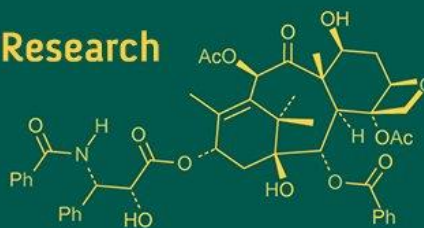


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Comparative antibacterial efficacy of aqueous and ethanolic extracts of *Psidium guajava* leaves against *Bacillus subtilis* and *Escherichia coli*

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Abstract

The hunt for potent plant-derived antibacterial medicines has accelerated due to the rising incidence of antimicrobial resistance. This study used the agar well diffusion method to compare the antibacterial activity of aqueous and ethanolic extracts of guava (*Psidium guajava*) leaves against *Bacillus subtilis* and *Escherichia coli*. Fresh leaves were powdered after being shade-dried. Standardized methods were used to make both aqueous and ethanolic extracts. Fourier transform infrared spectroscopy was used to evaluate the ethanolic extract, confirming the presence of phenolic chemicals, flavonoids, tannins, and terpenoids. At extract levels of 50, 100, and 150 microliters, antibacterial activity was evaluated. There was definite concentration-dependent action in both extracts. Nevertheless, against both test species, the ethanolic extract continuously generated greater zones of inhibition than the aqueous extract. The maximum activity was recorded with 150 microliters of the ethanolic extract against *Escherichia coli* (25 ± 0.8 mm). Penicillin shown modest effectiveness against *Escherichia coli* but high activity against *Bacillus subtilis*. These findings show that ethanol is a more effective solvent for guava leaf antibacterial phytochemical extraction. The results point to guava leaves' potential as a sustainable supply of natural antibacterial agents and validate their traditional medical usage.

Keywords: Guava leaves, antibacterial activity, ethanolic extract, aqueous extract, solvent effect, agar well diffusion, Fourier transform infrared spectroscopy

Introduction

Antimicrobial resistance (AMR) has emerged as one of the most significant global public health challenges of the twenty-first century, severely reducing the effectiveness of conventional antibiotics and complicating the management of bacterial infections (Almutairi *et al.*, 2025; Pereira *et al.*, 2023) ^[1, 2]. Globally, higher illness, mortality, longer hospital stays, and significant healthcare expenses have resulted from the quick establishment and dissemination of resistant bacterial strains. Finding new antimicrobial drugs has therefore emerged as a crucial area of scientific focus. Natural products, particularly those derived from medicinal plants, have gained renewed attention due to their diverse bioactive compounds that often act through multiple mechanisms, potentially reducing the likelihood of rapid resistance development (Biswas *et al.*, 2013; Oncho *et al.*, 2021; George, 2024) ^[19, 3, 15]. Both traditional and contemporary healthcare systems continue to rely heavily on medicinal plants, which have long been important sources of therapeutic chemicals. Among these is the widely grown tropical tree *Psidium guajava* L. (Myrtaceae), often known as guava, which is valued for its nutritional and therapeutic qualities. Guava leaves have long been used to heal wounds, inflammation, gastrointestinal issues, and a variety of microbial diseases (Oncho *et al.*, 2021; Pandhi *et al.*, 2022) ^[3, 12]. Flavonoids (like quercetin), phenolic compounds, tannins, terpenoids, glycosides, and essential oils are among the many bioactive secondary metabolites found in the leaves, many of which have antibacterial, antioxidant, and anti-inflammatory qualities (Huynh *et al.*, 2025; Almutairi *et al.*, 2025; Bilal *et al.*, 2024) ^[1, 9, 6]. *P. guajava* leaf extracts have been shown to have antibacterial activity against a variety of

pathogenic bacteria, such as *Escherichia coli*, *Staphylococcus aureus* and other clinically relevant microorganisms (Pereira *et al.*, 2023; Sarkingobir *et al.*, 2022; George, 2024)^[2, 16, 15]. However, the type and quantity of phytochemicals recovered depends on the extraction solvent (Dirar *et al.*, 2019; Kokilananthan *et al.*, 2022; Pandhi *et al.*, 2022)^[13, 11, 12]. Comparative analyses of aqueous versus ethanolic extracts from locally available *P. guajava* leaves against Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria under standardized circumstances are still few, despite their potential, especially in the Indian context. Thus, the current study compared the antibacterial activity of aqueous and ethanolic extracts of *Psidium guajava* leaves taken from Suramangalam, Tamil Nadu, against *B. subtilis* and *E. coli* using the agar well diffusion method. Additionally, the ethanolic extract was initially phytochemically characterized using Fourier Transform Infrared (FTIR) spectroscopy. The results should demonstrate guava leaves' potential as a sustainable supply of natural antibacterial agents and offer scientific support for their traditional use.

