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Phytochemical Screening, Antioxidant Test and Antimicrobial Test of Streptococcus Mutans and Candida Albicans from Extracts of Ultrasound Assisted Extraction of Red Betel Leaves (Piper Ornatum N. E. Br)

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Abstract

Red betel (Piper ornatum) is an ornamental plant with potential antibacterial and antioxidant properties due to its bioactive compounds, including alkaloids, flavonoids, steroids, triterpenoids, saponins, and tannins. This study aimed to determine the relationship between flavonoid content, antioxidant activity, and antimicrobial activity in red betel leaves. Ultrasound-Assisted Extraction (UAE) was employed using three solvents of varying polarity: n-hexane (non-polar), ethyl acetate (semi-polar), and ethanol (polar). Total flavonoid content was determined spectrophotometrically based on flavonoids' ability to form complexes with AlCl3. Antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Antibacterial and antifungal properties were evaluated through Minimum Inhibitory Concentration (MIC) and Zone of Inhibition (ZOI) tests. MIC was determined using the agar dilution method, while ZOI was measured using the disc diffusion method. Results showed that the 96% ethanol extract yielded the highest flavonoid content (4.748%) and the strongest antioxidant activity (IC50 = 47.185 ppm, classified as very strong). The n-hexane extract at 25% concentration exhibited the best antibacterial activity against Streptococcus mutans (ZOI = 17.43 mm), while the 96% ethanol extract at 25% concentration demonstrated the highest antifungal activity against Candida albicans (ZOI = 17.02 mm). In conclusion, red betel leaf extracts show promising potential as strong antioxidants and antimicrobial agents against C. albicans and S. mutans.

Keywords: Red betel, antioxidants, antimicrobial activity, Streptococcus mutans; Candida albicans; Flavonoids

1. Introduction

Dental caries represents a significant global public health challenge, with a prevalence ranging from 20% to 50% per 100,000 inhabitants across various countries in 2017 (Peres et al., 2019). This infectious disease is primarily caused by multiple microorganisms present in dental plaque, with Streptococcus mutans being recognized as the principal cariogenic agent responsible for the destruction of tooth structure (Matsumoto-Nakano, 2014). The increasing prevalence of antibiotic resistance poses a significant obstacle in the treatment of dental caries. A study conducted at the Makassar Hajj Hospital between January and March 2016 revealed alarming rates of antibiotic resistance among S. mutans isolates. In a sample of 10 patients, 100% resistance to amoxicillin and 85% resistance to ceftriaxone were observed (Shamrin et al., 2024). These findings underscore the urgent need for alternative therapeutic approaches to combat dental caries and mitigate the growing threat of antibiotic resistance in oral pathogens.

In addition to bacterial infections, fungal infections such as oral candidiasis remain prevalent in the oral cavity. Candida albicans is responsible for 85-95% of these infections, colonizing the tongue, oral mucosa, and palate (Chander, 2017). The treatment of candidiasis, particularly in HIV-infected patients, has been challenged by increasing antifungal resistance. Sharma et al. (2013) reported resistance rates of C. albicans to various antifungal agents: 34.07% to fluconazole, 10.99% to voriconazole, 7.69% to ketoconazole, 6.59% to itraconazole, 2.19% to clotrimazole, and 1.09% to amphotericin B. These emerging resistance patterns underscore the need for innovative alternatives to conventional antimicrobial agents. One promising approach is the development of traditional medicine, leveraging the availability of plants with potential pharmaceutical value (Emrizal et al., 2014). Puspa et al. (2018) identified red betel leaf (Piper

crocatum) as a medicinal plant with significant potential for alternative dental treatments. Traditionally, infusions of red betel leaves have been used as mouthwash to prevent halitosis and treat oral conditions such as canker sores and toothaches (Suri et al., 2021).

