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Antibacterial Activity of Fresh Aqueous Extracts of Guava (*Psidium guajava*) Leaves and Fruits against *Edwardsiella ictaluri* and *Flavobacterium columnare*

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Abstract

This study evaluated the antibacterial and bactericidal activities of fresh aqueous extracts prepared from guava (*Psidium guajava*) leaves and fruits against two important freshwater fish pathogens, *Edwardsiella ictaluri* and *Flavobacterium columnare*. For each bacterial species, three strains were revived from the bacterial collection maintained at the Faculty of Fisheries, Vietnam National University of Agriculture. They were confirmed by colony morphology, biochemical characteristics, and species-specific polymerase chain reaction (PCR). Fresh extracts were obtained by mechanically pressing 100g of guava leaves or fruits with 200mL of distilled water, yielding 163mL and 186mL of extract, respectively. Antibacterial activity was assessed using the agar well diffusion method. The inhibition zones produced by the fruit extract against *E. ictaluri* and *F. columnare* measured 21.4mm and 25.8mm, respectively, and were significantly smaller than those produced by the leaf extract (26.7mm and 30.8mm, respectively). Both extracts exhibited strong antibacterial activity, comparable to that of oxytetracycline (30µg). The minimum bactericidal concentrations (MBCs) of the fruit extract against *E. ictaluri* and *F. columnare* were 1:8 (12.5%) and 1:32 (3.13%), respectively. In contrast, the leaf extract showed bactericidal activity at lower concentrations, with MBCs of 1:16 (6.25%) for *E. ictaluri* and 1:64 (1.57%) for *F. columnare*. Overall, these findings demonstrate that fresh guava leaf extract, in particular, possesses strong antibacterial and bactericidal activities and has considerable potential for controlling bacterial diseases caused by *E. ictaluri* and *F. columnare* in freshwater aquaculture.

Keywords

Fresh aqueous extract of guava; antibacterial activity; agar diffusion assay; fish disease control; antibiotic alternative

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Introduction

In recent years, aquaculture, particularly freshwater fish farming, has developed rapidly and has become an important sector of agricultural production in Vietnam. However, the intensification of farming practices, including increased stocking densities and production scales, has been accompanied by growing incidences of infectious diseases, especially those caused by bacterial pathogens. Among them, *Edwardsiella ictaluri* and *Flavobacterium columnare* are recognized as two of the most common and destructive bacterial agents affecting freshwater fish, often causing high cumulative mortality rates leading to substantial economic losses for farmers worldwide (Declercq *et al.*, 2013; Dong *et al.*, 2015; Nhinh *et al.*, 2022). The economic losses associated with Edwardsiellosis in the United States have been estimated at up to USD 45.9 million annually (Kumar *et al*., 2024), whereas Columnaris disease is responsible for estimated annual losses of approximately USD 30 million in the aquaculture industry (Declercq *et al*., 2013).

Edwardsiella ictaluri has long been known as the causative agent of enteric septicemia of catfish (ESC) (Newton *et al.*, 1989). More recently, this pathogen has also been reported to cause severe disease outbreaks in red tilapia and Nile tilapia, with high virulence and cumulative mortality reaching up to 65%, and spreading across multiple farming regions, highlighting the serious threat posed by *E. ictaluri* to freshwater aquaculture (Dong *et al.*, 2019; Nhinh *et al.*, 2022). Meanwhile, *F. columnare* has been documented as a major pathogen of various freshwater fish species, including grass carp, tilapia, and channel catfish is capable of causing extremely high mortality, in some cases approaching 40% (Lu *et al.*, 2021; Elgendy *et al.*, 2022; LaFrentz *et al*., 2024). In addition, Declercq *et al*. (2013) reported that *F. columnare* exhibited resistance to several commonly used antibiotics, including enrofloxacin, flumequine, and oxolinic acid. Similarly, Malick *et al*. (2022) documented resistance to various β-lactams and other antimicrobial agents, such as cefuroxime, ampicillin, penicillin-G, cefalexin, and trimethoprim. The emergence and spread of antimicrobial resistance in *F. columnare* represent major challenges for effective disease management in aquaculture. According to Pham *et al.* (2015) and Ström *et al.* (2019), approximately 72.3-84% of surveyed farms in Vietnam used at least one antibiotic (ampicillin, amoxicillin, trimethoprim, erythromycin, florfenicol, doxycycline, oxytetracycline, enrofloxacin, ciprofloxacin, sulfamethoxazole) at some stage of the production cycle. Such extensive use of antibiotics has contributed to the development of antimicrobial resistance, environmental contamination, and antibiotic residues in aquatic products (Pérez-Sánchez *et al.*, 2018), thereby threatening consumer health (Milijasevic *et al.*, 2024) and negatively affecting the export value of Vietnamese aquaculture products.

