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# Extracting Hydroxychavicol and Evaluating the Antibacterial and Antifungal Properties of Betel Leaf Extract(*Piper Betle* L.)

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This study aimed to extract hydroxychavicol from betel leaf (*Piper Betle* L.) and assess its antibacterial and antifungal properties. The Soxhlet extraction method was used to obtain the extract, which was then isolated using column chromatography with silica gel. This process resulted in hydroxychavicol-enriched fractions with a purity of 74.94 %. The antibacterial and antifungal activities of the betel leaf extract were tested on several strains: *Streptococcus mutans, Streptococcus mitis, Candida albicans*, and *Malassezia furfur*, using the agar well diffusion method. At 20 mg/mL, *S. mutans* and *S. mitis* had inhibitory rings of 16.12 ± 1.27 mm and 21.87 ± 1.64 mm. The extract (50 mg/mL) produced a zone diameter of 15.67 ± 0.94 mm against *Candida albicans. M. furfur* was inhibited at an extraction (20 mg/mL), resulting in an inhibitory ring diameter of 21.63 ± 0.79 mm. Based on these results, Piper betel leaves have potential as sources of antibacterial and antifungal agents for scalp and oral care products.

## 1. Introduction

In today's society, increased sugar consumption and poor diet have led to a rise in dental diseases, primarily tooth decay and periodontitis. These conditions are typically caused by *Streptococci* bacteria found in dental plaque. These bacteria produce acids that erode tooth enamel, leading to tooth decay. In addition to dental issues, environmental factors can contribute to gynecological diseases in women and symptoms such as an itchy scalp. The latter can be caused by dandruff fungus, folliculitis, or multi-colored ringworm. Long-term use of antibacterial or antifungal agents, like fluoride, weak acids, antibiotics, and antifungals, can potentially lead to unforeseen consequences.

In Vietnam, betel leaves (*Piper betle* L.) are a common ingredient in folk medicine due to their beneficial properties [Nguyen et al., 2016]. *Piper betle* L., commonly known as the betel plant, is a species that thrives in various areas of Vietnam, with a particular prevalence in the North and North Central regions. This plant is also found in numerous other Asian countries, spreading its influence across borders.

In a notable study published by Subramani et al. (2023), it was revealed that the act of chewing betel leaves post-meal has a beneficial effect on oral health. Specifically, the act can bring about a neutralization effect on the pH of saliva. This leads to a significant decrease in the detrimental impact of acid on the teeth, which is a primary cause of demineralization. By mitigating this acid effect, the general health and strength of teeth can be improved. In a separate yet complementary study conducted by Ali et al. (2022), it was proven that the incorporation of betel leaves into toothpaste had a significant positive impact on dental health. The researchers found that this innovative toothpaste formulation greatly reduced the presence of dental plaque, which is known to be a common cause of various dental diseases. The toothpaste incorporation of betel leaves was also found to reduce gingival bleeding, a symptom associated with poor oral hygiene and gum diseases.

Betel plant leaves contain many of chemical components, including bioactive compounds such as alkaloids, phenols, flavonoids, tannins, saponins, glycosides, terpenoids, and steroids [Biswas et al., 2022]. According to Foo et al. (2017), over 70 types of medicinal substances were discovered when the supercritical carbon dioxide

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method was applied to betel leaves. Hydroxychavicol, beta-caryophyllene, and eugenol are the major components among them, as stated by Syahidah et al. (2017).

Among the plethora of compounds found in betel leaves, hydroxychavicol (also known as 4-allyl-catechol or 1-allyl-3, 4-dihydroxybenzene) stands out. This major phenolic compound exhibits a broad range of properties that are beneficial to health, including antibacterial, antioxidant, antifungal, anti-inflammatory, anti-cancer, and antidiabetic effects. Given its potent bactericidal and fungicidal effects, hydroxychavicol shows great promise as a potential agent in the prevention and treatment of various dental disorders [Sarma et al., 2018].

Research studies provide insights into the mode of action of hydroxychavicol's antibacterial properties. The compound appears to induce oxidative stress within bacterial cells. This oxidative stress leads to the generation of Reactive Oxygen Species (ROS), which are known to cause the loss of cell viability, effectively killing the bacteria [Sharma et al., 2009]. Further studies have demonstrated the compound's effectiveness against *Streptococci* oral bacteria, a common cause of many oral diseases. According to research by Ni Made Dwi Mara Widyani Nayaka et al. (2021), it has been shown that hydroxychavicol could fight Streptococci oral bacteria, in addition to this compound can also kill *C. albicans*.