Red betel (Piper crocatum), commonly known as sirih merah in Indonesia, is recognized not only for its ornamental value due to its exotic leaf shape and color but also for its potential medicinal properties. It is widely cultivated in home gardens for both aesthetic and herbal medicinal purposes (Anugrahwati et al., 2016). The therapeutic potential of red betel is attributed to its rich phytochemical profile, which includes bioactive compounds such as flavonoids, alkaloids, tannins, polyphenols, and essential oils (Lister, 2019; Safithri et al., 2012). Kusuma et al. (2017) further elucidated the bioactive constituents of red betel leaves, identifying flavonoids, alkaloids, steroids, tannins, saponins, chalcone anthocyanins, phenolics, and essential oils as key components contributing to its antimicrobial properties. The antimicrobial efficacy of red betel has been demonstrated in several studies. Heliawati et al. (2022) reported that a 100% concentration of 96% ethanol extract of red betel leaves, obtained through maceration, exhibited superior inhibitory effects against Streptococcus mutans compared to the positive control (0.2% Chlorhexidine). Additionally, Savitri et al. (2020) established that a 25% concentration of ethanolic red betel leaf extract represented the Minimum Inhibitory Concentration (MIC) against Candida albicans growth. These findings collectively underscore the potential of red betel as a source of natural antimicrobial agents, warranting further investigation into its applications in oral health and antifungal treatments.

One of the bioactive compounds that are beneficial for health is flavonoids. Flavanoids are phenolic compounds and are often isolated from plants with properties as antioxidants, antibacterials, and as cancer treatments (Dewi et al., 2018). In its ring structure, flavonoids can act as antioxidants that function as reducing agents, hydrogen donors, oxygen absorbers, and counteract superoxide radicals (Martins Gregório et al. (2016). By reducing and stopping the activity of free radicals from oxidation reactions caused by free radicals, antioxidants will prevent or slow down the damage that will be caused by free radicals (Azad et al. (2018). The purpose of this research is to qualitatively screen the compounds contained in red betel leaves and conduct antibacterial and antifungal tests on these compounds to obtain alternative antibiotics.

2. Materials and Methods

2.1. Materials

The red betel leaves used were from the IPB University Collection Baranangsiang Bogor and identified at the National Research and Innovation Agency (BRIN) at the Biology Research Center, Bogor. Pure culture of C albicans ATCC10231 fungus and S mutans bacteria from IPB Culture Collection. Other materials were aluminum foil, filter paper, spirtus, discs (Oxioid), Nutrient agar media (Merck), Potato Dextrose Agar media (Merck), 96% Ethanol, 70% Ethanol, Ethyl acetate, n-Hexane, Amoxicillin tablets (Mersi), Nystatin suspension 100.00 IU/ml (Novell), Tween 1%, Bouchardat reagent, Dragendorff, Mayer, hydrochloric acid (Merck), chloroform (Merck), concentrated sulfuric acid (Merck), anhydrous acetic acid (Merck), iron (III) solution (Merck), barium chloride (Merck), 1% H2SO4 (Merck), physiological NaCl, sterile distilled water, FeCl3, DPPH, Aluminum Chloride, Quercetin and other materials.

The tools used are autoclave (Hirayama), incubator (Nuve®), digital scale (Labpro), blender (Zppelin®), dropper pipette, measuring cup (pyrex), petri dish, test tube (pyrex), bunsen, ose needle, filter paper no.1 (whatman), ultrasonic (Branson m5800h), hot plate, vacuum rotary evaporator, tweezers, vernier, spatula, muffle furnace, oven (Memment®), desiccator (Iwaki®), laminar air flow, crucibles, Sonication ultrasonicator (Sonica®), rotary evaporator (IKA®), UV-Vis Spectrophotometry (Jasco V730®), dark bottle (brown bottle), digital balance (AND G-120®), furnace (Ney®) and other glassware.

2.2. Methods

2.2.1. Extraction

Red betel leaves as much as 5 kg were washed with running water, dried in an oven at 45 0C to dry. Then mashed and sieved using a 40 mesh sieve until it becomes powder. Then weighed as much as 600g of powder and added n-Hexan solvent in the ratio of material: solvent 1:10. The mixture of powder and solvent was then extracted using UAE frequency 40 KHz for 15 minutes at 40 0C. The filtrate obtained was filtered with Whatman no.1 filter paper and the powder dregs were air dried and re-extracted with ethyl acetate solvent in the same way. Furthermore, the pulp was dried again and extracted again with 96% ethanol solvent. Each filtrate was evaporated with a rotary evaporator at 40 0C until a thick extract was obtained.