In recent years, the use of herbal products as alternatives to antibiotics for controlling bacterial diseases in aquaculture has attracted increasing attention due to their potential efficacy, safety, and environmental friendliness. Guava (*Psidium guajava*), a widely distributed and easily cultivated plant in Vietnam (Jiménez-Escrig *et al.*, 2001) has been reported to contain various bioactive compounds beneficial to human and animal health. According to Kumar *et al.* (2021), guava leaves contain at least 13 bioactive compounds, including phenolic acids, flavonoids, terpenoids, glycosides, and saponins, many of which exhibit antibacterial activity against several bacterial species, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus faecalis*, *Staphylococcus aureus*, and *Bacillus subtilis*. In Vietnam, preliminary studies have demonstrated the antibacterial potential of guava leaf extracts against *Salmonella typhi* and *Pseudomonas aeruginosa* (Linh *et al.*, 2019). Moreover, several initial studies in veterinary and aquaculture contexts have suggested that guava leaf extracts can inhibit the growth of fish pathogenic bacteria; however, the number of studies remains limited and lacks systematic investigations.

Given the increasing impact of *E. ictaluri* and *F. columnare* on freshwater aquaculture in Given

the increasing impact of *E. ictaluri* and *F. columnare* on freshwater aquaculture in Vietnam, a comprehensive evaluation of the antibacterial activity of guava extracts against these two important pathogens is both necessary and practically relevant. Therefore, the present study aimed to investigate the antibacterial and bactericidal activities of fresh extracts prepared from guava leaves and fruits against *E. ictaluri* and *F. columnare* under laboratory conditions. The results of this study are expected to provide a scientific basis for developing plant-based products as alternatives or complements to antibiotics, thereby contributing to safer, more sustainable, and environmentally friendly disease management strategies in freshwater aquaculture.

Materials and Methods

Study materials

Young guava (*Psidium guajava*) leaves and immature fruits (whole fruits with diameters of 2-3cm) were collected from trees located at the Faculty of Fisheries, Vietnam National University of Agriculture. The plant materials were washed, air-dried, and mechanically processed to obtain fresh extracts for antibacterial assays. In addition, this study utilized three strains of each bacterial species, *Edwardsiella ictaluri* and *Flavobacterium columnare*, which were isolated from four commonly cultured freshwater fish species and collected across five provinces in Vietnam (**Table 1**), in order to diversify the sources of pathogenic bacteria.

Experimental methods

Bacterial preparation

Three strains of each bacterial species, *E. ictaluri* and *F. columnare*, previously isolated and preserved at the Faculty of Fisheries, Vietnam National University of Agriculture (**Table 1**), were selected for use in this study. The *E. ictaluri* strains were revived and cultured on tryptic soy agar (TSA), whereas the *F. columnare* strains were cultured on tryptone yeast extract salts (TYES) agar. The inoculated plates were incubated at 28°C for 24-48h.

Baterial identification

After revival, bacterial colonies displaying morphological features characteristics of *E. ictaluri* and *F. columnare*, as described by Nhinh *et al.* (2022) and Dong *et al.* (2015), respectively, were subjected to Gram staining to examine cell morphology and motility. Colonies that met the reference characteristics were subsequently purified by repeated subculturing. The purified isolates were further identified based on colony morphology and biochemical profiles, and their identities were conclusively confirmed by amplification of species-specific gene fragments using polymerase chain reaction (PCR).

(i) Identification based on morphology and biochemical characteristics

The biochemical characteristics of the *E. ictaluri* strains were examined using the oxidase and catalase tests following Crumlish *et al.* (2002) and the API 20E system according to the manufacturer's instructions. Results were read after 24h and interpreted using the API 20E profile index or APIWEB for preliminary identification at the genus/species level. For *F. columnare*, the catalase and oxidase tests, Congo red absorption, and the flexirubin pigment test using a 20% KOH solution were performed as described by Bernardet *et al.* (2002). The morphological and biochemical characteristics were compared with those of the *E. ictaluri* reference strains (Dong *et al.*, 2019) and *F. columnare* ATCC23463.

