption required for ANOVA analysis.

The ANOVA test results showed a significance value of $p = 0.001$ ($p < 0.05$), indicating that there were significant differences in inhibition zone diameters among the treatment groups. This finding suggests that variations in the concentration of the ethyl acetate fraction of kopasanda leaf extract significantly influenced its antibacterial activity against *Streptococcus mutans*..

Further analysis using the Tukey HSD test showed that the negative control differed significantly from all treatment groups of the ethyl acetate fraction. The ethyl acetate fractions at concentrations of 10%, 15%, and 20% demonstrated higher antibacterial activity compared with the negative control. However, the antibacterial activity of all ethyl acetate fraction concentrations remained lower than that of the positive control gentamicin. Increasing the fraction concentration resulted in increased antibacterial activity, as indicated by the larger inhibition zone diameters. Nevertheless, the antibacterial activity of the ethyl acetate fraction was still lower than that of the positive control gentamicin, which is a standard antibiotic with a more specific mechanism of action in inhibiting bacterial protein synthesis. Despite this, the findings of this study indicate that the ethyl acetate fraction of kopasanda leaf extract has promising potential as a natural antibacterial source that may be further developed for pharmaceutical applications based on natural products.

CONCLUSION

The ethyl acetate fraction of the ethanol extract of kopasanda leaves (*Chromolaena odorata* L.) demonstrated antibacterial activity against *Streptococcus mutans*, as evidenced by the formation of inhibition zones at concentrations of 10%, 15%, and 20%. Among the tested concentrations, the 20% fraction exhibited the strongest antibacterial activity, with an average inhibition zone diameter of 15.98 mm. These findings indicate that the ethyl acetate fraction of *Chromolaena odorata* leaves has promising potential as a natural antibacterial agent and may serve as an alternative source for the development of plant-based antibacterial compounds for the prevention of dental caries.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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