The research findings suggest that hydroxychavicol, a phenolic compound, could have significant benefits if integrated into formulations for scalp and oral care products. In response to the potential, this study has set an objective to isolate the hydroxychavicol fraction from the extract of the betel leaf, also focus on the ability of hydroxychavicol to combat bacterial and fungal strains that are known to cause skin and dental diseases. It could pave the way for the creation of innovative preparations designed to protect teeth and skin, driving forward the fields of dental and dermatological health.

## 2. Materials and methods

### 2.1 Experiment apparatus

All experiments were conducted in a biochemistry laboratory at the Faculty of Chemical Engineering, Ho Chi Minh City University of Technology. The solvents: ethanol, and methanol, were obtained from Merk Chemical Co., Inc. Microbial culture mediums such as Mueller Hinton Agar, Potato Dextrose Agar, and Dixon Agar were procured from Himedia, India. The extract was concentrated using a VWR® LED Digital Rotary Evaporator. The primary equipment used in this study included a biological safety cabinet (Esco LHG-6AG-F8), a 058 – DEVS Gerhardt Soxhlet, a Memmert incubator (both from Germany), an OD GENESYSTM30 Vis Spectrophotometer (Thermo Fisher Scientific, America), an Elmasonic S 100(H) Ultrasonic bath (Elma, Germany), and a GC – MS (Gas Chromatography-Mass Spectrometry) system (SCION SN 456-GS, Netherlands).

### 2.2 Materials and microbial strains

*Piper betle* L. leaves were sourced from the Tan Phat drug store in Ho Chi Minh City. These leaves were then washed and dried at 60 °C for 24 h. Later it was homogenized to a dimension of 3 x 3 mm and subsequently stored under cool and dry conditions.

### 2.3 Extraction Conditions for *Piper betle* L. were based on the study by Azahar et al. (2020).

*Piper betle* L. powder was extracted using a 99 % ethanol solvent via the Soxhlet method with a ratio of 1:30 (w/v) of ingredients to ethanol. The extraction process lasted for 6 h at 50 °C. The extract was then filtered using a Whatman filter No.1 ϕ 110 mm. Once the solvent had evaporated, the leftover residue was stored at 4 °C.

### 2.4 Hydroxychavicol Extraction from Betel Leaf Extract

Betel leaf extract was dissolved in a solvent system and applied to a glass column (2 x 30 cm) filled with silica gel 230 - 400 mesh (37 - 63 µm). The eluent solvent Dichloromethane: Ethyl acetate was used in a 99:1 ratio. Each resulting fraction was monitored using thin-layer chromatography (TLC). The compounds on the TLC were visualized using a 1 % vanillin: $H_2SO_4$ reagent and $FeCl_3$ reagent. Clean fractions were collected and evaporated to yield hydroxychavicol. The purity of the hydroxychavicol was then analyzed using a Gas Chromatography-Mass Spectrometry (GC – MS) system.

The microbial strains used in this study include: *Streptococcus mutans* ATCC 25175; *Streptococcus mitis* ATCC 49456; *Candida albicans* ATCC 10231; *Malassezia furfur* ATCC 14521.

### 2.5 Hydroxychavicol Analysis

An HP5 - MS capillary column of 30 m length, 0.25 mm inner diameter, and 0.25 µm film layer was used for the analysis. Helium, flowing at 1 mL/min, served as the carrier gas. The temperature was initially set to 40 ºC for 2 min, then raised to 160 ºC at 4 ºC/min and held for 6 min. Finally, it was ramped up at 15 ºC/min to 250 ºC and maintained for an additional 10 min. The sample ignition temperature was 250 ºC, and the detector temperature was 350 ºC. A 20 µL sample of hydroxychavicol was prepared, diluted with Dichloromethane/ Ethyl acetate

(9:1), and then 1 µL of the sample was injected for mass spectrometry testing. The Willey/Chemstation HP library was used to identify the components.