(ii) PCR confirmation

The bacterial strains were subjected to DNA extraction using the InstaGen Matrix kit (Bio-Rad, USA) according to the manufacturer's instructions. The species-specific primer pairs listed in **Table 2** were used for PCR amplification. Each PCR reaction mixture (20µL) contained 10µL GoTaq Green Master Mix (Promega, UK), 1.5µL of each forward and reverse primer (10µM), 3µL of bacterial DNA template, and 4µL of nuclease-free water. Amplification was performed using an Eppendorf Mastercycler (Hamburg, Germany) under the following conditions: initial denaturation at 95°C for 2min; 35 cycles of

Table 1. Information on bacterial strains used in this study

Bacterial species	Strain Code	Host species	Sampling location	Sampling time
F. columnare	Fc-CC-HN	Common carp	Ha Nam	2021
	Fc-RP-HB	Nile tilapia	Hoa Binh	2021
	Fc-TC-HN	Grass carp	Hanoi	2025
E. ictaluri	Ed-RP-HB-T15	Nile tilapia	Hoa Binh	2021
	Ed-CL-HD	Channel catfish	Hai Duong	2022
	Ed-CL-NĐ	Channel catfish	Nam Dinh	2022

Table 2. Primer pairs used for PCR identification of *Edwardsiella ictaluri* and *Flavobacterium columnare*

Bacterial species	Target gene	Primer sequence (5'→3')	Product size (bp)	References
E. ictaluri	fimbrial gene	F: GTAGCAGGGAGAAAGCTTGC R: GAACGCTATTAACGCTCACACC	470	Sakai *et al.* (2009)
F. columnare	ISR	F: TGCGGCTGGATCACCTCCTTTCTAGAGACA R: TAATYRCTAAAGATGTTCTTTCTACTTGTTTG	470	Welker *et al.* (2005)

denaturation at 95°C for 60s, annealing at 55°C for 60 s, and extension at 72°C for 60s; followed by a final extension at 72°C for 10min. DNA of the *E. ictaluri* reference strains (Dong *et al.*, 2019) and *F. columnare* ATCC23463 were used as positive controls for *E. ictaluri* and *F. columnare*, respectively, and nuclease-free water was used as a negative control. PCR products were separated by electrophoresis on 1.3% agarose gels in TBE buffer using a 100-bp DNA ladder. Electrophoresis was performed at 100V and 100mA for 30min, and gels were visualized using a gel documentation system (Analytik Jena, USA).

Preparation of fresh guava leaf and fruit extracts

Fresh extracts of the guava leaves and fruits were prepared based on the method described by Hoai *et al.* (2025), with minor modifications. Guava leaves at the mature but non-senescent stage, free from visible damage, pests, or fungal infection, and immature, intact guava fruits were collected. After washing and air-drying, the plant materials were weighed, mixed with distilled water, and processed using a screw press oil extractor (MISHIO MK-39). The masses of plant materials, volumes of distilled water added, and volumes of extracts obtained are summarized in **Table 3**. The ratios of plant material to water were selected to ensure homogeneous processing and to obtain the most concentrated fresh extracts possible. The extracts were dispensed into sterile 50mL Falcon tubes, stored at 2-4°C, and used within one week.

Agar well diffusion assay for antibacterial activity

The antibacterial activity of the fresh guava leaf and fruit extracts was evaluated using the agar well diffusion method against *E. ictaluri* (n = 3 strains) and *F. columnare* (n = 3 strains). The *E. ictaluri* strains were grown in nutrient broth (NB), while the *F. columnare* strains were cultured in TYES broth for 24h. Bacterial cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS). The bacterial suspensions were adjusted to approximately 1×10^8 CFU mL^{-1} using the McFarland standard (0.5). The suspensions were evenly spread onto TSA or TYES agar plates using sterile cotton swabs. Wells 6mm in diameter were punched into the agar, and 50µL of each extract was added to each well. Oxytetracycline (30µg; Oxoid, Thermo Scientific, USA) was included on each plate as a positive control. Each assay was performed in four replicates for each bacterial strain. Plates were incubated at 28°C for 24-36h, and the diameters of the inhibition zones were

Table 3. Amounts of plant materials, volumes of distilled water added, and volumes of fresh extracts obtained

Materials	Material weight (g)	Volume of distilled water added (mL)	Volume of extract obtained (mL)
Guava fruits	100	200	186
Guava leaves	100	200	163

measured to evaluate antibacterial activity. Based on inhibition zone diameters, antibacterial activity was classified as very strong, strong, moderate, or weak according to Davis & Stout (1971) and Pratiwi *et al.* (2023) (**Table 4**).