2.2.2. Extract Characterization

The three thick extracts produced were then characterized by organoleptic tests (color, aroma and taste), water content test and ash content test. The water content test was carried out using the gravimetric method, namely by weighing a sample of 1g placed in a cup that has been tared. Then dry in an oven at 105 0C for 5 hours, cooled in a desiccator and weighed again. Continue drying and weighing at a distance of 1 hour until the difference between the last 2 weighings is no more than 0.25% (MOH RI, 2017). The ash content test was carried out by weighing a sample of 1g and placing it in a previously weighed krus, then incinerated in a furnace with a temperature of 600 0C (NHS RI, 2017).

2.2.3. Phytochemical Screening of Red Betel Leaf

Simplisia and red betel leaf extracts obtained were then subjected to phytochemical screening including testing of alkaloid metabolite compounds, flavonoids, tannins, saponins, and triterpenoids/steroids using the color change reaction method (Banu & Cathrine 2015).

2.2.4. Flavonoid content determination

Determination of flavonoid content was done by AlCl3 colorimetric method. Preparation of quercetin standard curve was done by weighing 10 mg of quercetin into a 100 ml volumetric flask, then adding 70% ethanol to 100 ml (100 µg/ml mother liquor). To find the ideal stable time, absorption was measured by measuring the maximum wavelength at 10, 20, 30, 40, 50, and 60 minutes at a maximum wavelength of 380-780 nm (Chang et al., 2002). Furthermore, a series of standard solutions of 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml and 10 µg/ml were made in a 10 mL volumetric flask and added with 3 mL of 70% ethanol, then added 0.2 mL of 10% AlCl3, and 0.2 mL of 1M sodium acetate then added distilled water to the limit. The absorbance of the standard solution was measured using a UV-Vis spectrophotometer with the maximum wavelength obtained. The extract content (ppm) can be calculated using a linear regression equation, where the Y value is the absorbance of the extract. The linear regression equation (y = bx+a) was obtained by making a curve between the concentration of standard quercetin solution and the absorbance value obtained. In the extract, 50 mg of n-hexane, ethyl acetate and 70% ethanol extracts of red betel leaves were dissolved with 50 mL of 70% ethanol. Furthermore, 10 mL of solution was pipetted into a 50 mL volumetric flask, added with 15 mL of 70% ethanol, 1 mL of 10% AlCl3, 1 mL of 1 M sodium acetate, and distilled water to the limit. The solution was then pipetted back as much as 10 mL, added distilled water to the limit, and shaken well. The absorbance of the solution was measured with a UV-Vis spectrophotometer at the maximum wavelength after the solution was incubated at room temperature and ideal time (Chang et al., 2002). The flavonoid content in the extract could be calculated using the regression equation of the quinine standard curve with the formula:

$$\text{Concentration (\%)} = \frac{\text{C (ppm)x Volume (mL)x fp}}{\text{Sample mass}-(\text{sample mass x \% water concentration})} \text{ x } 100\%$$

C = Flavonoid Concentration (ppm), V = Extract Volume that Used (ml),
Fp = Dilution Factor

2.2.5. Antioxidant Activity Testing of Red Betel Leaf Extract

Antioxidant activity testing using the DPPH method (1,1 diphenyl-2-picrylhydrazyl), as much as 39.432 mg was put into a 100 mL volumetric flask and added methanol to the limit mark, then homogenized. Pipetted 1 mL of 1 mM DPPH solution into a 10 mL volumetric flask, then added methanol to the limit mark, then everything was then homogenized. Then the blank solution was incubated for thirty minutes at 25-30 °C. A wavelength of 500-600 was used to measure the absorption length. In a 100 ml volumetric flask, 10 mg of ascorbic acid was dissolved with methanol (100 ppm), pipet 1 mL of 100 ppm vitamin C standard solution, add 1 mL of 1 mM DPPH solution, and then dilute with methanol until the limit mark. Then mix everything until homogeneous. Peak wavelengths were measured starting from 10, 20, 30, 40, 50, 60 minutes used to measure absorbance. This is done to ensure a stable ideal absorption time (Javed, S., Mangla & Ahsan ,2022). Vitamin C standard series solutions were made with concentrations of 2, 4, 6, 8, and 10 ppm from a 100 ppm stock solution, put into a 10 mL volumetric flask, then added 1 mL of 1 mM DPPH solution, and diluted with methanol to the limit mark. Incubation was carried out at the optimum time, and the absorbance was measured by UV-Vis spectrophotometer with the maximum wavelength.

