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Research Article

Assessment of phenolic and flavonoid contents, tannins, and antifungal potential of heat-assisted aqueous *Piper betle* leaf extract

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Abstract

Piper betle leaf (betel leaf) has been traditionally used in Southeast Asian medicine to treat ailments such as sore throat, skin abscesses, and digestive disorders. Modern research has demonstrated that the leaf contains a wide range of bioactive compounds, including phenolic acids and essential oils such as eugenol, chavicol, and chavibetol, which possess notable antibacterial, antifungal, anti-inflammatory, antioxidant, and potential anticancer properties. This study evaluated the total phenolic content, flavonoid content, the tannin presence of the aqueous extract obtained from fresh young *P. betle* leaf using a heat-assisted extraction method as well as the antifungal efficacy of that extract. The antifungal activity was evaluated against two opportunistic yeast strains: *Candida albicans*, a representative of spoilage yeasts in food and dairy products, and *Candida auris*, a multidrug-resistant species. Antifungal activity was assessed in-vitro using the agar well diffusion method, and the extract exhibited inhibition zones diameter ranging from 1.37 ± 0.06 cm to 2.23 ± 0.06 cm, indicating clear growth suppression. The minimum inhibitory concentration (MIC) of the extract was determined to be 32 mg dry weight equivalent per mL of extract for *C. albicans* and 64 mg/mL for *C. auris*. The total phenolics and flavonoid content were 131.06 ± 2.92 µg GAE/mg and 8.48 ± 0.19 µg QUE/mg dry weight basis, respectively. Tannin was also found in betle leaves extract, indicated by the presence of a brownish green color when reacted with $FeCl_3$. These findings suggested that aqueous extracts of *P. betle* leaves offered promising antifungal properties against both foodborne and clinically relevant yeasts. The use of water as a solvent not only ensured safety and compatibility with food systems but also supported the development of sustainable, plant-based antifungal agents. Overall, this study contributes to the growing body of evidence supporting the application of traditional medicinal plants in modern food preservation, with *P. betle* offering a viable natural alternative to synthetic antifungal compounds.

Keywords: *Antifungal, phenolic, flavonoid, tannin, Piper betle aqueous extract*

1. INTRODUCTION

Piper betle L. (betel leaf) is a traditional medicinal plant widely used in Southeast Asia for its diverse therapeutic applications. Phytochemical investigations have identified key bioactive constituents such as hydroxychavicol, eugenol, and chavibetol as well as significant levels of total phenolic content (TPC) and total flavonoid content (TFC), which are closely associated with antifungal activity. According to Guilherme R. Teodoro et al (2015) phenolic acids-including gallic, caffeic, and cinnamic acids exert antifungal effects by increasing membrane permeability, disrupting mitochondrial functions, and inhibiting essential enzymes involved

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in fungal metabolism and biofilm formation [1]. In parallel, flavonoids exert antifungal effects through complementary mechanisms. As reviewed by Mohammed Saleh Al Aboody et al (2020) [2], flavonoids impair fungal membrane integrity, inhibit the biosynthesis of ergosterol and cell wall polysaccharides such as β-glucans and chitin, disrupt mitochondrial respiration, and promote intracellular accumulation of reactive oxygen species (ROS). This oxidative stress contributes to protein and lipid damage, mitochondrial collapse, and ultimately apoptosis. Furthermore, certain flavonoids have been shown to inhibit biofilm formation, reducing the virulence of *Candida albicans*. These compounds are associated with a broad spectrum of biological activities including antibacterial, antifungal, anti-inflammatory, and antioxidant effects [3].