### 2.6 Antimicrobial Activity of *Piper betle* L. extract by agar well diffusion method

All antimicrobial assays were conducted using the disk diffusion method on Mueller Hinton agar, with 6 mm wells punched into the agar plate. The experiment involved the use of betel leaf extract at varying concentrations (5-50 mg/mL). Gentamicin (0.5 mg/mL) was the negative control, while DMSO acted as the positive control. The antimicrobial effect was detected by the appearance of inhibition zones around the disks, with the diameters measured in millimeters.

The antifungal activity of this extract also used the disk diffusion method, this time on Potato Dextrose agar for *Candida albicans* and Dixon agar for *Malassezia* furfur. The positive control was Nystatin (200 µg/mL), and the negative control was DMSO.

## 3. Results and discussion

### 3.1 Extraction of hydroxychavicol from betel leaf extract

This involves the extraction of *Piper betle* L. powder using ethanol as a solvent and the Soxhlet extraction method. The extract from the betel leaf is dark green in color and has a strong, typical betel odor.

Thin layer chromatography results showed that the Dichloromethane/Ethyl acetate: 99/1 solvent system is suitable for separating substances in the betel extract. Hydroxychavicol has a retention factor Rf = 0.33. This outcome is in line with the study by Syahidah et al. (2017).

The betel leaf extract was processed through a chromatographic column using a solvent system of dichloromethane and ethyl acetate (99:1). During the chromatography process, two yellow streaks appeared, as shown in Figure 1. Fractions containing hydroxychavicol were evaluated using thin-layer chromatography to identify those with pure hydroxychavicol. The fraction with pure hydroxychavicol was identified as the one producing a yellow-orange streak against a 1 % Vanillin/$H_2SO_4$ reagent. This was further tested with $FeCl_3$ reagent, where hydroxychavicol would turn dark blue upon spraying.

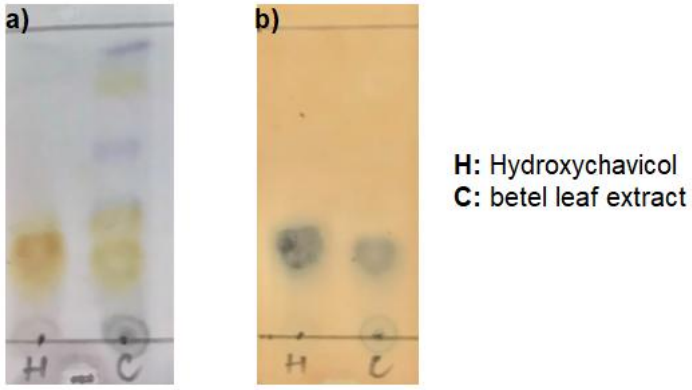


*Figure 1: Thin layer chromatography of hydroxychavicol and betel leaf extract (a) TLC using 1 % Vanillin/$H_2SO_4$ (b) TLC using $FeCl_3$*

### 3.2 Analysis of Hydroxychavicol by GC – MS

The hydroxychavicol obtained from column chromatography fractions is depicted in Table 1 and Figure 2.

*Table 1: Content of Hydroxychavicol and other substances in betel leaf extract*

| Entry | Retention time ($R_t$) | Compounds | Content (%) |
|---|---|---|---|
| 1 | 5.714 | *Cis*-4-Thujanol | 8.774 |
| 2 | 11.943 | Carvacrol | 6.187 |
| 3 | 16.186 | Hydroxychavicol | 74.944 |
| 4 | 22.186 | Bis (ethyl hexyl) sebacate | 8.842 |
| 5 | 23.182 | Diisooctyl adipate | 1.254 |