Red betel leaf extract n-hexane, ethyl acetate and 70% ethanol, weighed as much as 50 mg each, then dissolved with methanol then in a 50 mL volumetric flask each made a concentration series of 20, 40, 60, 80 and 100 ppm of the parent solution into a 10 mL volumetric flask, then added 1 mL of 1 mM DPPH solution and added methanol to the limit mark and homogenized. The series of test solutions were stored for an optimum time at room temperature and then measured the absorbance at the maximum wavelength that had been obtained. The percentage value of DPPH inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{Blank\ Absorbance - Sample\ Absorbance}{Blank\ Absorbance} \text{ x } 100\ \%$$

Then a plot of the extract concentration with the inhibition value is made on the linear regression equation, so that the equation is obtained (y = bx + a), where y (% inhibition) = 50 and x shows IC_50. The IC_50 value states the concentration of sample solution needed to reduce 50% of DPPH free radicals (Molyneux, 2004).

2.2.6. Antimicrobial Testing

2.2.6.1 Phytochemical Screening of Red Betel Leaf

Pure cultures of microbes were cultured in PDA (Potato Dextrose Agar) media for C. albicans fungi and NA (Nutrient Agar) media for S. mutans bacteria. Then incubated at 37 0C for 24 hours for bacteria and for 2 x 24 hours for fungi. Then a suspension of C. albicans and S. mutans cultures was prepared, by mixing with NaCl 0.9%, then the turbidity was equalized with the Mc farland 0.5 standard (Kusumahastuti et al., 2024; Miladiarsi et al., 2023). Make a standard solution of Mc Farland 0.5 by mixing 0.05 ml of 1% Barium Clorida in distilled water 9.95 ml of 1% H2SO4 (Berliana & Pujiyanto (2020).

2.2.6.2 Minimum Inhibitory Concentration (MIC) testing

MIC testing of S. mutans and C. albicans using the agar dilution method. MIC testing was carried out by preparing a Petri dish filled with 15 ml of NA medium, then mixed extracts with concentrations of 2.5, 5, 7.5, and 10% in 1% tween, then into which a bacterial suspension of 0.2 ml was inserted and homogenized to form a number 8. Then incubated for 24 hours at 37 0C. MIC testing of C. albicans was carried out similarly to S. mutans testing, only the media used was PDA media and the incubation period was 2 x 24 hours. MIC is indicated when observations are made after completion of incubation, the test medium remains clear and there are no fungal or bacterial colonies, this indicates inhibition of microbial growth (Hoque et al., 2019).

2.2.6.3 Diameter of Inhibition Zone (DIZ) Testing

The Diameter of Inhibition Zone (DIZ) test, also known as the Kirby-Bauer disk diffusion method, was employed to evaluate the antibacterial and antifungal activities of red betel (Piper crocatum) leaf extracts. This method involves measuring the clear zone formed around paper disks impregnated with the test substance (Dyah Astuti et al., 2020). Paper disks were immersed in red betel leaf extract for 15 minutes at concentrations determined by the Minimum Inhibitory Concentration (MIC) results. For Streptococcus mutans testing, amoxicillin (100 ppm) served as the positive control. For Candida albicans testing, nystatin (100,000 IU/mL) was used as the positive control. A 1% Tween solution was employed as the negative control for both microorganisms. The diameter of the clear zone surrounding each disk was measured to quantify the antimicrobial activity of the extracts against the tested microorganisms.

Cultures were incubated in an incubator at 37 0C for 24 hours for S. mutans bacteria and 48 hours for C. albicans fungi. The parameter measured was the DIZ value in the form of a clear zone formed around the disk using a vernier caliper. The clear zone formed indicates the presence of antimicrobial power in the test solution against S. mutans bacteria and C. albicans fungi (Jaleel et al,. (2024). Data analysis using the SPSS 26 application with a complete randomized design (CRD). Data in this study in the form of numerical variables that have two factors were tested using Two Way ANOVA (Analysis of Variance) with a confidence level of 95% (α <0.05). If the treatment given shows a significant difference, it is continued with Duncan's further test to determine which treatment is most effective in having similarities with the control. In this study, the categorization of antimicrobial activity follows CLSI (2013) where DDH> 20 mm is categorized as susceptible, DIZ 15-19 mm is Intermediate, and DIZ < 14 mm is categorized as Resistant.

