BIOMA p-ISSN: 0126-3552 e-ISSN: 2580-9032

DOI:10.21009/bioma.v21i1.50131 Research article

Reproducibility of antibacterial effects of ethanol extracts from *Piper ornatum N.E.Br.*, and *Piper betle* L. (Piperaceae), against common acne, *Propionibacterium acnes* (Gilchrist, 1900) Douglas & Gunter, 1946 (Propionibacteriaceae)

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ABSTRAK

Daun sirih merah dan hijau bermanfaat sebagai obat tetapi perlu diuji kemampuan antibakteri untuk dijadikan bahan baku pembuatan serum anti-jerawat. Penelitian ini bertujuan untuk mengetahui efek antibakteri daun sirih merah dan hijau terhadap respon sensitivitas bakteri Propionibacterium acnes*. Penelitian eksperimental ini menggunakan sampel berupa daun sirih merah dan hijau dengan prosedur meliputi penyiapan sampel, maserasi, skrining fitokimia secara kualitatif, dan uji senyawa antibakteri ekstrak etanol sampel 70%, 80%, 90% dan 100% dengan metode Kirby Baeur. Kontrol positif berupa antibiotik klindamisin 30 µg dan kontrol negatif berupa aquades steril. Hasil rendemen ekstrak kental daun sirih merah dan hijau sebesar 13,5% dan 15%. Pemberian ekstrak kental daun sirih merah 70%, 80%, 90%, dan 100% mampu menghambat* Propionibacterium acnes *dengan diameter zona hambat sebesar 16 mm, 18 mm, 18,5 mm,*dan *19 mm sedangkan sirih hijau sebesar 18 mm, 18 mm, 21 mm, 23 mm. Pemberian ekstrak kental daun sirih merah dan hijau efektif dalam menghambat pertumbuhan* P.acnes *dan potensial dijadikan bahan baku formulasi serum anti-jerawat.*

Kata kunci: *Sirih merah; sirih hijau;* P.acnes*; skrining; fitokimia*

ABSTRACT

Red and green betel leaf plants are useful as medicine but need to be tested for antibacterial ability to be used as raw materials for making anti-acne serum. This study aims to determine the antibacterial effect of red and green betel leaves on the sensitivity response of Propionibacterium acnes *bacteria. This experimental study used samples of red and green betel leaves with procedures including sample preparation, maceration, qualitative phytochemical screening, and testing of antibacterial compounds in ethanol extracts of 70%, 80%, 90% and 100% samples using the Kirby Baeur method. Positive control was clindamycin 30 µg antibiotic and negative control was sterile distilled water. The yield of thick extracts of red and green betel leaves was 13.5% and 15%. The administration of 70%, 80%, 90%, and 100% thick extract of red betel leaves was able to inhibit* Propionibacterium acnes *with inhibition zone diameters of 16 mm, 18 mm, 18.5 mm, and 19 mm while green betel was 18 mm, 18 mm, 21 mm, 23 mm. The administration of thick extracts of red and green betel leaves is effective in inhibiting*

the growth of P. acnes *and has the potential to be used as raw material for anti-acne serum formulations.*

Keywords: *Red betel; green betel; P. acnes; screening; phytochemical*

INTRODUCTION

One of the problems with the use of medicinal plants in Indonesia is the practice of using them in a society where the information is still based on empirical experience (Yuslianti et al., 2016). Statistical data from a compilation of various studies in Indonesia states that there are 30.000 species of plants in Indonesia, of which 7.000 are medicinal plants and 300 species have been used for traditional medicinal practices (Hakim, 2015; Siahaan & Aryastami, 2018; Qamariah et al., 2018; Nasution et al., 2018). However, this practice is only carried out based on empirical experience or carried out from generation to generation, which is inherited and maintained by oral narrative (Jawa La & Kurnianta, 2019). Whereas according to World Health Organization (WHO) (2013), the use of medicinal plants both in the form of decoctions and herbal medicines is highly recommended in treating infectious diseases; however, these recommendations have been scientifically proven through a process of scientification and standardization of their efficacy in-vitro and in-vivo. The existence of scientific evidence will provide information regarding the efficacy of medicinal plants so that they can be used as a source of data to develop them as modern pharmaceutical products. Referring to the challenges of medicinal plants that require scientific evidence, all the properties of medicinal plant raw materials that are to be developed into pharmaceutical innovation products must be scientifically proven. One of very common health problem, for youngsters especially, is the common acne, causes by the bacterium *Propionibacterium acnes* (Gilchrist, 1900) Douglas & Gunter, 1946. Common acne is not a fatal problem; however, its common incidence causes the need in standard traditional medication. Traditionally, people used various species of plant available in Indonesia, such as cucumber, lavender, basil, neem and aloe. Some species had been investigated scientifically, as *sambiloto*, *Andrographis paniculata* (Purwoko et al, 2020) and *sungkai*, *Peronema canescens* (Novianti & Wirnawati, 2024).