Materials and Methods

Plant material collection and preparation

In an urban agricultural zone in Suramangalam, Tamil Nadu, India, fresh, mature *Psidium guajava* L. (Myrtaceae) leaves were harvested from healthy, five to seven-year-old trees in January 2025. Approximately 500 g of undamaged leaves were collected and taken to the laboratory in sterile polyethylene bags within 2 h after collection (Oncho *et al.*, 2021; Pandhi *et al.*, 2022)^[3, 12].

In the lab, leaves were thoroughly rinsed under running tap water for five minutes to get rid of superficial debris, immersed in 0.1% (w/v) sodium hypochlorite (NaOCl) for two minutes to disinfect the surface, and then cleaned three times with sterile distilled water. The leaves were shade-dried in a single layer on perforated trays at room temperature (28 to 35 °C) and 60 to 70% relative humidity for five to seven days (Kokilananthan *et al.*, 2022; Mowes *et al.*, 2025)^[11, 7]. In order to prevent thermal degradation, dried leaves were ground into a fine powder using a pre-sterilized electric grinder in brief 30-second bursts with one-minute cooling intervals. Prior to extraction, the powder was kept at 4 °C in sealed glass containers (Dirar *et al.*, 2019; Turay *et al.*, 2025)^[13, 14].

Extraction of bioactive compounds

The effectiveness of ethanolic and aqueous solvents was compared using two extraction techniques. The aqueous extract was made by suspending 10 g of leaf powder in 100 mL of sterile distilled water (1:10 w/v) and shaking the mixture on an orbital shaker at 100 rpm for 32 hours at 25-28 °C (Pereira *et al.*, 2023)^[2]. For the ethanolic extract, 10 g of leaf powder was macerated in 50 mL (99.9%; 1:5 w/v) under dark conditions at room temperature for 4 days with intermittent shaking every 6 h (Almutairi *et al.*, 2025)^[11]. A rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) was used to condense both extracts under decreased pressure at 40 °C after they had been filtered through Whatman No. 1 filter paper (Ramchandran, 2020;

Imtara *et al.*, 2025)^[10, 8]. The concentrated extracts were transferred to sterile sealed amber glass vials and kept at 4 °C until further examination. The ratio of the dried extract weight to the initial dry leaf powder weight was used to calculate the extraction yield (%) (Dirar *et al.*, 2019; Pandhi *et al.*, 2022)^[13, 12].

Fourier Transform Infrared (FTIR) spectroscopy

An Alpha II FTIR spectrometer with an attenuated total reflectance (ATR) module (Bruker Optik GmbH, Germany) was used to characterize the functional groups found in the ethanolic extract. 32 scans were used to acquire spectra in the wavenumber range of 4000-500 cm⁻¹ at a resolution of 4 cm⁻¹ (Huynh *et al.*, 2025)^[9].

Test organisms and inoculum preparation

Test organisms included standard reference strains of *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (ATCC 6633) (Biswas *et al.*, 2013; George, 2024)^[19, 15]. Cultures were maintained at 4 °C on nutritional agar slants. A loopful of each strain was transferred into nutritional broth and incubated aerobically at 37 °C for 24 hours to create working inocula. Sterile saline was used to adjust turbidity to a 0.5 McFarland standard (~1.5 x 10² CFU/mL) (Efoli-Bam *et al.*, 2024; Sarkingobir *et al.*, 2022; Remmy-Martin *et al.*, 2026)^[4, 16, 18].

Antibacterial activity assay

The agar well diffusion method was used to assess antibacterial activity (Biswas *et al.*, 2013; Oncho *et al.*, 2021)^[19, 3]. Standardized bacterial solutions were consistently swabbed onto Mueller-Hinton agar (MHA; HiMedia, India) plates. Wells (6 mm diameter) were bored using a sterile cork borer (Chaturwedi *et al.*, 2025). Stock solutions of the extracts were produced at 100 mg/mL in their respective solvents. The wells received 5, 10, and 15 mg of crude extract each from aliquots of 50, 100, and 150 µL (Bilal *et al.*, 2024)^[6]. The positive control was Penicillin G (100 µg/mL; HiMedia, India), and the negative controls were ethanol and sterile distilled water (Gutierrez-Montiel *et al.*, 2025). After 30 minutes of pre-diffusion at room temperature, the plates were incubated aerobically at 37 °C for 24 hours. A ruler was used to measure zones of inhibition (ZOI) to the closest millimeter. Each susceptibility test was performed independently in triplicate (n=3).

