

# Antibacterial activity of plant extracts obtained by two methods against pathogens of clinical importance in veterinary medicine

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Submitted: 2025-01-16

Accepted: 2025-09-13

Published: 2025-11-18

Additional information and declarations  
can be found on page 10

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## Abstract

This study aimed to determine the antibacterial activity of different plant extracts against ATCC-type pathogens of importance to veterinary medicine. Extracts were obtained using the Soxhlet and hydroalcoholic extraction method. Each requires different conditions, and these methods will likely afford different biological activities. Hence, results were compared to determine the influence of the extraction process, particularly against the antibacterial activity of the extracts of *Origanum vulgare*, *Rosmarinus officinalis*, *Psidium guajava*, *Thymus vulgaris*, *Eysenhardtia polystachya*, *Cinnamomum zeylanicum*, and *Syzygium aromaticum*. All extracts were assessed for antibacterial activity against gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli*, *Salmonella enterica* serotype *Typhi*). The agar well diffusion method was performed, and MICs were determined by the broth microdilution method. From all plant primary extracts tested, results show that the antibacterial action vs. gram-positive bacteria was greatest for the hydroalcoholic extracts of *Origanum vulgare*, *Thymus vulgaris*, and *Psidium guajava*, and again, *Psidium guajava* and *Eysenhardtia polystachya* for the Soxhlet extracts. Only the Soxhlet extract of *Eysenhardtia polystachya* showed a well-defined antibacterial action vs. gram-negative bacteria, although results do not exceed the reference drug utilized (enrofloxacin). It is proposed that research on the use of the mentioned extracts alone or combined with antibiotics in veterinary medicine follows, as well as the isolation and quantification of secondary metabolites present.

**Keywords:** Veterinary medicine; Antibacterial; Extracts; Hydroalcoholic-extraction; Soxhlet.

## Cite this as:

Mendoza J, Gutiérrez L, Alfonseca E, Gracia J, Tapia-Pérez G, Sumano H, Sánchez Z. Antibacterial activity of plant extracts obtained by two methods against pathogens of clinical importance in veterinary medicine. *Veterinaria México OA*. 2025;12. doi: 10.22201/fmvz.24486760e.2025.1466.

## Study contribution

Restrictions on the use of antibiotics due to the development of resistance are one of the reasons why the search for new treatment alternatives against important pathogens in veterinary medicine is more common. The study of the use of plants with antibacterial activity has been increasing; however, the complexity of the composition of the extracts makes it necessary to study not only the plants but also the methods for obtaining their bioactive compounds. In this study, plant extracts obtained by two different methods were evaluated for their ability to inhibit the growth of pathogenic bacteria. It was shown that hydroalcoholic extracts are the most effective, especially against gram-positive bacteria. Since the extracts with good results have a low minimum inhibitory concentration, their use as an adjuvant in the treatment of infections caused by gram-positive bacteria, especially *Staphylococcus aureus*, is proposed.

## Introduction

Infectious diseases caused by bacteria remain a major threat to veterinary food production units and public health in general. Resistant veterinary pathogens to antibiotic treatment are becoming increasingly common.<sup>(1, 2)</sup> Also, bacterial resistance in veterinary medicine may eventually impact public health<sup>(3, 4)</sup> as bacterial resistance in livestock threatens the ecological niche shared with humans.<sup>(4–6)</sup> The antibacterial drug resistance of pathogens significantly burdens the economic viability of food production.<sup>(7)</sup> Antimicrobial resistance (AMR) is recognized as one of the top ten threats to public health and is currently causing nearly 2 million human deaths per year. If all contributing causes are not addressed, it has been estimated that this figure could increase substantially to 10 million deaths by 2050.<sup>(8, 9)</sup>

AMR has been associated with the selective pressure caused by the extensive and often indiscriminate use of antimicrobials in human and veterinary medicine. In veterinary medicine, some precautions have already been taken. For example, the use of growth promoters has been canceled or, at least, severely restricted in many countries worldwide.<sup>(10–12)</sup> Nevertheless, the pressure of intensive production of animals for human consumption has led to the increasingly accentuated use of antibiotics for therapeutic purposes.<sup>(13, 14)</sup> The AMR problem is fueled by scarce or marginally successful research that seeks to discover or develop new drug entities with antibacterial activity.<sup>(15)</sup> However, the task is urgent. In the field of veterinary production, special attention has been given to phytobiotic elements.<sup>(16–18)</sup>