Regarding food contamination, *Candida albicans* has been occasionally isolated from goat milk, cheese, and other dairy matrices [4] whereas *Candida auris*, although primarily linked to clinical outbreaks, has recently been detected on the surface of stored apples [5]. Their occurrence in food-related environments highlights the need to evaluate safe, plant-derived antifungal agents under extraction conditions that reflect practical and accessible applications. Notably, no prior study has examined the antifungal activity of aqueous *P. betle* extract against *C. auris*, presenting an opportunity to expand current knowledge. Recent research has explored the extraction of *P. betle* using a variety of organic solvents such as ethanol, methanol, and acetone, which have been shown to yield high levels of phytochemicals and enhanced antifungal efficacy [6]. However, in the present study, aqueous extraction was selected as the method of choice due to its eco-friendly, non-toxic, and food-safe nature. Water, being a green solvent, offers significant advantages for applications in food preservation where safety and environmental sustainability are paramount [7]. This approach aligns with the growing interest in natural, safe, and biodegradable antimicrobial agents for use in food systems. In previous studies, the aqueous extraction of *P.betle* leaves was typically performed at relatively low temperatures for extended periods, often using specialized equipment [8]. However, it remains unclear whether *P. betle* retains its antifungal properties when extracted with hot water using simple, readily available tools. Therefore, this study aimed to determine the phytochemical profile (TPC, TFC, and tannins) of heat-assisted aqueous *P. betle* extract and to evaluate its in vitro antifungal activity against *Candida albicans* and *Candida auris*.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Betel leaves (*Piper betle* L.) were collected in April 2025 from Quang Trach, Quang Xuong district, Thanh Hoa province, Vietnam. Only mature leaves with petioles removed were selected for extraction. Young apical leaves were excluded. After collection, the leaves were washed thoroughly with clean water to remove dust and surface contaminants, drained to eliminate excess moisture, and used immediately for extraction.

The standard strains of *Candida* used in this experiment are *Candida albicans* ATCC 10231 and *Candida auris* B11222-AR384 from the collection of Applied Microorganism Laboratory. Both strains were maintained on Yeast-Peptone-Dextrose (YPD-HiMedia) agar dish. Other media used were YPD broth (yeast extract - 10 g/L, peptone - 20 g/L, glucose - 20 g/L and Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/mL methylene blue (Cas 28983-56-4).

2.2. Plant extraction

Bioactive compounds in *P. betle* leaves were extracted using water, boiling at water boiling point referred to Ngo Thi Mai Vi et al [9]. The cleaned leaves were weighed to 100 g and cut into small pieces about 1x1cm before added into a 500 mL beaker with 200 mL distilled water. At this stage, betel leaf was heated on an infrared cooker at 1000W to 100°C and boiled in water for 45 min. During extraction, the beaker was covered with aluminum foil and stirred occasionally using a clean glass rod to enhance efficiency while minimizing heat loss. The residue was removed by a sieve, and the liquid was filtered through filter paper. Then the piper betel leaf extract (BLE) was concentrated on the infrared cooker at 1000W with the aluminum cover until 20 mL left. The concentrated extract is considered as the stock solution and stored at 4°C for further analysis.

On the other hand, the fresh leaves were dried in a drying cabinet at 70°C until constant mass and recorded the dry weight.

2.3. Analysis of bioactive components in extract

2.3.1. Total phenolics content

The total phenolics of the extracts were determined using the Folin-Ciocalteu method described by Taukoorah et al [10] with minor adjustments. The plant extract was diluted with double-distilled water 1000 times before the test. The test sample (100 µL) was reacted with Folin-Ciocalteu reagent (500 µL) in a test tube. After 4 min, 400 µL of sodium carbonate 7.5% w/v was added. The mixture was allowed to react for 30 min in the dark at room temperature. The absorbance was measured at 760 nm by a spectrophotometer against a blank that did not contain plant extract. The experiment was conducted in triplicate, and gallic acid was prepared at various concentrations ranging from 20-200 µg/mL to construct the standard calibration curve. (**Figure 1**). The total phenolic content was expressed as µg gallic acid equivalent per mg of dry weight (µg GAE/mg dw).