Results

Plant material and extraction yield

Fresh *Psidium guajava* leaves weighing around 500 g were shade-dried to produce 420 g of dry leaf powder (Figure 1). The powder was light greenish-brown, finely grained, and preserved the typical aromatic odor of guava leaves. Aqueous extraction of 10 g powder gave ~80 mL of dark reddish-brown extract with a yield of 8% (w/w). 1.3-1.5 g of viscous dark greenish-brown concentrate, or 13-15% (w/w) recovery, were obtained via ethanolic extraction. The ethanolic extract demonstrated better extraction efficiency than the aqueous extract.



Fig 1: Preparation of *Psidium guajava* leaf material for extraction: (a) fresh mature leaves, (b) shade-dried leaves, and (c) finely powdered leaf sample.

Fourier Transform Infrared (FTIR) analysis

The ethanolic extract's FTIR spectrum showed a number of distinctive absorption bands that were suggestive of the main bioactive phytochemicals (Figure 2). O-H stretching of flavonoids and phenolic compounds was identified as a wide band at 3332 cm^{-1} . Bands at $2977\text{--}2932\text{ cm}^{-1}$ suggested aliphatic C-H stretching, whereas the peak at 1642 cm^{-1}

corresponded to C=O and aromatic C=C stretching of flavonoids and tannins. C-O stretching of glycosides and other oxygenated compounds was suggested by additional bands in the fingerprint region ($1276\text{--}1044\text{ cm}^{-1}$) (Table 1). These functional groups suggest the existence of phenolics, flavonoids, tannins, terpenoids, and other oxygenated phytochemicals linked with antibacterial activity.

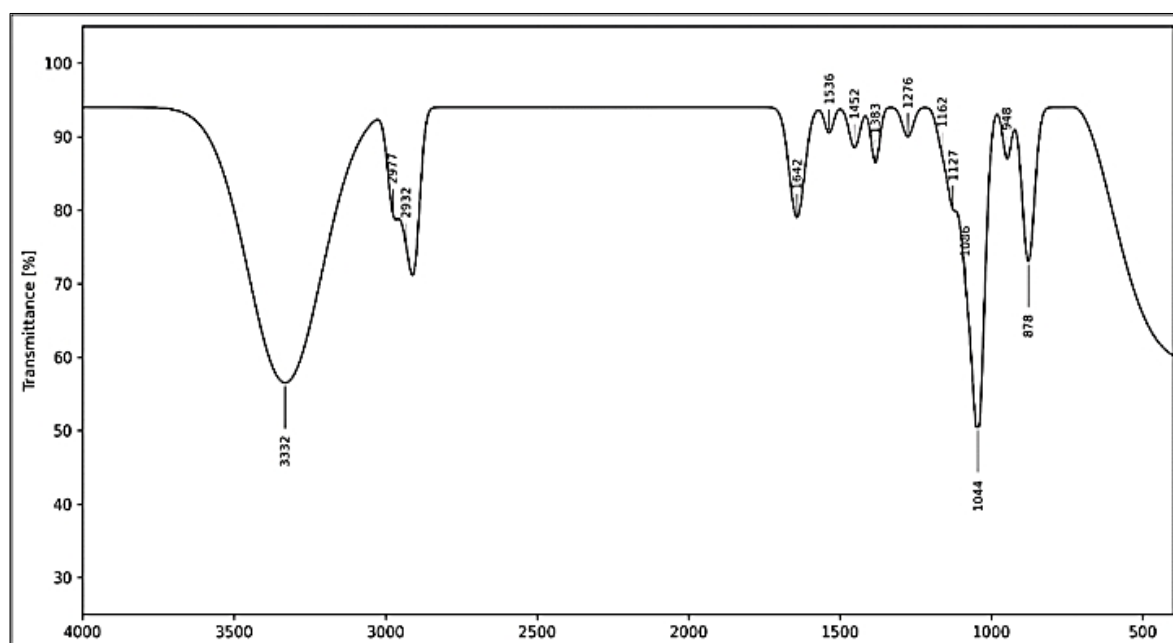


Fig 2: Fourier transform infrared (FTIR) spectrum of the ethanolic leaf extract of *Psidium guajava* showing the characteristic absorption bands of major phytochemical functional groups.