One of the medicinal plants whose efficacy requires scientific evidence is red (*Piper ornatum N.E.Br.*) and green betel (*Piper betle* L.). Both red and green betel are Indonesian native plants, with the green is widespread throughout Indonesian archipelago and had been utilized by Indonesian for centuries. The other species, the red betle is a native of Sulawesi (POWO, 2025) but usually utilized by Indonesian as ornamentals, with increasingly utilization as traditional medicine, nowadays. Indonesians often uses these two plants as antimicrobials that cause bad breath, vaginal discharge, inflammation, anti-acne, and cough (Anindita et al., 2023). If the red and green betel leaf extracts prove to be effective as antibacterial agents, the two plants are potentially entering the manufacture of innovative anti-acne products. Biswas et al (2022) reported that the use of betel leaves is more effective as an anti-acne (inflammatory skin disease), as evidenced by the results of effective inhibition against *S. aureus* and *P. acnes* bacteria.

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The selection of red and green betel as raw material candidates for making anti-acne products is based on scientific evidence from several previous studies, including research (Rinanda et al., 2012) which showed that administration of 45% and 60% red betel leaf methanol extract was able to produce a diameter of the inhibition zone in *Staphylococcus Methicillin Resistance* (MRSA) of 13.6 mm and 15.7 mm. Soleha et al. (2015) who reported that administration of 50% and 100% ethanol extract of green betel leaves were able to inhibit the growth of *S. aureus* by 15.1 mm and 16.3 mm. Jamil et al. (2021) proved that giving 75% green betel leaf ethyl acetate extract was able to inhibit the growth of *Staphylococcus aureus* by 16.65 mm; Rameshwari & Priya (2020) suggested that the antibacterial activity of betel leaves with water, methanol, and ethyl acetate solvents would be more effective when combined with other natural materials, such as guava leaves which produced an interval of inhibition zone diameter for various pathogenic bacteria of 19 mm - 29 mm. Beside *Staphylococcus*, Nayaka *et al* (2021) reported that betel leaf extract treatment was able to inhibit the growth of *P. acne* with a MIC value of 1-4%.

According to Rahardjo et al. (2018), the colour detection results for the secondary metabolites of red and green betel leaves extracted by maceration with ethanol and water solvents were positive for the presence of alkaloids, flavonoids, tannins, polyphenols, saponins, and essential oils. Djohan et al. (2022) stated that the interaction of these various phytochemical compounds was able to prevent the growth of *S. aureus* and *S. epidermidis* bacteria. Parfati & Windono (2016) added that the effect of betel leaf as an antibacterial was more effective if given at concentrations above 60%. Based on these, it is advisable that the method of choice in extracting red and green betel leaves is ethanol solvent with the maceration method, while the effective concentration in inhibiting acne-causing bacteria such as *S. aureus* and *S. epidermidis* is above 60%. Furthermore, we aim at elaborating the existing research in green and red betel by testing the effects of ethanol extract at levels of 70%, 80%, 90%, and 100% using the maceration method, on *P. acnes* bacteria. The novelty of this research compared to other research lies in the extract concentration and test bacteria used. The purpose of this study was to determine the antibacterial effect of red and green betel leaves on the sensitivity response of *P. acnes.* The result of this research is also aimed at proving the reproducibility and repeatability of the efficacy of red and green betel leaves on various types of bacteria. If they produce effective properties, red and green betel leaves are potential to be recommended as raw materials for anti-acne serum formulations.

METHODOLOGY

Tools and materials

Materials needed in this study included red and green betel viscous extracts as independent variables, pure culture of *P acnes* ATCC: 11827 (microbiology laboratory, University of Indonesia) as the dependent variable, Clindamycin antimicrobial susceptibility discs 30 µg (Oxoid, Germany) as positive control, and sterile distilled water as a negative control. Equipment used includes automatic autoclaves (Hirayama HG-80, 76L, Japan), Laminar Air Flow (LAF) (ESCO, Singapore), hot plates and stirrers (IKA C-MAG HS7, Germany), incubators (Memmerth IN-30, Germany), rotary

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evaporator (IKA-RV-3 V, Germany), 6 Hole water bath (HH, China), digital analytical balance (Acuplus, China), P100 micropipette (Socorex, Switzerland), loop needle (ROFA, Indonesia), micropipette and vortex mixer VM 300 (Gemmy, Taiwan).