2.3.2. Determination of flavonoid content

The flavonoid content was evaluated following the aluminum chloride colorimetric method performed by Tripti Singh *et al* (2024) [11] with some modifications. Quercetin (10-160 µL/mL) and BLE were diluted separately in ethanol 50%. One microliter of the diluted extract (100 µL) was mixed with 300 µL of ethanol, 20 µL of $AlCl_3$ 10% (w/v), 20 µL of CH_3COONa 1 M, and 560 µL of double distilled water. The absorbances at 415 nm were read after 30 min compared to a blank consisting of 100 µL double distilled water instead of plant extract. The procedure was repeated three times. The standard curve was conducted using quercetin diluted in 50% ethanol. (**Figure 2**). The total phenolic content was expressed as µg quercetin equivalent per mg of dry weight (µg QUE/mg dw).

2.3.3. Detection of tannin

Tannins were qualitatively detected based on their ability to form colored complexes with ferric ions (Fe^{3+}). Hydrolyzable tannins typically produced a bluish-black precipitate, while condensed tannins yielded a dark green coloration. For the qualitative assay, 2–3 drops of ferric chloride solution were added to 1 mL of the plant extract, visual evaluation was used to assess the color change. The experiment was performed three times independently [12].

2.4. Evaluation of antifungal activity

2.4.1. Determination of antifungal activity by well diffusion method

Initially, pure fungal strains were cultured on YPD agar plates and inspected to ensure the absence of contamination. The antifungal activity was appraised using the procedure based on the methods described by S. Magaldi *et al*. [13] and the CLSI M44-A standard [14]. A loop of each strain was incubated overnight in YPD broth at 35 ± 2°C, and 150 rpm. Then the inoculum suspension was adjusted using 0.85% sodium chloride to obtain the turbidity of 0.5 McFarland (relatively 1–5 × 10^6 CFU/mL [14]).

In the following step, a sterile cotton swab was dipped into the suspension, pressed against the tube wall several times to remove excess liquid. Afterward, the swab was used to spread the yeast on the whole prepared medium agar plates. There were 3 wells cut off in a plate, each 5 mm in diameter, then 15, 20, 25 µL of BLE were injected separately into each well. Sterile 0.85% sodium chloride was used as negative control. All the plates were incubated in an incubator at 35 ± 2°C for 24h. The diameter of the inhibition zone was measured using a caliper.

2.4.2. Determination of Minimum Inhibitory Concentration (MIC) and spot on agar petri dish

The minimum inhibitory concentrations (MICs) of betel leaf extract (BLE) against *Candida auris* and *Candida albicans* were determined using a broth microdilution assay adapted from CLSI M27-A4 (2022) [15], with YPD broth were used as the culture medium instead of RPMI 1640 medium.

Betel leaf extract as an antifungal agent was serially diluted in distilled water using a two-fold dilution scheme in a 96-well plate. Control wells included: (i) a negative control containing YPD medium only, (ii) a positive growth control with fungal inoculum but no BLE, and (iii) an extract-only control (BLE + YPD, no fungal inoculum) to account for background color interference due to the natural pigmentation of the extract. The plates were incubated at 35 ± 2°C for 24 h.

After incubation, 3 µL from each well was spotted onto YPD agar plates and incubated for an additional 24 h at 35 ± 2°C to verify fungal viability and confirm MIC results via direct colony observation. The MIC was defined as the lowest concentration of BLE that resulted in complete or significant inhibition of visible fungal growth, both in the broth wells and on the agar plates, relative to the positive control. All experiments were conducted in triplicate to ensure reproducibility.

3. RESULTS AND DISCUSSION

3.1. Plant extraction

The aqueous extract obtained from fresh betel leaf displayed a dark brown color with a pungent and slightly spicy scent. The initial 100 g fresh leaves yield 20.78 g of dry leaves. Subsequently, 20 mL betel leaf extract included that amount of dry material, equivalent to a 1039 mg dw/mL solution.