Table 1: Major FTIR absorption bands and probable functional group assignments in the ethanolic leaf extract of *Psidium guajava*.

Wavenumber (cm^{-1})	Functional Group	Vibrational Mode	Probable Phytochemicals
3332	O-H	Stretching	Phenols, flavonoids
2977-2932	C-H	Stretching	Aliphatic hydrocarbons, terpenoids
1642	C=O / C=C	Stretching	Flavonoids, tannins
1536-1452	Aromatic C=C	Stretching	Phenolic compounds
1276-1044	C-O / C-O-C	Stretching	Glycosides, phenols, alcohols

Antibacterial Activity

Both aqueous and ethanolic extracts revealed clear, concentration-dependent antibacterial activity against *Bacillus subtilis* and *Escherichia coli*, as determined by the agar well diffusion assay (Figure 3). The quantitative comparison of the mean zones of inhibition is provided in Figure 4 and Table 2. At every tested concentration, the

ethanolic extract continuously generated greater zones of inhibition than the aqueous extract. $150\text{ }\mu\text{L}$ of the ethanolic extract showed the strongest antibacterial activity against *E. coli* ($25 \pm 0.8\text{ mm}$). Penicillin G had restricted action against *E. coli* but greater zones of inhibition against *B. subtilis*. For the negative controls, no zones of inhibition were seen.

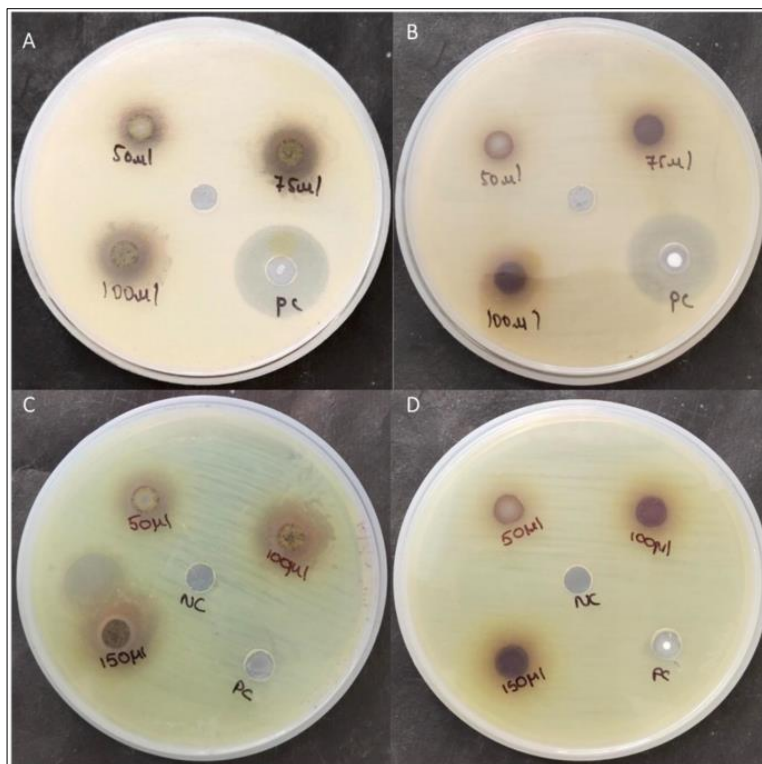


Fig 3: Representative agar well diffusion assay showing the antibacterial activity of *Psidium guajava* leaf extracts against *Bacillus subtilis* and *Escherichia coli*: (a) *B. subtilis*-ethanolic, (b) *B. subtilis*-aqueous, (c) *E. coli*-ethanolic, and (d) *E. coli*-aqueous.

Table 2: Antibacterial activity of *Psidium guajava* leaf extracts against *Bacillus subtilis* and *Escherichia coli*, expressed as mean zone of inhibition (mm $\pm$ SD) from three independent experiments (n = 3).