Although standardizing a final commercially available preparation can be challenging. Plants' abundance of active principles is often linked to geographic areas and weather conditions. Plants produce specialized secondary metabolites to accomplish diverse functions, i.e., to attract pollinators, as defensive substances against parasites and predators, or to resist diseases.<sup>(19–21)</sup> Hence, raw plant-derived material to prepare a given herbal remedy needs more uniformity. However, it is safe to state that the use of commercially viable phytobiotics for controlling and treating bacterial infections is a project that may crystallize in the coming years as extraction methods are standardized, so plant-based raw materials become chemically homogeneous.<sup>(22, 23)</sup>

The extraction of phytobiotics is a crucial process in recovering active principles. Nevertheless, numerous methods can be found in the literature, i.e., maceration, boiling, percolation, hydro distillation, Soxhlet, ultrasound, and microwave.<sup>(24–26)</sup> The basic principle is solid-liquid extraction with solvents and leaching. However, the main factors that can influence the extraction yield and selectivity are the solvent, time, temperature, solid-liquid ratio, and particle size of the plant material.<sup>(27, 28)</sup> Hence, it is essential to consider the characteristics of a given extraction to assess its efficacy as a source of antibacterial molecules. Considering the above, this work aimed to an-

alyze the antibacterial activity of plant extracts obtained by two different methods, assessing the potential phytobiotic activity inhibiting the growth of ATCC-type strains of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* subsp. *Enterica* serotype *Typhi*, *Bacillus cereus*, and *Bacillus subtilis* are examples of pathogens of clinical relevance in veterinary medicine.

## Materials and methods

### Bacterial cultures

The antibacterial activity of the Soxhlet and hydroalcoholic extracts was evaluated against strains of *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 10536, *Salmonella enterica* subsp. *Enterica* serovar *Typhimurium* ATCC 14028, *Bacillus subtilis* ATCC 6633, and *Bacillus cereus* ATCC 11778.

### Plant material

Oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), guava (*Psidium guajava*), and thyme (*Thymus vulgaris*) plants, bark of blue-wood (*Eysenhardtia polystachya*), cinnamon (*Cinnamomum zeylanicum*), and clove flowers (*Syzygium aromaticum*) were obtained from a local botanical market. The leaves were air-dried, and each of the plant materials was ground. Milled plant material was extracted by the Soxhlet method and hydroalcoholic extraction.

### Preparation of bacterial cultures

The bacteria were cultured on Mueller-Hinton agar for 18–24 h at 37 °C. The colonies were suspended in 5 mL of sterile physiological solution at a concentration of 10<sup>8</sup> CFU (turbidity = 0.5 McFarland).

### Extraction method

Soxhlet extraction was carried out with 30 g of each plant, following the methodology described by Alara with a feed-to-solvent ratio of 1:10 g/mL,<sup>(29)</sup> however, for this study the solvent was modified, using water and a heating mantle was used to keep the system under reflux (94 °C); the extraction cycles lasted around 1.5 h. At the end of the process, the extract was allowed to cool to room temperature of 26 °C. Finally, all extracts were filtered through Whatman No. 1 and stored at -20 °C until use.

Hydroalcoholic extraction was carried out with an ethanol/deionized water mixture (70 % v/v) using 30 g of each plant material. The samples were extracted at a room temperature of 26 °C and solid-to-liquid ratio (1:10 g/mL).<sup>(30)</sup> In this study, the extract was obtained by simple filtration with Whatman No. 1 paper, and the plant material was manually compressed to obtain all the remaining solvents. The extract was concentrated in a rotary evaporator and finally stored in amber bottles at 8 °C until use.

### Antimicrobial activity of plant extracts by agar well diffusion method

The agar diffusion assay was performed based on Balouiri, to assess the antimicrobial profile of plant extracts. Briefly, bacterial inoculum was spread evenly with a sterile cotton swab on a Mueller-Hilton agar plate in sterile Petri dishes. One hundred microliters of each extract were added

to 7 mm diameter wells plunged in the agar gel using as a reference value 100 µL of a solution of enrofloxacin (Sigma Aldrich, CAS Number: 93106-60-6) at a concentration of 2.5 µg/mL. The plates were incubated for 24 h at 37 °C under aerobic conditions. After incubation, confluent bacterial growth was observed. The diameter of bacterial growth inhibition was measured in mm using a digital Vernier caliper. Only clear halos with no growth around the well were considered, and all tests were performed in triplicate.<sup>(31)</sup>