3.2. Analysis of bioactive components in extract

3.2.1. Total phenolics content

The total phenolic concentration recorded was 131.06 ± 2.92 µgGAE/mg dw, which is consistent with the range between 105 mg GAE/g and 192 mg GAE/g reported by Tripti Singh (2024) [11]. Moreover, U. Taukoorah (2016) also claimed that the number of phenolic compounds in betel extract was 128.89 ± 0.36 µg GAE/mg in aqueous extract while the highest value was 186.26 ± 0.73 µg with acetone as the extract solvent [10].

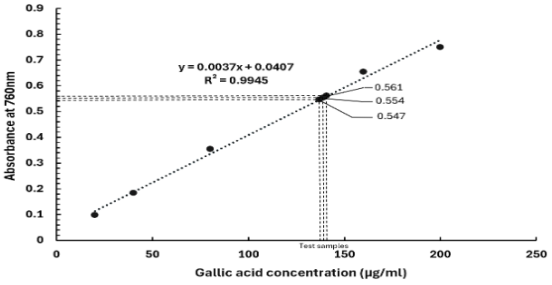

Figure 1. *Standard curve of total phenolic content with measured value of the 1000 times diluted samples*

3.2.2. Determination of flavonoid content

The total flavonoid content of the BLE was experimented using the aluminum chloride colorimetric method. The results were expressed as micrograms of quercetin equivalent per milligram of dry betel leaves (µg QUE/mg). The flavonoid content was found to be 8.41 ± 0.11 µg QUE/mg dw. This indicates a relatively high concentration of flavonoid level in the extract, which may contribute to its observed antifungal activity. The result of this research higher than total flavonoid of Hoang Thuy Duong et al (2021) [8] research on aqueous betel leaf extract (5.287 mg QE/g). Otherwise, the extract of betel leaf with other solvents indicated higher concentration of flavonoid (29.58 mg/g in petroleum ether; 46.08 mg/g in acetone, and 31.08 mg/g in methanol) [16] which may be attributed to differences in solvent polarity and extraction efficiency. Organic solvents such as ethanol or methanol are known to more effectively solubilize flavonoid compounds compared to water, leading to higher extraction yields. These solvents can penetrate plant cell walls more efficiently and dissolve both polar and moderately non-polar flavonoids, resulting in increased recovery of these bioactive compounds.

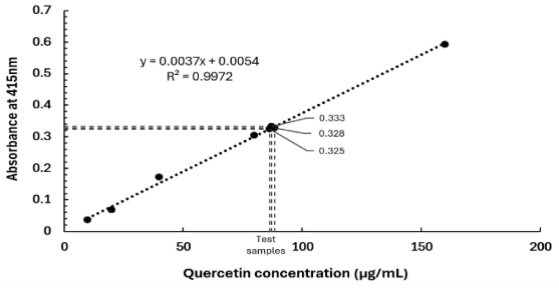

Figure 2. *Standard curve of flavonoid content with measured value of the 100 times diluted samples*

3.2.3. Detection of tannin

After the addition of ferric chloride solution to the extract, the color of the test tube changed to brownish green, indicating the presence of tannin in the extract (**Figure 3**). This result suggests that betel leaves contain polyphenolic compounds capable of forming complexes with ferric ions, a typical property of tannins. As tannins are reported for their antimicrobial and antifungal properties [17], their detection in 100-time diluted BLE may partially explain the observed antifungal activity against both *Candida* species tested.