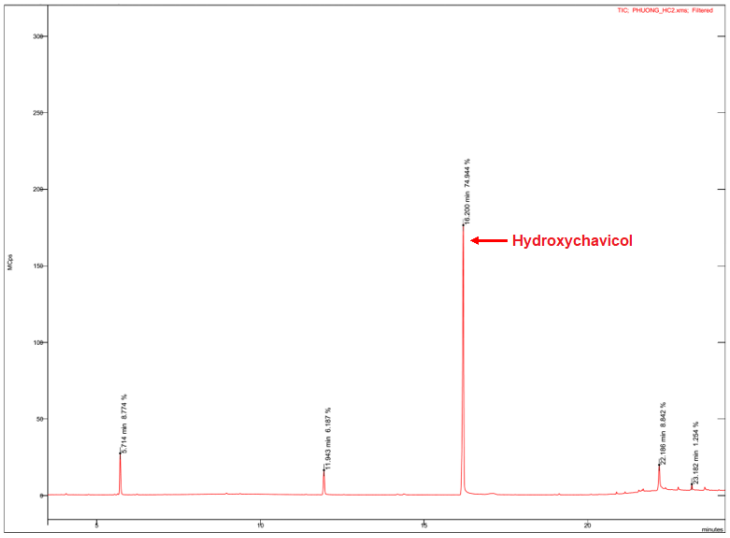


*Figure 2: Gas chromatography-mass spectrometry of hydroxychavicol in betel extraction.*

### 3.3 Investigation into the antibacterial properties of betel leaf extract

This experiment was conducted to evaluate the antibacterial potency of betel leaf extract, a commonly used natural remedy in traditional medicine, against certain bacterial strains. The extract's antibacterial activity was determined through the application of the well-established and globally recognized agar disk diffusion method. In this study, the betel leaf extract was tested at various concentrations including 50 mg/mL, 20 mg/mL, 10 mg/mL, and 5 mg/mL. In the experiments, DMSO, the antibiotic gentamicin (500 µg/mL) served as the negative, positive control. The results of these experiments, which are a testament to the antibacterial potency of betel leaf extract, are depicted graphically in Figure 3 and tabulated in Table 2.

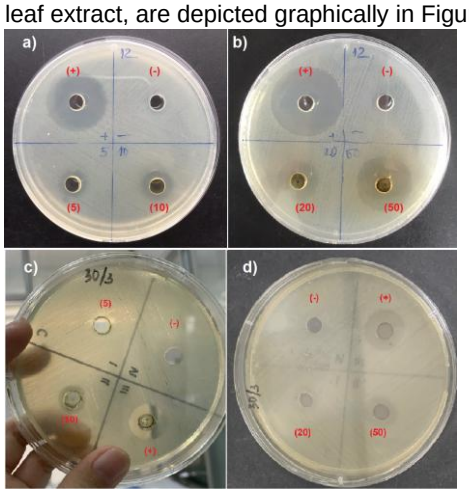


*Figure 3. Inhibition of Streptococcus mitis (a, b) and Streptococcus mutans (c, d) bacteria by betel leaf extract.*

The findings derived from the study are quite revealing. They indicate that the betel leaf extract can inhibit the growth of both *Streptococcus mitis* and *Streptococcus mutans* bacteria (5 - 20 mg/mL). This clearly demonstrates the extract's moderate inhibitory capability against these bacterial strains. Most notably, at a concentration of 50 mg/mL, the extract significantly inhibited the growth of the *Streptococcus mitis* strain. This was evidenced by the formation of an antibacterial ring with a diameter larger than 21 mm, which is an indication of strong antibacterial activity.

In a related study conducted by Singh et al. (2018), they investigated the principal mechanism of Hydroxychavicol's antibacterial activity against *E. coli* bacteria, a common harmful bacterium. Their research suggests that the antibacterial effect of hydroxychavicol, a key component in betel leaf, is due to the induction of oxidative stress within bacterial cells. Reactive oxygen species are produced, leading to the loss of cell

viability [22]. This mechanism could potentially explain the antibacterial activity observed in our study, further validating the traditional use of betel leaf as a natural antibacterial agent.

*Table 2: Diameters of the inhibitory zones for Streptococcus mitis and Streptococcus mutans in both betel leaf extract and Gentamicin (500 µg/mL)*

| Bacterial strains | The concentration of betel leaf extract (mg/ mL) | Inhibition zone of Gentamicin (mm) | Inhibition zone of DMSO (mm) | Inhibition zone of Betel leaf extract (mm) |
|---|---|---|---|---|
| *Streptococcus mitis* | 50 | 26.87 ± 1.58 | – | 21.87 ± 1.64 |
| | 20 | | | 15.60 ± 0.29 |
| | 10 | | | 12.03 ± 0.48 |
| | 5 | | | 11.23 ± 0.33 |
| *Streptococcus mutans* | 50 | 19.45 ± 0.20 | – | 16.87 ± 1.40 |
| | 20 | | | 12.60 ± 0.90 |
| | 10 | | | 8.03 ± 1.18 |
| | 5 | | | – |

Note: ' – ' represents no antibacterial ring appears.; The results represent the average diameter (in mm) of the antibacterial ring, plus or minus the standard deviation (SD).