Determination of the minimum bactericidal concentration (MBC)

Extracts that exhibited at least moderate antibacterial activity in the agar diffusion assay were further evaluated to determine their minimum bactericidal concentrations (MBCs) following a modified protocol based on Hoai *et al.* (2025). The extracts were serially diluted 2-fold in sterile PBS to obtain dilution ratios ranging from 1:2 to 1:256. These ratios corresponded to the final extract concentrations ranging from 50% to 0.195% (v/v), calculated based on the proportion of extract volume in the total mixture after dilution. Bacterial cultures of *E. ictaluri* and *F. columnare* were cultured in NB and TYES broth, respectively, at 28°C for 24h. Bacterial cells were harvested, washed, resuspended in sterile PBS, and adjusted to approximately 1×10^8 CFU mL^{-1} (McFarland standard 0.5). For each bacterial strain, a series of 15-mL Falcon tubes containing 10mL of each diluted extract and one tube containing PBS as a negative control were prepared. Each treatment was performed in triplicate. Each tube was inoculated with 10µL of bacterial suspension to obtain an initial bacterial concentration of approximately 10^5 CFU mL^{-1}, thereby minimizing changes in the extract concentration. The mixtures were gently vortexed and incubated at 28°C for 6h, a period selected to allow sufficient contact between bacterial cells and the extract for bactericidal action while minimizing potential regrowth or degradation of active compounds, as recommended in previous antimicrobial assays of plant-derived extracts. After incubation, 50µL aliquots from each tube were aseptically spread onto TSA (for *E. ictaluri*) or TYES agar (for *F. columnare*) plates and incubated at 28°C for 24-48h. The MBC was defined as the lowest extract concentration at which no visible bacterial growth was observed on agar plates after subculture, indicating complete bacterial killing.

Statistical analysis

Inhibition zone diameters obtained from the agar diffusion assays were compiled and analyzed using descriptive statistics and two-way analysis of variance (ANOVA). Differences among treatment means were compared using Tukey's honestly significant difference (HSD) test, with statistical significance set at $P \leq 0.05$. Results are presented as means ± standard deviations. All statistical analyses were performed using R software version 4.5.2 (R Core Team, 2026) in RStudio version 2026.01.0 (RStudio Team, 2026).

Results and Discussion

Identification of bacterial strains

All six bacterial strains (three of *Edwardsiella ictaluri* and three of *Flavobacterium columnare*) were successfully revived and re-identified based on morphological, biochemical, and molecular characteristics (**Figure 1, Table 5**). The revived *E. ictaluri* strains formed small, circular, smooth, slightly convex, creamy-white colonies, and morphological examination revealed they were Gram-negative, motile, short-rod-shaped (**Figures 1A and 1B**). These results are similar to those of the *E. ictaluri* strains reported in previous studies by Huyen & Oanh (2020), and Nhinh *et al.* (2022). Their biochemical profiles showed high similarity to those of the reference *E. ictaluri* strain isolated from red tilapia as

Table 4. Classification of antibacterial activity based on inhibition zone diameters

Inhibition zone diameter	Antibacterial activity level
≤ 5mm	Weak
5-10mm	Medium
11-20mm	Strong
≥ 21mm	Very strong

reported by Dong *et al.* (2019) (**Table 5**). The PCR confirmation further demonstrated that all three of the revived *E. ictaluri* strains produced the expected species-specific amplicon of approximately 470 bp, identical in size to that of the positive control strain (*E. ictaluri*) (Dong *et al.*, 2019) (**Figure 1**).

Similarly, the three revived *F. columnare* strains displayed colony and cellular morphologies consistent with those described by Hoai *et al.* (2022). Biochemical identification was based on key diagnostic traits characteristic of *F. columnare*, including positive reactions for oxidase, catalase, flexirubin pigment production, Congo red absorption, and gelatin hydrolysis, together with negative results for Voges-Proskauer, glucose, ribose, arabinose, mannitol, sorbitol, lactose, trehalose, inulin, raffinose, glycogen, citrate utilization, indole production, and NaCl 1% (**Table 5**). Although the biochemical characteristics of *F. columnare* may exhibit strain-level variability, no notable differences were observed between the tested isolates and the reference strain (*F. columnare* ATCC23463) for the diagnostic markers employed. Molecular identification further confirmed these results, as all three isolates produced the species-specific PCR amplicon (~470bp), identical to that of the reference strain (**Figure 1**). Collectively, these findings confirm the identity of the isolates as *F. columnare* and support their use in the subsequent antibacterial and bactericidal assays.