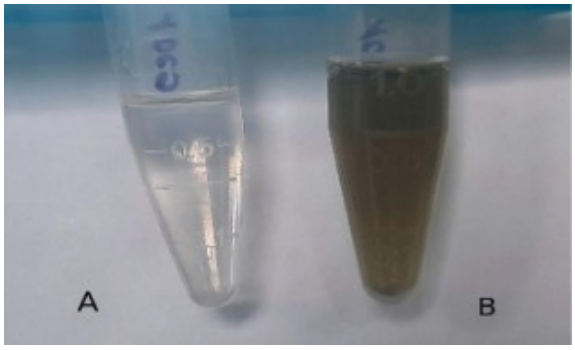

Figure 3. *Qualitative test for tannins in diluted betel leaf extract (1:100)*
A: Betel leaf extract diluted 100 times, without $FeCl_3$;
B: Betel leaf extract diluted 100 times, with $FeCl_3$ added.

This finding is in line with previous reports on the phytochemical composition of betel leaves. Deka et al (2022) [18] have also reported the existence of tannin in betel leaf ethanolic extract. U. Taukoorah, (2016) conducted the quantitative assessment of tannin in extracts of *P. betle* using catechin as standard reagent and revealed the amount of tannin in aqueous extract was 7.22 ± 0.48 µg catechin equivalent/mg [10].

3.3. Evaluation of antifungal activity

3.3.1. Determination of antifungal activity by well diffusion method

The antifungal effectiveness of aqueous betel leaf extract was evaluated by agar well-diffusion assay. The diameter of the inhibition zones formed after 24 h of incubation on both yeast strains was recorded, summarized in **Table1** and presented as a column chart in **Figure 4**.

Table 1. *Inhibition zone diameters of heat-assisted aqueous Piper betle leaf extract against Candida spp.*

Volume of betel leaves extract	**Mean diameter of inhibition zone (cm)**	
	C. auris B11222-AR384	*C. albicans* ATCC 10231
15 µL	1.37 ± 0.06	1.43 ± 0.06
20 µL	1.73 ± 0.06	1.76 ± 0.06
25 µL	2.07 ± 0.06	2.23 ± 0.06
Control (0µL)	0	0

The results in **Table 1** illustrates that as the volume of BLE increased from 15 µL to 25 µL, the diameter of inhibition zones expanded correspondingly, and there seems to be no significant difference between the two strains ($p > 0.05$) (**Figure 4**). In particular, the largest zone was obtained at 25µL wells with 2.07 ± 0.06 cm on *C. auris* and 2.23 ± 0.06cm on *C. albicans* (**Table 1**). In addition, the smallest diameters of antifungal activity zone were in 15µL wells, where the number recorded was 1.37 ± 0.06cm and 1.43 ± 0.06cm against *C. auris* and *C. albicans,* respectively. The slight difference in inhibition zones may be due to the amount of dry material equivalent in each well, relating to the volume of betel leaf extract added.

The inhibition zone diameters varied depending on the fungal strain and the volume of extract applied. For *C. albicans*, the inhibition zones ranged from 1.43 ± 0.06 cm to 2.23 ± 0.06 cm. Otherwise, *C. auris* exhibited clearance zones with a bit decrease, ranging from 1.37 ± 0.06 cm to 2.07 ± 0.06 cm (**Table 1**).

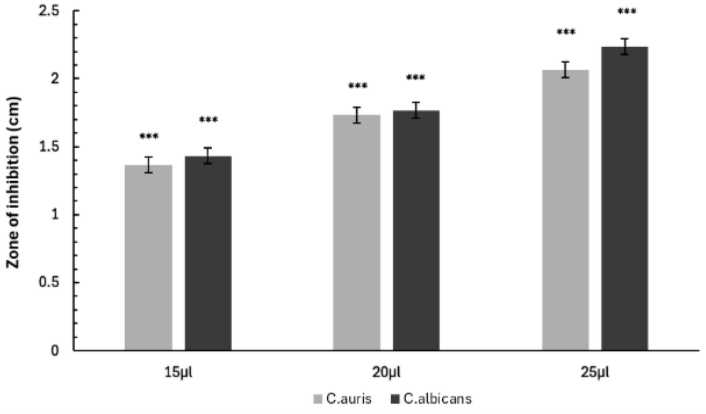

Figure 4. *Diameter of inhibition zones of betel leaves extract against C. albicans ATCC 10231 and C. auris B11222-AR384 obtained by well-diffusion method. *** ($p < 0.001$)*

Compared to the negative control (sterile 0.85% sodium chloride) which showed no inhibition zone (0 cm) for either fungal strain (**Figure 5**), all volume of BLE presented statistically significant inhibition ($p < 0.001$), indicating that the antifungal effect was directly attributable to BLE.