Research design

This study used an experimental design that tested the effect of thick extracts of red and green betel leaves with concentrations of 70%, 80%, 90%, and 100% on the Growth of *Propionibacterium acnes.* The concentration variations of red and green betel viscous (solvent-free) extracts in this study were 70%, 80%, 90%, and 100% (g/ml). The positive control was in the form of 30 µg clindamycin antibiotic discs. Clindamycin was chosen as a positive control because it is a group of antibiotics that is most widely used in Indonesia for acne therapy. Beside positive control, we use negative control, 30 µl sterile distilled water. All treatments were dropped as much as 30 µl on a sterile blank disc with a diameter of 6 mm.

Plant Identification

Plant identification is quite problematic for the red betel. It is normally misidentified on the internet as *Piper crocatum*, which is however, an alien species from Mexico. Further examination combining the herbarium collection of BRIN (BO), UI (UIDEP) and UNJ (JUNJ) resulted on a Sulawesi species of *Piper ornatum* N.E.Br. Therefore, in this publication, red betel used in this study is referred to as *P. ornatum.*

Preparation of leaf samples

The preparation of samples of natural materials in this study included taking samples of 2 kilograms (kg) of red betel from Srimulyo village, Bantul, Yogyakarta, and green betel from Mangunjaya District, Bekasi (**FIGURE 1**).

FIGURE 1. A. Red betel leaf. B. Green betel leaf

Extraction by Maceration.

Extraction by maceration was carried out by weighing 500 grams of red and green betel leaf powder each, then placing it in an Erlenmeyer containing 1000 ml of 70% ethanol, and then tightly closing it using aluminium foil. Soak for 3 days with

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occasional stirring until the solvent is completely mixed. The remaining liquid from the filtration is filtered again using filter paper Whatman No. 1. The maceration process is repeated for 5 days in the same way until a colourless liquid extract is obtained. Evaporation was carried out by removing the solvent using a rotary evaporator (temperature 40 ºC, pressure 197 Mbar; speed 40 rpm) for ± 2 days. After 2 days, heating was carried out on a water bath at 50 ºC to obtain the optimal viscous extract (Anindita et al., 2022). The extraction process is controlled so that the yield was satisfactory (**TABLE 1**).

TABLE 1. Percentage of red and green betel leaf extracts

Sample	Powder Weight (g)	Viscous Extract Weight (g)	Extract Yield Value (%)
Red betel leaf	13,5 g	100 g	13,5%
Green betel leaf	15 g	100 g	15%

Phytochemical Screening Test.

Phytochemical screening of concentrated extract results included tests for flavonoids, tannins, saponins, essential oils, and alkaloids. Alkaloid tests are performed with Meyer's, Dragendorf's, and Wagner's reagents, while flavonoid test with Mg and HCL reagents, tannin test with $FeCl_3$ reagents, and saponin test with distilled water reagent and HCl.

Preparation of *P. acnes* Suspension.

The preparation of the *P.acnes* suspension was carried out by taking several samples of pure culture of the *P.acnes* subculture, putting it in 0.9% NaCl, and then homogenising it using a vortex. The homogeneous suspension results were compared to the turbidity level with a 0.5 Mc Farland tube (equivalent to a bacterial suspension of 1.5 x 10^8 CFU/mL).

Kirby Baeur test (paper disc diffusion).

A bacterial inhibition test was carried out using the Kirby-Bauer method using a 4-quadrant streak plate technique on the surface of MHA media using a sterile cotton swab and left for ± 5 minutes. Then, attach 5 blank discs containing ethanol extracts of red and green betel leaves with concentrations of 70%, 80%, 90%, and 100%, subsequently let it stand for ± 15 minutes. Each petri dish had 3 replicates, which were incubated for 24 hours at 37 ◦C. After 1x24 hour incubation, the presence/absence of a clear zone formed around the treatment disc was measured using a ruler. The results of measuring the diameter of the inhibition zone were compared with CLSI (2020) the to see the sensitivity category of the test bacteria in response to each treatment disc.

Data analysis.

Data analysis was carried out by processing the variable results in tabular form and then analysed using a quantitative descriptive test to see the sensitivity category of the test bacteria in responding to the treatment discs.

DOI: 10.21009/bioma.v21i1.50131

RESULTS AND DISCUSSION

Content of red and green betel extract

TABLE 2 presents the content of red and green betel maceration extraction using 70% ethanol are flavonoids, alkaloids, saponins, tannins, and essential oils. It means that the four classes of plant secondary metabolites are contained in both green and red betel. However, whether green betel has higher content of flavonoids or other secondary metabolites compared to red betel, or vice versa, is not known. This extract screening shows that both green and red betel contain potent active content for antibacterial activity.