Antibacterial activity of fresh guava leaf and fruit extracts against Edwardsiella ictaluri and Flavobacterium columnare

The inhibition zone diameters produced by the fresh guava leaf and fruit extracts against *E. ictaluri* were 26.7mm and 21.4mm, respectively, whereas those against *F. columnare* were 30.8mm and 25.8mm, respectively (**Figure 2**). For both extracts, the inhibition zones against *F. columnare* were significantly larger (P <0.05) than those against *E. ictaluri*, indicating that *F. columnare* was more susceptible to the guava extracts under the tested conditions. Moreover, for both bacterial species, the leaf extract produced significantly larger inhibition zones than the fruit extract (P <0.05), indicating stronger inhibitory activity.

The inhibition zone diameter of oxytetracycline (30µg) against *F. columnare* was 32.8mm, which was comparable to that produced by the leaf extract and significantly larger than that produced by the fruit extract. In contrast, against *E. ictaluri*, oxytetracycline produced an inhibition zone of 24.2mm, which was significantly larger than that of the fruit extract (21.4mm) but significantly smaller than that of the leaf extract (26.7mm) (P <0.05) (**Figure 2**). Notably, the inhibition zones produced by both the leaf and fruit extracts against both bacterial species were significantly larger than those of the negative control (PBS, 0mm) and were classified as "very strong" antibacterial activity according to the criteria proposed by Davis & Stout (1971) and Pratiwi *et al.* (2023), as all the inhibition zones exceeded 21mm. These results clearly indicate that fresh guava leaf and fruit extracts possess strong antibacterial activity against *E. ictaluri* and *F. columnare*. This finding highlights the considerable potential of guava extracts as alternatives or complements to antibiotics for the prevention and control of bacterial diseases such as enteric septicemia of catfish and Columnaris disease, which have caused high mortality and substantial economic losses in several freshwater fish species, including rainbow trout, tilapia, and catfish (Declercq *et al.*, 2013; Dong *et al.*, 2015; Huyen

& Oanh, 2020). According to Kumar *et al.* (2021), guava leaves and fruits contain various phenolic and flavonoid compounds, particularly quercetin and tannins, which confer their antibacterial properties. These bioactive compounds have been reported to disrupt bacterial cell membranes, promote the generation of reactive oxygen species, inhibit biofilm formation, interfere with DNA replication, impair energy metabolism, and suppress bacterial toxin production (Mickymaray, 2019). The fresh guava extracts evaluated in the

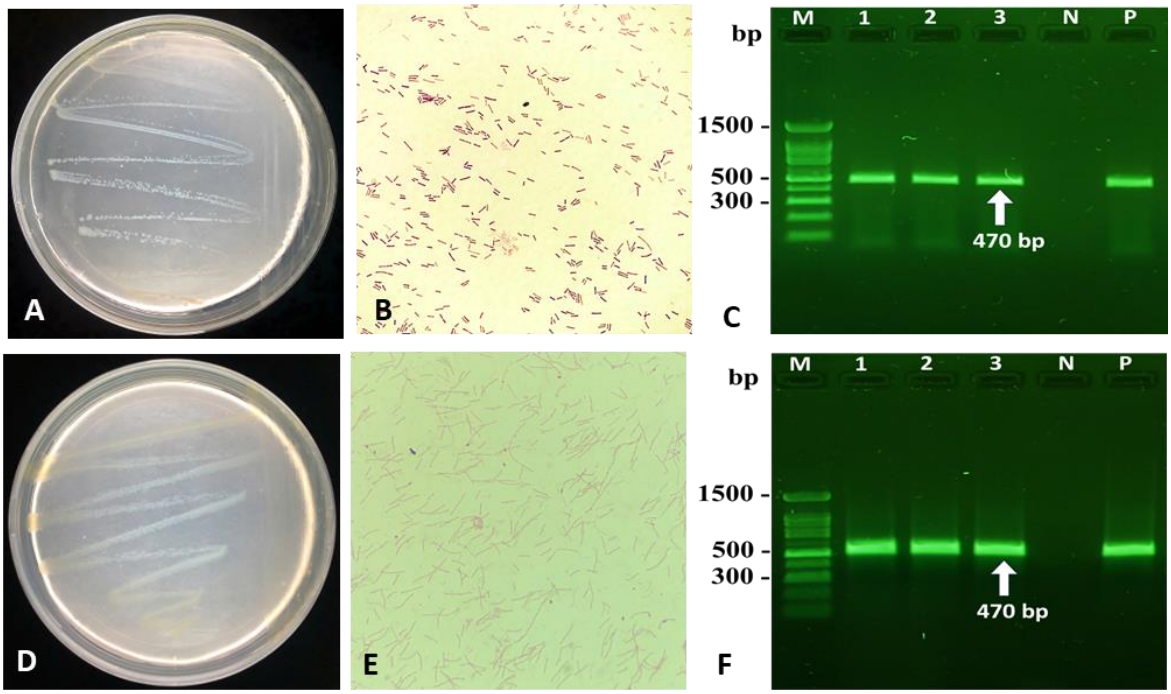


Figure 1. Colony morphology, cellular morphology, and PCR identification of *Edwardsiella ictaluri* and *Flavobacterium columnare*. A-C: Colony morphology, cellular morphology, and PCR results of *E. ictaluri*. D-F: Colony morphology, cellular morphology, and PCR results of *F. columnare*. M: DNA marker; P: positive control; N: negative control.