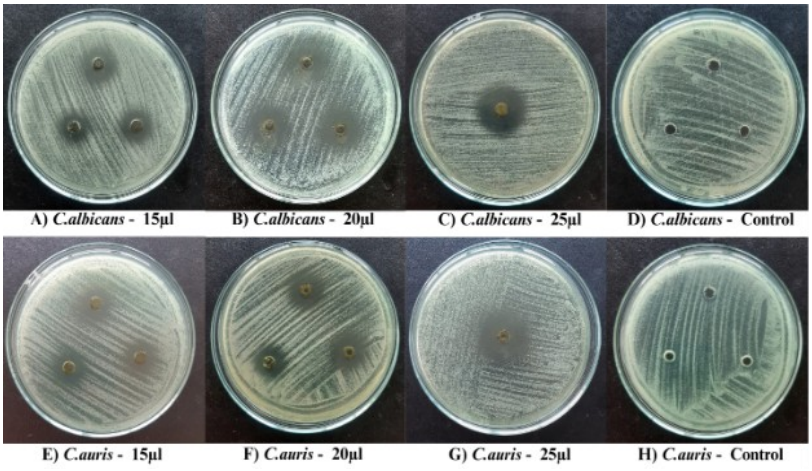

Figure 5*. Inhibition zone of betel leaf extract against C. auris and C. albicans after 24h*

The dose-dependent increase in inhibition zone diameter supports the hypothesis that the extract's antifungal efficacy is dependent on extraction ratio. Overall, the data demonstrated that the aqueous extract of fresh *Piper betle* leaves possessed measurable antifungal activity against both tested *Candida* species, with slightly higher potency observed against *C. albicans*.

Hoang Thuy Duong *et al.* (2021) reported the diameter of inhibition zone was 0.33 mm against *C. albicans* using water-extracted betle leaves via ultrasound-assisted extraction [8] and Sivareddy *et al* (2019) observed inhibition zone diameter ranging from 5 to 22 mm against *C. albicans* when using ethanol extract of mature *Piper betel* leaves [6]. About *C. auris*, there aren't any articles which have investigated the growth inhibition activity of betle leaf extract. These findings in this article revealed that betle leaf extract still has antifungal activity even when extracted at high temperature.

3.3.2. Determination of Minimum Inhibitory Concentration (MIC) and spot on agar petri dish

Following 24 h of incubation at 35 ± 2°C on 96 - well plate, two strains were assessed both visually in the 96-well microtiter plate and via spot-inoculation of 3 µL aliquots from each well onto YPD agar. **Figure 6** and **Figure 7** indicated that the aqueous extract of fresh *Piper betle* leaves exhibited notable antifungal activity against *Candida albican* and *Candida auris*.

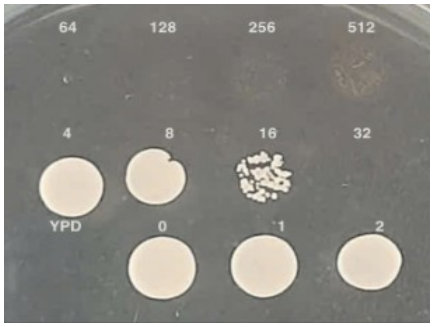

Figure 6. *MIC values via the growth of Candida albicans ATCC 10231 colonies with different concentrations of betel leaf extract*