### *Determination of the Minimum Inhibitory Concentration (MIC)*

The minimum inhibitory concentration of the extracts obtained by Soxhlet method (aqueous extracts) and hydroalcoholic extraction (hydroalcoholic extracts) to inhibit bacterial growth was performed using the modified method described by CLSI. Tenfold dilutions of an initial volume of 200 µL of the extracts were made in 96-well plates. Each dilution was inoculated with the different bacteria suspended in nutrient broth at a concentration of 10<sup>6</sup> CFU/mL, until reaching a final volume of 200 µL. Nutrient broth without extract, inoculated with each bacteria, was used as a bacterial growth control, and nutrient broth with each extract without bacterial inoculation was used as a negative control for bacterial growth. After 24 h of incubation at 37 °C, 30 µL of 0.02 % (w/v) resazurin solution in distilled water was added and incubated again for an hour. The change from blue to pink color was recorded as an indication of bacterial viability. The MIC value corresponds to the well before the color change from blue to pink.<sup>(32)</sup>

### *Determination of Minimum Bactericidal Concentration (MBC)*

The bactericidal capacity of aqueous and hydroalcoholic extracts was performed by inoculating 100 µL in nutrient broth with agar from the wells corresponding to the MIC, and thereof with a higher concentration until no growth was achieved. The plates were incubated for 24 h at 37 °C. The value of the minimum bactericidal concentration of the extracts was considered the lowest concentration capable of inhibiting total growth for each microorganism.<sup>(32)</sup>

### *Statistical analysis*

Descriptive statistics analyzed MIC values. The statistical differences in the antibacterial activity determined by the inhibition zones and the susceptibility of the different microorganisms tested with the extracts obtained by the two methods were analyzed using the following model:

$$Y_{ijklm} = \mu + B_i + E_j + M_k + (BE)_{ij} + (BM)_{ik} + (EM)_{jk} + (BEM)_{ijk} + \varepsilon_{ijklm}$$

where,

$Y_{ijklm}$  is the m-th inhibition zone of the i-th bacteria produced by the j-th extract and the k-th extraction method,

$B_i$  it is the i-th bacteria ( $i = 1, 2, 3, 4, 5$ ),

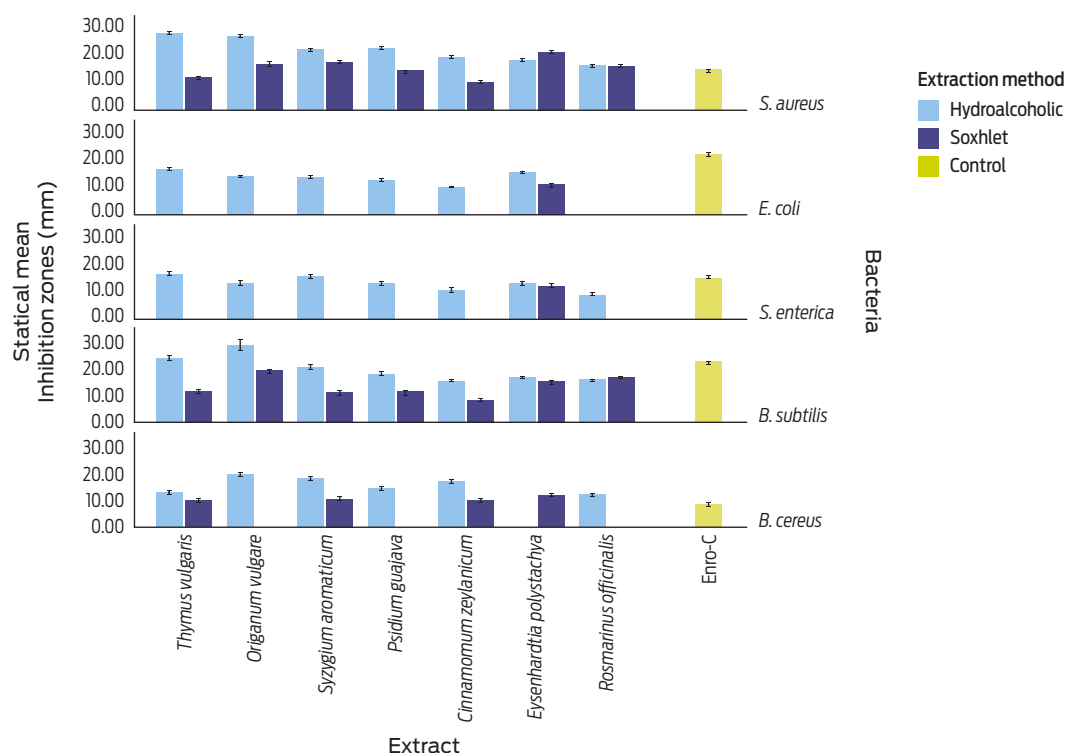
$E_j$  it is the j-th extract ( $J = 1, 2, 3, 4, 5, 6, 7, 8$ ),