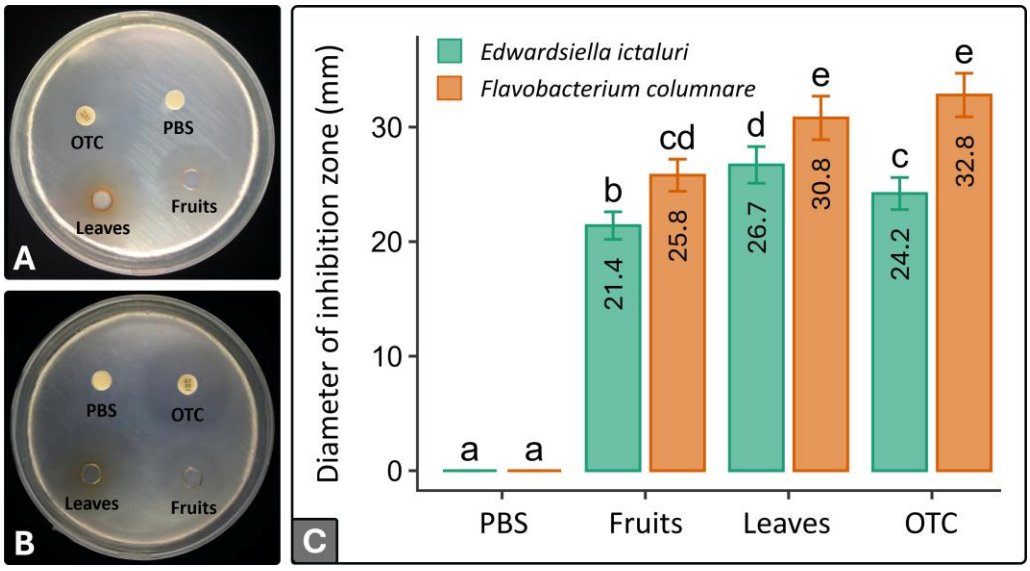


Figure 2. Inhibition zones produced by fresh guava extracts against *Edwardsiella ictaluri* and *Flavobacterium columnare* using the agar well diffusion method. A: Inhibition zones against *E. ictaluri*. B: Inhibition zones against *F. columnare*. C: Mean inhibition zone diameters (mm). PBS: phosphate-buffered saline (negative control).

Table 5. Biochemical characteristics of *Edwardsiella ictaluri* and *Flavobacterium columnare* used in this study

Biochemical characteristics	*E. ictaluri* this study	*E. ictaluri* Dong *et al.* (2019)	*F. columnare* this study	*F. columnare* ATCC23463
Gram staining	-	-	-	-
Bacterial morphology	Rod-shaped	Rod-shaped	Slender rod	Slender rod
Motility	+	+	Gliding	Gliding
Oxidase	NT	NT	+	+
Catalase	NT	NT	+	+
Congo red	NT	NT	+	+
Flexirubin	NT	NT	+	+
NaCl 1%	NT	NT	-	-
β-galactosidase (ONPG)	-	-	NT	NT
Arginine dihydrolase (ADH)	-	-	NT	NT
Lysine decarboxylase (LDC)	+	+	NT	NT
Ornithine decarboxylase (ODC)	-	-	NT	NT
Citrate utilization (CIT)	-	Variable	-	-
Hydrogen sulfide (H_2S)	-	-	NT	NT
Urease (URE)	-	-	NT	NT
Tryptophan deaminase (TDA)	-	-	NT	NT
Indole production (IND)	NT	NT	-	-
Voges-Proskauer (VP)	-	+	-	-
Gelatinase (GEL)	-	-	+	+
D-glucose (GLU)	+	+	-	-
D-mannitol (MAN)	-	-	-	-
Inositol (INO)	-	-	NT	NT
D-sorbitol (SOR)	-	-	-	-
D-rhamnose (RHA)	-	-	NT	NT
D-sucrose (SAC)	-	-	NT	NT
D-melibiose (MEL)	-	-	NT	NT
Amygdalin (AMY)	-	-	NT	NT
L-arabinose (ARA)	-	-	-	-
Ribose (RIB)	NT	NT	-	-
Lactose (LAC)	NT	NT	-	-
Trehalose (TRE)	NT	NT	-	-
Raffinose (RAF)	NT	NT	-	-
Inulin (INU)	NT	NT	-	-
Glycogen (GLYG)	NT	NT	-	-