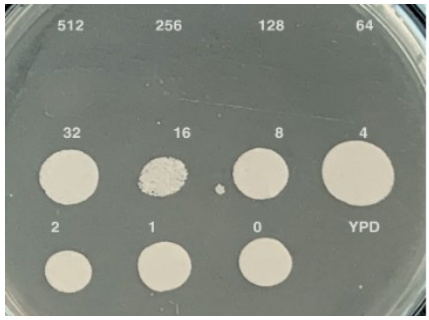

Figure 7. *MIC values via the growth of Candida auris B11222-AR384 colonies with different concentrations of betel leaf extract*

As illustrated in **Figure 6** and **Figure 7**, the MIC value was determined and presented in **Table 2**. The dry weight was used to calculate the unit for the MIC test since it is a more popular unit when comparing the results indirectly with other articles. The initial 100g fresh leaves gave 20.78 g dry weight and 20 mL extract. Thus, the extract yield was 20% and 1 mL of extract corresponded approximately to 1.039 g dry weight equivalent.

Table 2*. The MIC value of betel leaves extract against C. auris and C. albicans*

Strain	**MIC value** (mg dwe/mL)
Candida albicans ATCC 10321	32
Candida auris B1122-AR384	64

Himratul-Aznita W.H. et al investigated antifungal activity of a *Piper betle* crude aqueous extract prepared through a 5-6 h extraction process followed by freeze-drying, against oral *Candida* species. They reported an MIC value of 12.5 mg/mL against *C. albicans* [19]. Similarly, Le Van Kim Anh *et al* examine d refined betel leaf extract using water and ethanol 96% and found an MIC value of 2 mg/mL [20]. Compared to these findings, the present study recorded higher MIC values. This difference could be attributed to variations in extraction solvent, temperature, and duration, which strongly influence the yield and stability of phenolic and flavonoid compounds. Nonetheless, the results confirm that the heat-assisted aqueous extract of betel leaves retains considerable antifungal potential even under simple extraction conditions without the use of organic solvents, though prolonged extraction time may be required to enhance its efficacy.

To the best of our knowledge, this is the first study to report the inhibitory activity of *Piper betel* aqueous extract against *Candida auris*. Given that *C. albicans* and *C. auris* have been detected in dairy products and stored fruits, these findings suggest that this simple, eco-friendly extract of *Piper betle* may be applicable in food preservation. Although its MIC values are higher than those of solvent or low-heat extracts, the extract still shows measurable antifungal activity after high-temperature processing, indicating sufficient stability of key bioactives. This study provides evidences supporting the use of heat-assisted aqueous extract of *P.betle* leaves as a natural antifungal agent in food shelf-life extension and represents its inhibitory activity against C. auris using an aqueous preparation.

4. CONCLUSION

This study demonstrated that the heat-assisted aqueous extract of fresh *Piper betle* leaves possessed significant antifungal activity against both Candida albicans ATCC 10231 and *Candida auris* B11222-AR384. The extract exhibited clear inhibition zones in the agar well diffusion assay and showed notable MIC values. It retained significant levels of phenolic compounds (131.06 ± 2.92 µg GAE/mg), flavonoids (8.41 ± 0.11 µg QUE/mg), as well as the presence of tannins, which are likely contributors to the antifungal effects observed. The use of water as an extraction solvent ensures safety, environmental compatibility, and practical applicability in food systems. The findings supported the potential of *P. betle* aqueous extract as a natural antifungal agent for food preservation, especially in controlling yeast contamination on food surfaces. In future work, detailed chemical profiling of the more concentrated extract will be conducted to identify and quantify specific antifungal constituents. This will provide a scientific foundation for the formulation of a commercial antifungal product derived from *P. betle*, aimed at replacing or reducing synthetic preservatives in the food industry. With its bioactivity, safety, and sustainability, *P. betle* extract represents a promising candidate in the development of plant-based solutions for enhancing food shelf life and safety

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