### 3.4 Antifungal ability of betel leaf extract

The extract's antifungal properties were tested on two strains: *Candida albicans* and *Malassezia*. This examination was carried out at different concentrations of betel leaf extract. The approach used for this experimentation was the disc diffusion method.

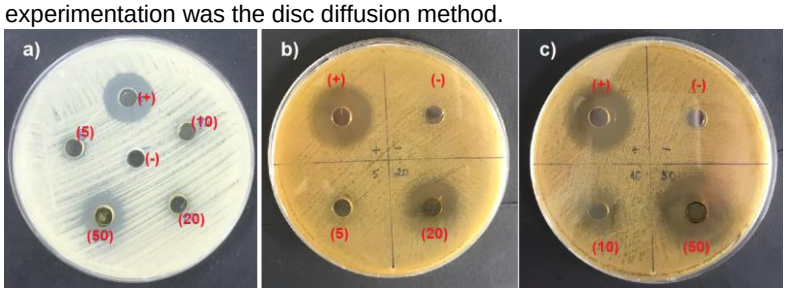


*Figure 4: The inhibition zone of betel leaf extract (5-50 mg/mL) on Candida albicans (a); betel leaf extract (5; 20 mg/mL) on Malassezia furfur (b) and betel leaf extract (10; 50 mg/mL) on Malassezia furfur (c).*

DMSO was used as the negative control in this experiment. On the other hand, nystatin, at a concentration of 200 µg/mL, served as the positive control. These controls were crucial in providing a benchmark for understanding the effectiveness of the betel leaf extract (refer to Figure 4 and Table 3 for more details).

*Table 3: Diameter of inhibition zone for Candida albicans and Malassezia furfur using betel leaf extract and nystatin (200 µg/mL)*

| Fungal strains | The concentration of betel leaf extract (mg/ mL) | Inhibition zone of Gentamicin (mm) | Inhibition zone of DMSO (mm) | Inhibition zone of Betel leaf extract (mm) |
|---|---|---|---|---|
| *Candida albicans* | 50 | 17.83 ± 0.24 | – | 15. 67 ± 0.94 |
| | 20 | | | – |
| | 10 | | | – |
| | 5 | | | – |
| *Malassezia furfur* | 50 | 22.45 ± 0.72 | – | 28.77 ± 0.56 |
| | 20 | | | 21.63 ± 0.79 |
| | 10 | | | 16.03 ± 1.18 |
| | 5 | | | – |

Note: ' – ' represents no antifungal ring appears.; The results represent the average diameter (in mm) of the antifungal ring, plus or minus the standard deviation (SD).

The results gathered from this testing painted a promising picture. The extract showcased the capability to inhibit the growth of both *Candida albicans* and *Malassezia furfur* species. This implies that the betel leaf extract could be a potential candidate for treating infections caused by these organisms.

However, it was observed that under the given experimental conditions, the betel leaf extract only inhibited *C. albicans* at a concentration of 50 mg/mL. In contrast, the extract started inhibiting *M. furfur* from a lower concentration itself, starting from 10 mg/mL. This indicates a higher sensitivity of *M. furfur* towards the betel leaf extract compared to *C. albicans*.Further supporting this observation was the measurement of the antibacterial ring's diameter. For the extract solution of 50 mg/mL, the diameter was approximately 15.67 mm for *C. albicans*. In contrast, for *M. furfur*, the diameter was significantly larger, approximately 28.77 mm. This larger diameter for *M. furfur* suggests that it is more sensitive to the betel leaf extract compared to *C. albicans*.

## 4. Conclusions

This comprehensive study delved into identifying the optimal conditions necessary for the extraction of high concentrations of polyphenols from the betel plant, a plant known for its significant health benefits. The findings indicated potent antibacterial and antifungal properties of the polyphenols. This suggests that betel leaves, when processed appropriately, could be used in the development and production of oral care products. These products could effectively prevent tooth decay, a common ailment affecting a large portion of the population. Additionally, the antifungal properties could contribute to the formulation of shampoos designed to maintain scalp health. By inhibiting fungal growth, these shampoos could potentially prevent the occurrence of dandruff, promoting overall hair and scalp health.

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