TABLE 2. Results of phytochemical screening of viscous extracts of red and green betel leaves

Secondary Metabolites	Extract Test Results		Reagen	evidence
	Red betel leaf	Green betel leaf		
Flavonoids	+	+	Concentrated HCL + Mg	Dark red colour
Alkaloids	+	+	*Wagner*	Brown precipitate
Saponins	+	+	Aquadest	Forms stable foam
Tannins	+	+	$FeCl_3$ 1%	Bluish black or greenish colour
Essential oil	+	+	Ethanol 96%	Presence of residue and characteristic odour

The antibacterial activity of red and green betel extract

TABLE 5. Comparison test results of the effects of giving red and green betel leaves on growth of *P. acnes*

Treatment	Mean Inhibition Zone Diameter (mm)			
	Red betel leaf	Result	Green betel leaf	Result
Aquadest	0 mm	Resistant	0	Resistant
Clindamycin	25.7 mm	Sensitive	25.6	Sensitive
70%	16 mm	intermediate	18 mm	Intermediate
80%	18 mm	intermediate	18 mm	Intermediate
90%	18,5 mm	intermediate	21 mm	Sensitive
100%	19 mm	Sensitive	23 mm	Sensitive

TABLE 5 is the result of the effect of condensed extracts of red and green betel leaves on the growth of *P. acnes* as indicated by the diameter of the inhibition zone around the treatment disc. The inhibition zone is a clear area around the disc measuring 6 mm, which has been suspended with various treatment solutions. The presence of a clear zone indicates no growth of *P. acnes*, so the higher the diameter of the inhibition zone, the more effective the concentration of the test solution is in inhibiting the growth of *P. acnes*. The administration of red betel leaf condensed extract with a concentration of 70%, 80%, 90%, and 100% can inhibit the reproductive power of *P. acnes* with the diameter of the inhibition zone respectively 16 mm, 18 mm, 18.5 mm, and 19 mm, while the green betel of 18 mm, 18 mm, 21 mm, and 23 mm. It is evident that green betel is better antibacterial than red betel, which is shown by the sensitive category of sensitive responses to green betel leaf extract with a concentration of 90% and 100%, while red betel only at a concentration of 100%. The results in **TABLE 5** were obtained using the Kirby Bauer method, which is visualised in **FIGURE 2**.

DOI: 10.21009/bioma.v21i1.50131

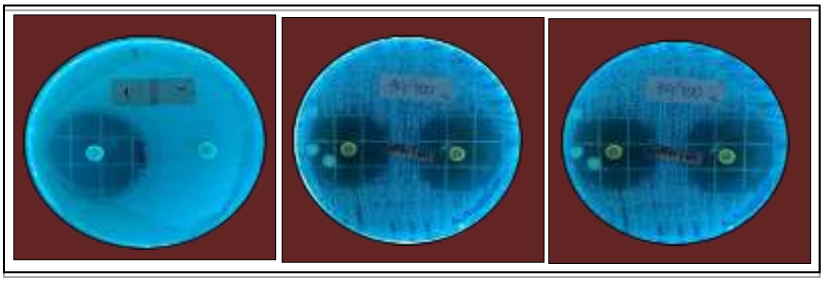

Figure 2. A. Positive and negative controls. B. extract of red betel leaves. C. extract of green betel leaves

The ability of red and green betel leaf extract shown in **FIGURE 2** is also shown in the results of the study by Patil et al (2016) which proved that red and green betel leaf extracts with concentrations of 1.5%, 3%, and 4% were able to produce diameters of inhibition zones on *Staphylococcus aureus* bacteria ranging from 16.9 mm to 18.4 mm in the intermediate category; Pratiwi dan Muderawan (2016) who used 1.25%, 2.5%, 5% red betel leaf extract produced *S. aureus* inhibition zones of 6.82 mm, 10.5 mm, 14.5 mm and *Escherichia coli* of 8 mm, 10.3mm, 12.5mm ; Rahardjo dkk (2018) tested 50% and 100% red betel leaf extract against *Staphylococcus epidermidis* with the results of inhibition zone diameters of 5.63 mm and 9.2 mm with the weak category. Another study conducted by Rinanda et al (2012) proved that red betel leaf essential oil with a concentration of 5%, 10%, 20%, 40%, and 80% was able to produce *S. aureus* growth inhibition zones of 8.75 mm, 10.25 mm, 11 mm, 16.25 mm and *E. coli* of 11 mm, 13 mm, 14.25 mm, 14.75 mm, 19.25 mm. This comparison revealed that our result in this research produced higher zone of inhibition, up to 23.5 mm in 100% extract concentration. However, our result is unable to match the activity of Clindamycin which inhibtion zone was the highest at 25.7 mm. The average diameter of the inhibition zone for *P.acnes* growth after administration of red betel leaf viscous extract ranged from 16 mm to 18 mm. In contrast, green betel leaf ranged from 18 mm to 23 mm with intermediate and sensitive categories. In this study, the classification of the sensitivity response category of *P.acnes* to thick extracts of red and green betel leaves referred to standard Yuslianti et al. (2016) regarding the effect of the antibiotic clindamycin as a positive control for the sensitivity of *P.acnes* bacteria, that is, if the diameter of the inhibition zone ≥ 18 mm is classified as sensitive, 13-17 mm is in the intermediate category, and ≤ 12 mm is in the resistant category.