3. Results and Discussion

3.1 Characteristics of Simplicia and Extracts

The red betel leaves used in this study were identified as Piper ornatum N. E. Br. (Piperaceae family) by the National Research and Innovation Agency (BRIN) at the Biology Research Center, Bogor (identification number 588/UN2.F3.11/PDP.02.00/ 2023). From 5 kg of fresh red betel leaves, 1,469 g of simplicia powder was obtained (29.8% yield), characterized by a brownish-green color, distinctive aromatic odor, and slightly spicy bitter taste (Figure 1).

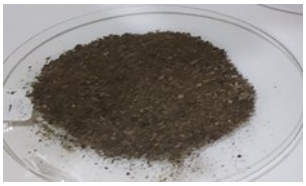

Figure 1. Simplicia Powder

Ultrasound-Assisted Extraction (UAE) of the simplicia powder yielded thick extracts with a dark green color, viscous texture, and a characteristic aroma of red betel leaves combined with the respective solvent used (Figure 2).

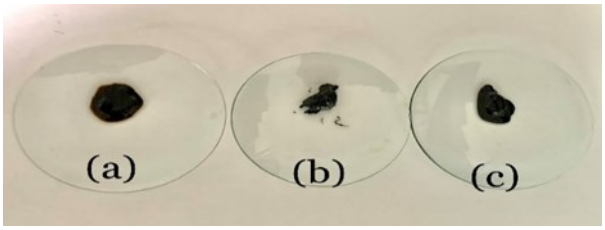

Figure 2. Thick Extracts of Red Betel Leaves, (a) 96% Ethanol Extract, (b) n-Hexane Extract, (c) Ethyl Acetate Extract

Yield is defined as the ratio of the dry weight of the product to the weight of the raw material (Yuliani et al., 2019). The yield value is directly related to the amount of bioactive compounds extracted during the extraction process (Myint). Our extraction results demonstrate that different types of solvents produce varying extract yields. This observation aligns with Senduk et al. (2020), who stated that the extraction yield of bioactive compounds is influenced by both the bioactive content in the simplicia and the solvent used. Furthermore, Myint noted that higher extraction yields correlate with higher amounts of extracted substances. The yield calculations from the extraction process of 600 g of red betel leaf simplicia powder using different solvents are presented in Table 1.

Table 1. The results of simplisia characterization and red betel leaf extracts with different solvents

Ingredients Type	Yield (%)	Requirement (%)	water content (%)	Requirement (%)	Ash Level (%)	Requirement (%)
Powder	29.8	>10	4.00 ± 0.41	≤ 10	2.83 ± 0.29	≥ **4.09**
n-Hexane Extract	8.93	≥ 10	6.43 ± 0.42	≤ 22.2	3.91 ± 0.06	≥ 5.9
Ethyl Acetate Extract	10.23		7.31 ± 0.29		3.53 ± 0.01	
Ethanol Extract	11.76		8.27 ± 0.49		5.00 ± 0.36	

Farmakope Herbal Indonesia, (2017)

The lowest extract yield was observed for n-hexane (8.93%), while the highest was for ethanol (11.76%). This difference is attributed to the varying boiling points of the solvents: ethanol (78.32 °C), ethyl acetate (77c°C), and n-hexane (69 °C). During extraction at 40 °C and concentration at 40 °C, n-hexane evaporates faster than ethanol and ethyl acetate. Solvents with higher boiling points generally yield higher extracts (Kanifah, 2015). The moisture content of simplicia and extracts met the Indonesian Herbal Pharmacopoeia 2017 requirements (≤ 10% for simplicia, ≤ 22.2% for extracts). Excessive moisture can promote rapid microbial growth and hydrolysis of active ingredients (Handayani et al., 2017). The total ash content results also met the requirements for simplicia (14%) and thick extracts (3.9% to 17.4%) (DepKes RI, 2013).