Organism	Extract Type	50 μ L	100 μ L	150 μ L	Penicillin (Control)
<i>B. subtilis</i>	Ethanolic	18 $\pm$ 1.2	20 $\pm$ 0.8	23 $\pm$ 1.0	28 $\pm$ 0.9
<i>B. subtilis</i>	Aqueous	14 $\pm$ 0.9	15 $\pm$ 1.1	20 $\pm$ 0.7	27 $\pm$ 1.2
<i>E. coli</i>	Ethanolic	23 $\pm$ 1.0	24 $\pm$ 0.6	25 $\pm$ 0.8	10 $\pm$ 1.1
<i>E. coli</i>	Aqueous	13 $\pm$ 1.3	15 $\pm$ 0.9	18 $\pm$ 1.0	11 $\pm$ 0.7

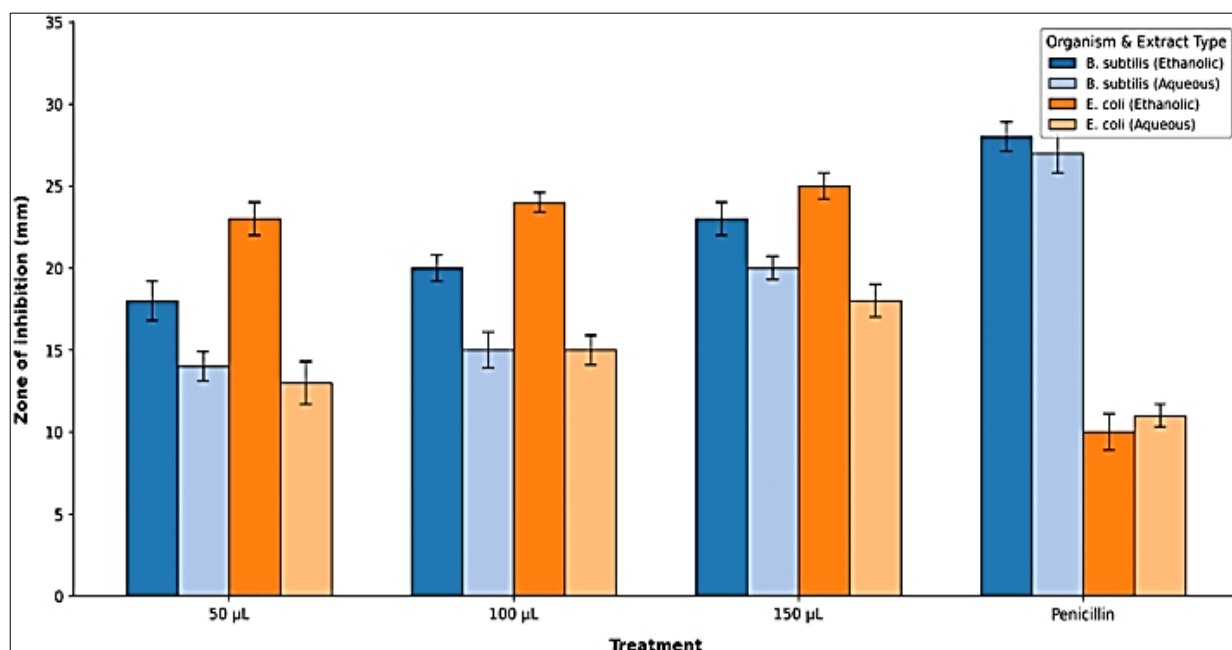


Fig 4: Comparison of the mean zones of inhibition produced by aqueous and ethanolic *Psidium guajava* leaf extracts against *Bacillus subtilis* and *Escherichia coli* at different extract concentrations and the positive control (Penicillin).

Discussion

The present study comparatively examined the antibacterial activity of aqueous and ethanolic extracts of *Psidium guajava* leaves against *Bacillus subtilis* and *Escherichia coli*

using the agar well diffusion method. Both extracts displayed clear concentration-dependent action, with the ethanolic extract consistently providing greater zones of inhibition. These findings are consistent with a number of