In this study, the positive control used the antibiotic clindamycin 30 µg, which could prevent the growth of *P.acnes* with an inhibition zone diameter of 25.6-25.7 mm with a sensitive category. Clindamycin is an antibiotic that is commonly used to treat acne. The working ability of clindamycin is to inhibit protein synthesis in *P.acnes*; besides that, clindamycin is also able to bind to 23S rRNA in the 50S ribosomal subunit and deactivate the peptidyl transferase enzyme. In *P. acnes*, the peptidyl transferase enzyme functions to react with the peptide bond between the new amino acid that is still attached to the tRNA and the last amino acid that is being developed during protein synthesis. Therefore, the blockade of the peptidyl transferase enzyme spontaneously results in the cessation of protein synthesis in *P. acnes*.

DOI: 10.21009/bioma.v21i1.50131

The advantages of this study are the use of concentrations of 70%, 80%, 90%, and 100%, which complement the concentration data from previous studies, and perhaps beneficial to the advancement of traditional or household medication application of easily obtainable plant materials. It means that when people who want to apply the readily available red and green betel for their own common acne, they can easily pick 10 leaves of green and red betel, crush them and apply them, without need to dilute or mix, directly to the acne-prone areas of the face.

In addition, the use of high concentrations refers to their use in the community and does not have the potential to cause bacterial resistance. The use of red and green betel so that they can be used to see differences in the performance of the two plants as antibacterial agents, the selection of *P. acnes* as a test bacterium that has not been widely tested in previous studies, and the results of this study were able to complete information regarding the effective efficacy of red and green betel leaves as antibacterials so that they are potential candidates for natural ingredients for pharmaceutical product manufacture. However, this study has limitations, including the use of the Kirby Bauer method for antibacterial testing, which cannot be used as a guideline for clinicians, the identification of essential oils both qualitatively and quantitatively, and the phytochemical screening of secondary metabolites has not been carried out quantitatively, and the structural damage of bacteria has not been examined by electron microscopy due to the administration of red and green betel.

CONCLUSIONS

The results of the phytochemical screening of viscous extracts of red and green betel leaves taken from Bekasi and Yogyakarta showed positive for the presence of flavonoids, tannins, alkaloids, saponins, and essential oils. The presence of phytochemicals contained in the thick extract of red betel leaves with concentrations of 70%, 80%, 90%, and 100% can inhibit the growth of *P. acnes* with an average diameter of the inhibition area ranging from 16 mm-18 mm, while green betel is ranging from 18 mm- 23 mm in the intermediate and sensitive categories. As both red and green betel leaf extracts are effective as antibacterial agents for *P.acnes,* these two plants can be used as candidates as raw materials for the formulation of anti-acne pharmaceutical products.

AUTHOR CONTRIBUTIONS

RA: project conception, & methodology; MF: data analyses, & original manuscript draft; MI: manuscript review, & editing; IKP : phytochemical screening of analysis; & MUB : calculation and determination of extract dosage

ACKNOWLEDGMENTS

The author would like to thank STIKes Mitra Keluarga for giving permission and providing facilities and infrastructure that support of this research. We thank Alex Tianara (UIDEP), Rani Asmarayani (BO) and Agung Sedayu (JUNJ) for their examination on the identity of red betel (*P. ornatum*).

DOI: 10.21009/bioma.v21i1.50131

CONFLICTS OF INTEREST STATEMENT

There are no conflicts to declare

DISCLOSURES AND ETHICS

As there is no use of animal test subject, animal test and/or application of our leaf extract to any animal and human, there is no ethics declaration needed.

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