3.2 Phytochemical Screening of Red Betel Leaf Simplisia and Extracts

Phytochemical screening was carried out qualitatively on simplisia powder and extracts. This phytochemical screening was carried out to determine the presence of secondary metabolite compounds of alkaloid, flavanoid, saponin. steroids and tannins. The results of phytochemical screening showed that the simplisia powder contained alkaloids, flavanoids, saponins. steroids and tannins (Table 2). The screening results of the three types of red betel leaf extracts produce different compound contents. N-Hexane extract contains alkaloids and steroids, ethyl acetate extract contains alkaloids, saponins. steroids, tannins and ethanol extract contain Alkaloids, flavanoids, saponins. steroids, tannins.

n-Hexane is a non-polar compound so it will attract non-polar compounds such as alkaloids and steroids. Ethyl acetate is a semi-polar compound so it can attract semi-polar compounds such as alkaloids, saponins. steroids, tannins. Ethanol

is polar so it will attract polar compounds such as alkaloids, flavanoids, saponins. and tannins. The difference in secondary metabolites in the screening results of the three betel leaf extracts is due to differences in the polarity of each solvent that can attract these compounds. The withdrawal of metabolite compounds is based on the principle of like dissolve like, namely polar solvents will dissolve polar compounds, and vice versa for non-polar solvents (Damayanti & Ervilita, 2019). The results of phytochemical screening of simplisia and red betel leaf extract are presented in Table 2.

Table 2. Phytochemical Screening Results of Simplisia and Red Betel Leaf Extract.

Compound	Reactant	Extract Result			
		Powder	n-Hexane	Ethil Acetate	Ethanol
Alkaloids	Dragendorff	+	+	+	+
	Bouchardat	+	+	+	+
	Mayer	+	+	+	+
Flavonoids	Mg	+	-	-	+
Saponins	Distilled Water	+	-	+	+
Steroids	$C_4H_6O_3$	+	+	+	-
Tannins	$FeCl_3$	+	-	+	+

The differences in secondary metabolites among the extracts are due to the varying polarity of the solvents, based on the principle of "like dissolves like" (Damayanti & Ervilita, 2019).

3.3 Minimum Inhibitory Concentration (MIC) Test Results

The MIC test aims to assess the susceptibility or resistance of specific microorganisms to antimicrobial agents in vitro (Kowalska-Krochmal & Dudek-Wicher, 2021). It serves as a preliminary evaluation to determine the concentration range exhibiting antimicrobial activity. Table 4 presents the MIC test results for n-hexane, ethyl acetate, and ethanol extracts of red betel leaves.

This approach provides valuable insights into the antimicrobial potency of red betel leaf extracts and establishes a foundation for further investigations into their potential therapeutic applications. The MIC data offer a quantitative measure of the extracts' effectiveness against target microorganisms, facilitating comparisons between different extraction solvents and guiding future research on optimizing antimicrobial formulations derived from red betel leaves.

Table 3. Testing Result (MIC) of Red Betel Extract

Targetted Microbes	**Extracts**	**MIC (%)**
C. albicans	n-Hexane	7.5
	Ethyl Acetate	7.5
	Ethanol 96%	7.5
S. mutans	n-Hexane	5
	Ethyl Acetate	5
	Ethanol 96%	10

The MIC test against Candida albicans revealed that n-hexane, ethyl acetate, and 96% ethanol extracts all exhibited the same MIC value of 7.5%. For Streptococcus mutans, n-hexane and ethyl acetate extracts demonstrated a lower MIC of 5%, outperforming the ethanol extract, which showed an MIC of 10%. These findings can be compared to research by Rahmawaty et al. (2009), which reported that ethanolic extracts of red betel leaf obtained through maceration had an MIC of 25% against Staphylococcus aureus (Gram-positive) and 6.25% against Escherichia coli (Gram-negative). Based on these MIC results, concentrations of 7.5%, 15%, and 25% were selected for the inhibition zone diameter (ZID) tests against C. albicans for all three extracts. For S. mutans, concentrations of 5%, 15%, and 25% were used for n-hexane and ethyl acetate extracts, while 10%, 15%, and 25% were used for the ethanol extract.

3.4 Inhibition Zone Diameter (IZD) Test Results

Two-way ANOVA analysis revealed significant effects of both solvent type and concentration on IZD values ($p < 0.05$) for both C. albicans and S. mutans. Furthermore, an interaction between concentration and solvent type was observed. Subsequent Duncan's test indicated that the 25% ethanol extract of red betel leaf was most effective against C. albicans, while the 25% n-hexane extract was most effective against S. mutans (Table 5). Figure 4 illustrates the antifungal activity of n-hexane, ethyl acetate, and 96% ethanol extracts against C. albicans. Similarly, Figure 5 displays the antibacterial activity of these extracts against S. mutans. These results highlight the differential efficacy of various solvent extracts against fungal and bacterial pathogens, underscoring the importance of solvent selection in the

development of antimicrobial formulations from red betel leaf. The observed variations in antimicrobial activity across different extracts and concentrations provide valuable insights for future research and potential therapeutic applications.