$M_k$  it is the k-th extraction method ( $k = 1, 2$ ),

$(BE)_{ij}$ ,  $(BM)_{ik}$ ,  $(EM)_{jk}$  y  $(BEM)_{ijk}$  are the double and triple interactions between the main factors,

$\varepsilon_{ijklm}$  is the distributed random error  $N(\mu, \sigma_\varepsilon)$ .

The software used was SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA) with a significance level of  $P < 0.001$ .



**Figure 1.** Grouped bars of statistical mean of inhibition zone by extract by extraction method. All components of the model were significant ( $P < 0.001$ ); the R-squared was 0.986, and the observed power for all effects in the model was one.

## Results

### Antibacterial activity of plant extracts obtained by different extraction methods

Soxhlet method and hydroalcoholic extraction were applied to oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*), guava (*Psidium guajava*), and thyme (*Thymus vulgaris*) air-dried plant material as well as the bark of blue-wood (*Eysenhardtia polystachya*), cinnamon (*Cinnamomum zeylanicum*) and clove flowers (*Syzygium aromaticum*) samples. All extracts were tested against gram-positive bacteria: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, and *Bacillus cereus* ATCC 11778, and gram-negative bacteria: *Escherichia coli* ATCC 10536 and *Salmonella enterica* subsp. *Enterica* serovar. *Typhimurium* ATCC 14028. Effectiveness was measured by comparing the inhibition zone obtained from the extracts and a reference antibacterial drug at a known concentration (Enrofloxacin, 2.5 µg/mL).

As shown in the results (Figure 1), all hydroalcoholic extracts showed antibacterial activity by inhibiting the growth of the microorganisms, however they were more effective against gram-positive than against gram-negative bacteria. Used for this test, in some cases equaling or improving the effectiveness shown by the reference, as is the case of *Thymus vulgaris* with *S. aureus*, *S. enterica*, and *B. subtilis*, *Origanum vulgare* with *B. subtilis*, and most of the extracts with *B. cereus*, except for *Eysenhardtia polystachya* which did not show any inhibition zone with this microorganism.

The hydroalcoholic extracts of *Eysenhardtia polystachya* and *Thymus vulgaris* had the largest inhibition zone for *E. coli*. However, they did not surpass the standard, whose inhibition zone was  $20.73 \pm 0.43$  mm. The activity of *Thymus vulgaris* and *Syzygium aromaticum* against *S. enterica* were the highest as compared to the rest of the hydroalcoholic extracts with no sig-

nificant difference when compared with enrofloxacin. *B. cereus* had the largest inhibition zone with *Origanum vulgare* and *Syzygium aromaticum* ( $P < 0.001$ ). In addition, the effectiveness of many extracts against this latter microorganism by all the hydroalcoholic extracts obtained (*Cinnamomum zeylanicum*, *Psidium guajava*, *Thymus vulgaris*, and *Rosmarinus officinalis*) were particularly efficient, exhibiting greater inhibition zone as compared with enrofloxacin.

On the other hand, all Soxhlet extracts inhibited the growth of *S. aureus* and *B. subtilis*; however, for *B. cereus*, only *Origanum vulgare*, *Psidium guajava*, and *Rosmarinus officinalis* had no effect, highlighting that the rest of the tested extracts (*Eysenhardtia polystachya*, *Syzygium aromaticum*, *Cinnamomum zeylanicum*, and *Thymus vulgaris*) showed a more significant inhibition zone than the reference. The antibacterial activity of *Eysenhardtia polystachya* against *S. aureus* stands out. It was statistically significantly higher than the rest of the Soxhlet extracts and the antibacterial used as a reference ( $P < 0.001$ ). *Origanum vulgare* showed the most significant inhibition of *B. subtilis*, but did not exceed the reference values or the hydroalcoholic extract inhibition. Only *Eysenhardtia polystachya* showed a slight activity against *E. coli* and *S. enterica* serotype *Thyphi*.