previous research (Almutairi *et al.*, 2025; Dirar *et al.*, 2019; Kokilananthan *et al.*, 2022; Pandhi *et al.*, 2022)^[1, 13, 11, 2] and provide compelling evidence for the impact of solvent polarity on the recovery of bioactive phytochemicals. The plant material processing method used, which involved shade drying for five to seven days and then mechanical grinding, worked well, producing 420 g of dry powder from 500 g of fresh leaves. This strategy is in line with common techniques for preserving thermolabile chemicals that have been documented in the literature (Kokilananthan *et al.*, 2022; Pandhi *et al.*, 2022; Turay *et al.*, 2025)^[11, 12, 14]. A yield of around 8% (w/w) was obtained via aqueous extraction (1:10 w/v, 32 h shaking), whereas 13-15% (w/w) was obtained from ethanolic maceration (1:5 w/v, 4 days) followed by rotary evaporation. The higher yield attained with ethanol is in line with research by (Almutairi *et al.*, 2025; Dirar *et al.*, 2019; Pandhi *et al.*, 2022)^[1, 13, 12], which showed that ethanol was more effective at extracting lipophilic and semi-polar components. FTIR examination of the ethanolic extract revealed strong absorption bands corresponding to O-H (3332 cm⁻¹), C-H (2977-2932 cm⁻¹), C=O/C=C (1642 cm⁻¹), and C-O (1276-1044 cm⁻¹) stretching vibrations, indicating the presence of phenolics, flavonoids, tannins, and terpenoids. These functional groups match spectrum patterns reported in earlier guava leaf investigations and are known contributors to antibacterial activity (Huynh *et al.*, 2025; Bilal *et al.*, 2024; Almutairi *et al.*, 2025)^[9, 1, 6]. The ethanolic extract outperformed the aqueous extract at all tested concentrations in the antibacterial testing, producing inhibition zones ranging from 18-23 mm against *B. subtilis* and 23-25 mm against *E. coli*. These results are similar to those for ethanolic guava leaf extracts published by (George *et al.*, 2024; Oncho *et al.*, 2021; Biswas *et al.*, 2013)^[19, 3, 15]. Better solubilization of membrane-disrupting substances like quercetin and terpenoids is probably the reason for the ethanolic extract's higher performance (Huynh *et al.*, 2025; Bilal *et al.*, 2024)^[9, 6]. Interestingly, both extracts continued to exhibit potent activity against the Gram-negative *E. coli*, with the ethanolic extract producing zones as large as 25 mm. Given the outer membrane barrier of Gram-negative bacteria, this discovery is noteworthy and supports findings (Pereira *et al.*, 2023; Sarkingobir *et al.*, 2022; Gutierrez-Montiel *et al.*, 2025)^[16, 2]. Penicillin G demonstrated limited and weakly discernible zones against *E. coli*, indicating the strain's inherent resistance, although it caused strong suppression against *B. subtilis*. On the other hand, significant efficacy against *E. coli* was maintained by the guava leaf extracts, especially the ethanolic formulation. This differential response suggests that the phytochemicals in *P. guajava* exert multi-target effects (membrane permeabilization, enzyme inhibition, and efflux modulation) that differ from the single-target mechanism of β -lactam antibiotics (Almutairi *et al.*, 2025; Bilal *et al.*, 2024; Gutierrez-Montiel *et al.*, 2025)^[1, 6]. The current study's evident concentration-dependent increase in zone diameter is consistent with previous findings (Oncho *et al.*, 2021; Bilal *et al.*, 2024; Pereira *et al.*, 2023)^[3, 2, 6]. All of these findings highlight how crucial extract concentration and solvent choice are to optimizing antibacterial activity. The findings of this study provide scientific validation for the traditional medicinal use of guava leaves and reinforce the potential of *P. guajava* as a sustainable source of natural antimicrobials, especially in regions like Tamil Nadu where the plant is abundant

(Pandhi *et al.*, 2022; Imtara *et al.*, 2025)^[8]. These plant-derived extracts provide interesting multi-target alternatives in the face of growing antimicrobial resistance (Almutairi *et al.*, 2025; Pereira *et al.*, 2023; George, 2024)^[1, 2, 15]. Nevertheless, the present study has several limitations due to its preliminary character. The agar well diffusion method, which offers qualitative and semi-quantitative data on antimicrobial potential, was mostly used to assess antibacterial activity. Although this method is commonly approved for first screening (Biswas *et al.*, 2013; Oncho *et al.*, 2021)^[19, 3].

Conclusion

In summary, the ethanolic extract of *Psidium guajava* leaves showed better antibacterial activity against *Bacillus subtilis* and *Escherichia coli* than the aqueous extract. The results demonstrate guava leaves as a promising, sustainable source of natural antibacterial agents and confirm the significance of solvent selection in phytochemical extraction. These results contribute to the expanding data supporting the exploitation of locally available medicinal herbs in the fight against antibiotic resistance.

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