NT: not tested; (+) positive; (-) negative.

the present study exhibited markedly stronger antibacterial activity than extracts from garlic and turmeric reported by Susanti *et al.* (2021), which produced inhibition zones ranging from 8 to 15mm against *E. ictaluri*. In addition, the antibacterial activity of the guava leaf extract against *F. columnare* was higher than that reported for extracts from aquatic plants by Novita *et al.* (2021), which showed inhibition zones of only 7.1-11.5mm. Therefore, guava

leaves and fruits represent promising raw materials for the development of herbal products for aquaculture, potentially reducing antibiotic use and the risk of antimicrobial resistance in freshwater systems.

Determination of the minimum bactericidal concentration (MBC)

Given the strong antibacterial activity of fresh guava leaf and fruit extracts against *E. ictaluri* and *F. columnare*, determining their minimum bactericidal concentrations (MBCs) is essential to provide a basis for practical application in disease control. The results showed that the MBCs of the leaf extract against both bacterial species were lower than those of the fruit extract. Specifically, the leaf extract retained bactericidal activity against *E. ictaluri* and *F. columnare* at dilution ratios of 1:16 (6.25%) and 1:64 (1.57%), respectively, whereas the fruit extract exhibited bactericidal activity only at dilution ratios of 1:8 (12.5%) and 1:32 (3.13%), respectively. In contrast, all the negative control tubes (PBS) showed normal bacterial growth (**Table 6**; **Figure 3**). These results indicate that the fresh guava leaf extract

Table 6. Minimum bactericidal concentrations (MBCs) of fresh guava leaves and fruit extracts against *Edwardsiella ictaluri* and *Flavobacterium columnare*

Bacterial species	Extract type	Dilution ratio of the original extract (%)							PBS (control)
		1:2 (50%)	1:4 (25%)	1:8 (12.5%)	1:16 (6.25%)	1:32 (3.13%)	1:64 (1.57%)	1:128 (0.78%)	
E. ictaluri	Fruits	-	-	-	+	+	+	+	+
	Leaves	-	-	-	-	+	+	+	+
F. columnare	Fruits	-	-	-	-	-	+	+	+
	Leaves	-	-	-	-	-	-	+	+

Note: (+) bacterial growth observed; (-) no bacterial growth observed.