In addition, *Eysenhardtia polystachya*, *Syzygium aromaticum*, *Cinnamomum zeylanicum*, and *Thymus vulgaris* showed activity against *B. cereus*. However, they were not statistically different from each other ( $P < 0.001$ ). Nevertheless, there was a statistically greater difference when comparing their values with enrofloxacin, exceeding the diameter of the inhibition zones of this reference antibacterial drug ( $P < 0.05$ ).

Table 2 shows the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of plant extracts tested against gram-positive bacteria (*S. aureus*, *B. subtilis*, and *B. cereus*) and gram-negative bacteria (*E. coli* and *S. enterica*), determined by two-fold dilutions. For the hydroalcoholic extracts, *Syzygium aromaticum* showed the highest dilution for *B. subtilis* and *B. cereus*, followed by *Thymus vulgaris* and *Origanum vulgare* against *S. aureus*, and finally, *Psidium guajava* against *B. subtilis*. Although dilutions obtained with other herbs to achieve inhibition of tested bacteria were not as high as the former extracts, they proved to be competitive i.e., *Syzygium aromaticum* and *Psidium guajava* against *S. aureus*; *Origanum vulgare* and *Rosmarinus officinalis* vs. *B. subtilis*, and *Origanum vulgare* and *Psidium guajava* vs. *B. cereus*.

Thus, all of them can be viewed as having remarkable antibacterial activity, requiring small concentrations of each extract to show inhibitory effect against these gram-positive bacteria. In contrast, for *E. coli* and *S. enterica*, only *Origanum vulgare* and *Syzygium aromaticum* had a mild inhibitory effect (1:64 and 1:32, respectively). Concerning the analysis of MICs and MBCs in Soxhlet extracts, the highest dilution was 1:128 for *Eysenhardtia polystachya* against gram-positive bacteria. For the two gram-negative bacteria studied *Eysenhardtia polystachya*, *Syzygium aromaticum*, *Rosmarinus officinalis*, and *Psidium guajava* exhibited inhibitory activity also at 1:128, with the former extract being the only one showing a mild degree of inhibition activity against *E. coli*.

**Table 2.** Minimum inhibitory concentration and minimum bactericidal concentration of plants extracted with hydroalcoholic and Soxhlet method against five species

|                | Extract                         | <i>S. aureus</i> |       | <i>E. coli</i> |      | <i>S. enterica</i> |      | <i>B. subtilis</i> |       | <i>B. cereus</i> |       |
|----------------|---------------------------------|------------------|-------|----------------|------|--------------------|------|--------------------|-------|------------------|-------|
|                |                                 | MIC              | MBC   | MIC            | MBC  | MIC                | MBC  | MIC                | MBC   | MIC              | MBC   |
| Hydroalcoholic | <i>Thymus vulgaris</i>          | 1:256            | 1:256 | 1:8            | 1:8  | 1:8                | 1:8  | 1:32               | 1:32  | 1:32             | 1:32  |
|                | <i>Origanum vulgare</i>         | 1:256            | 1:256 | 1:64           | 1:64 | 1:64               | 1:64 | 1:128              | 1:128 | 1:128            | 1:128 |
|                | <i>Syzygium aromaticum</i>      | 1:128            | 1:128 | 1:32           | 1:32 | 1:32               | 1:32 | 1:512              | 1:512 | 1:256            | 1:256 |
|                | <i>Psidium guajava</i>          | 1:128            | 1:128 | 1:4            | 1:4  | 1:4                | 1:4  | 1:256              | 1:256 | 1:128            | 1:128 |
|                | <i>Cinnamomum zeylanicum</i>    | 1:32             | 1:32  | 1:8            | 1:8  | 1:16               | 1:16 | 1:16               | 1:16  | 1:8              | 1:4   |
|                | <i>Eysenhardtia polystachya</i> | 1:16             | 1:16  | 1:16           | 1:16 | 1:16               | 1:16 | 1:32               | 1:32  | 1:16             | 1:16  |
|                | <i>Rosmarinus officinalis</i>   | 1:8              | 1:8   | 1:16           | 1:16 | 1:32               | 1:16 | 1:128              | 1:128 | 1:32             | 1:32  |
| Soxhlet        | <i>Thymus vulgaris</i>          | 1:64             | 1:64  | 1:8            | 1:4  | 1:4                | 1:2  | 1:16               | 1:16  | 1:8              | 1:8   |
|                | <i>Origanum vulgare</i>         | 1:64             | 1:64  | 1:4            | 1:4  | 1:2                | 1:2  | 1:32               | 1:32  | 1:16             | 1:16  |
|                | <i>Syzygium aromaticum</i>      | 1:128            | 1:128 | 1:8            | 1:8  | 1:4                | 1:4  | 1:128              | 1:128 | 1:32             | 1:32  |
|                | <i>Psidium guajava</i>          | 1:32             | 1:32  | 1:2            | 1:2  | 1:2                | 1:2  | 1:128              | 1:128 | 1:64             | 1:32  |
|                | <i>Cinnamomum zeylanicum</i>    | 1:8              | 1:4   | 1:2            | 1:2  | 1:2                | 1:2  | 1:8                | 1:4   | 1:16             | 1:16  |
|                | <i>Eysenhardtia polystachya</i> | 1:128            | 1:128 | 1:32           | 1:32 | 1:16               | 1:16 | 1:128              | 1:128 | 1:128            | 1:128 |
|                | <i>Rosmarinus officinalis</i>   | 1:128            | 1:128 | 1:4            | 1:2  | 1:8                | 1:4  | 1:4                | 1:4   | 1:128            | 1:128 |