Table 4. IZD Average (mm) of Red Betel Leaf Extract

Targetted Microbes	Extract	Concentration (%)	IZD ± SD	Category
C. albicans	n-Hexane	7.5	$9.45^{b} \pm 0.074$	*Resistant*
		15	$10.78^{c} \pm 0.178$	*Resistant*
		25	$13.52^{f} \pm 0.112$	*Resistant*
		K+	$33.17^{i} \pm 0.236$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	-
	Ethyl Acetate	7.5	$10.43^{c} \pm 0.18$	*Resistant*
		15	$12.18^{e} \pm 0.25$	*Resistant*
		25	$15.33^{g} \pm 0.15$	*Intermediate*
		K+	$33.20^{i} \pm 0.13$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	-
	Ethanol 96%	7.5	$11.28^{d} \pm 0.21$	*Resistant*
		15	$13.70^{f} \pm 0.45$	*Resistant*
		25	$\mathbf{17.02^{h} \pm 0.14}$	***Intermediate***
		K+	$33.09^{i} \pm 0.21$	*Susceptible*
		K-	$0.00^{a} \pm 0.00$	-
S. mutans	n-Hexane	5	$12.62^{c} \pm 0.13$	*Resistant*
		15	$14.57^{f} \pm 0.23$	*Resistant*
		25	$\mathbf{17.43^{h} \pm 0.20}$	***Intermediate***
		K+	$19.19^{i} \pm 0.05$	*Intermediate*
		K-	$0.00^{a} \pm 0.00$	-
	Ethyl Acetate	5%	$12.10^{b} \pm 0.23$	*Resistant*
		15%	$13.99^{e} \pm 0.40$	*Resistant*
		25%	$16.09^{g} \pm 0.29$	*Intermediate*
		K+	$19.22^{i} \pm 0.18$	*Intermediate*
		K-	$0.00^{a} \pm 0.00$	-
	Ethanol 96%	10	$12.03^{b} \pm 0.09$	*Resistant*
		15	$12.99^{d} \pm 0.09$	*Resistant*
		25	$15.83^{g} \pm 0.05$	*Intermediate*
		K+	$19.07^{i} \pm 0.10$	*Intermediate*
		K-	$0.00^{a} \pm 0.00$	-

Note: Numbers followed by the same superscript letter in the same column show no significant difference

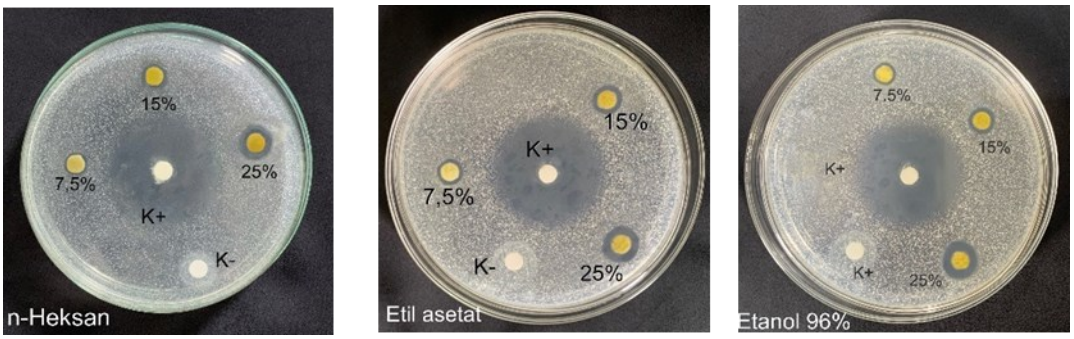

Figure 3. IZD Test Results of Red Betel Leaf Extract against C. albicans fungus.