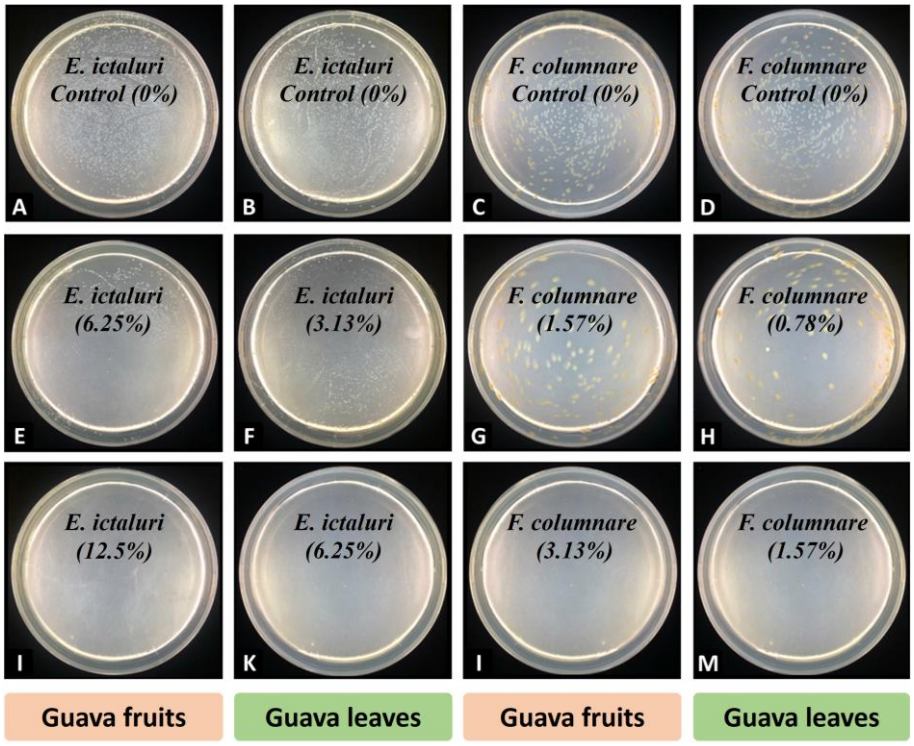


Figure 3. Determination of the minimum bactericidal concentration (MBC) of fresh guava leaf and fruit extracts against *Edwardsiella ictaluri* and *Flavobacterium columnare*. A-D: Negative controls without extract supplementation. E-H: Dilution levels at which bacterial growth was still observed. I-M: Dilution levels at which no bacterial growth was observed.

has stronger bactericidal activity against both tested pathogens than the fruit extract, consistent with the results from the agar diffusion assay.

When expressed in terms of fresh plant material equivalents, the MBCs of the fruit extract against *E. ictaluri* and *F. columnare* were 67.2 and 16.8mg fresh fruit per mL, respectively, whereas the corresponding MBCs of the leaf extract were 38.3 and 9.6mg fresh leaf per mL. The MBC values obtained in this study are higher than those reported by Yamanaka *et al.* (2008), who showed that guava leaf extracts inhibited *Vibrio vulnificus*, *V. mimicus*, *V. parahaemolyticus*, and *Aeromonas sobria* at concentrations of 256, 2048, 1024, and 1024 µg mL^{-1}, respectively. This difference may be attributed to the use of a fresh aqueous pressing method in the present study, compared with more advanced extraction techniques employing organic solvents in the study by Yamanaka *et al.* (2008).

In addition, guava leaf extracts have been shown to inhibit these bacterial species due to the presence of bioactive compounds with antibacterial and immunostimulatory properties, which are beneficial for the health of both humans and aquatic animals (Das & Goswami, 2019; Kumar *et al.*, 2021). Therefore, the antibacterial effect observed for the fresh extracts in the present study is likely attributable to the combined or synergistic actions of multiple compounds naturally present in guava leaves and fruits, particularly polyphenolic compounds, rather than to the activity of any single isolated constituent.

Although the fresh pressing method requires a relatively large amount of raw material, it is simple, cost-effective, and does not require expensive equipment or organic solvents. However, the relatively high MBC values observed suggest that the direct application of fresh extracts to pond water for disease control would be economically impractical and could lead to organic pollution from the decomposition of plant-derived organic matter. In contrast, several pioneering studies have demonstrated that dietary supplementation with guava extracts can effectively control bacterial infections or enhance immune responses in aquatic animals, with promising results in tilapia (Abdel-Tawwab & Hamed, 2020; Ceballos-Francisco *et al.*, 2020), Indian carp (Giri *et al.*, 2015), catfish (Dumpala *et al.*, 2023), tiger shrimp (Yin *et al.*, 2014), and mud crab (Yue *et al.*, 2023). Therefore, incorporating fresh guava extracts, particularly leaf extracts, into aquafeeds should be considered a more practical and economically feasible approach for controlling bacterial diseases in aquaculture.

Despite the lower plant material-to-water ratio used for leaf extraction compared with fruit extraction, the leaf extract exhibited significantly stronger bactericidal activity against both tested pathogens (P <0.05). This finding is consistent with the conclusion of Kamel (2001), who suggested that minimum inhibitory and bactericidal concentrations are closely related to the concentration and purity of bioactive compounds in plant extracts. Furthermore, Ceballos-Francisco *et al.* (2020) reported that dietary supplementation with guava leaf extracts reduced the abundance of pathogenic bacteria in tilapia, further supporting the potential of guava leaves as a functional feed additive. Overall, the present results demonstrate that fresh guava leaf and fruit extracts, particularly leaf extracts, exhibit strong bactericidal activity and considerable potential for preventing and controlling bacterial diseases caused by *E. ictaluri* and *F. columnare* in freshwater aquaculture.

Conclusions

This study demonstrates that fresh aqueous extracts of guava (*Psidium guajava*), particularly from leaves, exhibit strong antibacterial and bactericidal activities against *Edwardsiella ictaluri* and *Flavobacterium columnare* under laboratory conditions. The guava leaf extract consistently showed higher activity than the fruit extract and displayed effects comparable to oxytetracycline in the agar diffusion assay. These findings highlight the potential of guava leaf extract as a promising plant-based alternative or complementary approach for controlling bacterial diseases in freshwater aquaculture. Further in vivo studies and field evaluations are required to confirm its practical applicability and effectiveness under farming conditions.

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