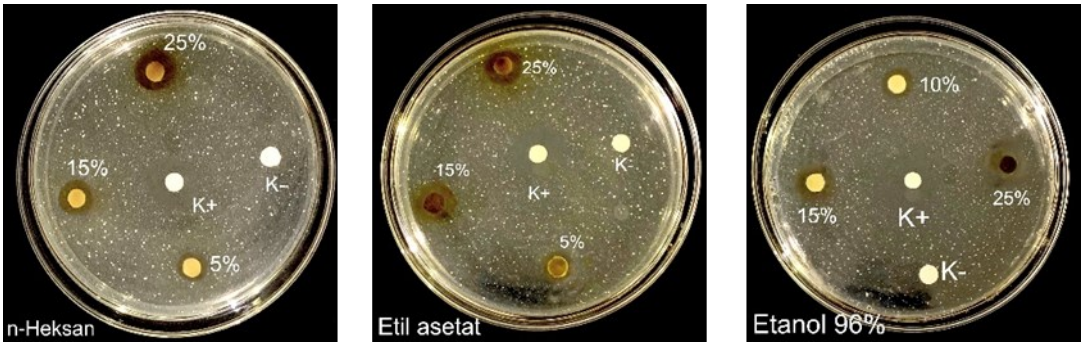

Figure 4. IZD Test Results of Red Betel Leaf Extract against S. mutants Bacteria

The average inhibition zone diameters (IZD) for antifungal activity against Candida albicans revealed that only the ethyl acetate and 25% ethanol extracts fell into the 'intermediate' category. According to Cukic (2013), this classification indicates that these extracts retain the ability to inhibit fungal growth and potentially exhibit fungicidal properties. The results suggest that polar solvents are more effective in extracting bioactive compounds from red betel leaves that possess antifungal activity against C. albicans. These findings align with research conducted by Shivakumar et al. (2023), which demonstrated that ethanol extracts of white tea (Camellia sinensis) exhibited superior antifungal activity against C. albicans compared to extracts obtained using ethyl acetate and n-hexane. This corroborates the observation that polar solvents may be more suitable for extracting compounds with antifungal properties from plant materials. The preferential extraction of antifungal compounds by polar solvents highlights the importance of solvent selection in optimizing the isolation of bioactive components from red betel leaves. This insight may guide future research and potential applications of red betel leaf extracts as natural antifungal agents, particularly against C. albicans.

Research by Anugrahwati (2016) demonstrated that ethanolic extracts of red betel leaf, obtained through maceration, exhibited antibacterial activity against Streptococcus mutans at concentrations of 10, 20, 40, and 80%. Using the well diffusion method, the average inhibition zones for each concentration were less than 5 mm. Similarly, Amiludin (2023) employed the same methodology and reported that ethanolic extracts of red betel leaf at concentrations of 60, 70, 80, 90, and 100% effectively inhibited S. mutans growth, with inhibition zones <10 mm. These findings suggest that non-polar solvents (n-hexane) may be more effective than polar (ethanol) and semi-polar (ethyl acetate) solvents for extracting bioactive compounds with antibacterial activity against S. mutans. The variability in results across studies can be attributed to several factors, including extraction methods and solvents used. Moreover, Rozirwan et al. (2023) noted that the growing conditions and environment of the plant material significantly influence the quality of the resulting extracts. The superior performance of n-hexane in extracting antibacterial compounds effective against S. mutans highlights the importance of solvent selection in optimizing the extraction of bioactive compounds from red betel leaves. This observation underscores the need for careful consideration of extraction parameters in future studies and potential applications of red betel leaf extracts as natural antibacterial agents.

4. Conclussion

Based on the results of this study, the following conclusions can be drawn: The highest flavonoid content was obtained from the 96% ethanol extract, with a value of 4.748 ± 0.091%; The most potent antioxidant activity was observed in the 96% ethanol extract, with an IC50 value of 47.185 ± 0.017 ppm, categorized as very strong; The most effective antibacterial activity against Streptococcus mutans was demonstrated by the n-hexane extract at a concentration of 25%, with an inhibition zone diameter of 17.43 mm; and The most potent antifungal activity against Candida albicans was exhibited by the 96% ethanol extract at a concentration of 25%, with an inhibition zone diameter of 17.02 mm. These findings highlight the potential of red betel leaf extracts as sources of natural antioxidants and antimicrobial agents, with the choice of solvent significantly influencing the extraction efficiency and bioactivity of the resulting extracts.

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