The five species were: *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar. *Typhimurium*, *Bacillus subtilis*, and *Bacillus cereus*.

Two-fold dilutions from the extracts determined MICs and MBCs.

## Discussion

The accelerated increase in bacterial resistance to antibiotics, the growing demand for animal-derived foods, and restrictions on the use of antimicrobials have turned efficient animal production into a major challenge. Considering that plants contain a variety of phytochemical compounds, the use of plant-based medicinal preparations with antibacterial activity could be incorporated as an alternative to antibiotics or adjunct therapy for infectious diseases in farm animals. Thus, as conventional antimicrobials become less effective, these compounds may offer mechanisms of action that help mitigate the emergence and hinder the dissemination of resistance.

In particular, the plant species used in this study have also been shown to have antibacterial activity, both in humans and animals. For example, the antibacterial effect of *Rosmarinus officinalis*,<sup>(33)</sup> *Psidium guajava*,<sup>(34)</sup> *Eysenhardtia polystachya*,<sup>(35)</sup> *Origanum vulgare*,<sup>(36)</sup> and *Cinnamomum zeylanicum* against *S. aureus* and *E. coli* has been reported.<sup>(37)</sup> *Syzygium aromaticum* against *S. aureus* and *B. subtilis*,<sup>(38)</sup> and *Thymus vulgaris* against *S. aureus*, *E. coli*, *S. typhimurium*, and *B. cereus*.<sup>(39, 40)</sup> The antibacterial activity of secondary metabolites such as terpenoids, phenolic compounds, alkaloids, and others has been demonstrated in multiple studies.<sup>(41, 42)</sup>

In this study, the results obtained with hydroalcoholic extraction allow us to regard as outstanding the antibacterial activity of all the extracts against *Staphylococcus aureus* ATCC 6538 and *Bacillus cereus* ATCC11778, taking the inhibition zones of enrofloxacin as reference ( $\pm 15.93$  and  $\pm 9.28$  mm, respectively), and considering that it is a highly active antibacterial drug with a breaking point ranging from 2.5  $\mu\text{g/mL}$  vs. 0.5  $\mu\text{g/mL}$ .

The best antibacterial activity was shown by *Thymus vulgaris* (*S. aureus*  $\pm 27.13$  mm, *B. subtilis*  $\pm 22.18$  mm, *B. cereus*  $\pm 12.05$  mm, and *S. enterica*  $\pm 16.18$  mm) and *Origanum vulgare* (*S. aureus*  $\pm 25.68$  mm, *B. subtilis*  $\pm 26.86$  mm, and *B. cereus*  $\pm 18.36$  mm). As it has been shown, the effectiveness of these extracts is mainly due to the presence of carvacrol and thymol, found in considerable quantities. Additionally, these plants have antioxidant and antibacterial properties.<sup>(43–46)</sup>

Coinciding with our results, it has been shown that the essential oils of *Thymus vulgaris* and *Origanum vulgare* possess excellent activity against *S. aureus* with inhibition zones of 24 to 35 mm and 34 to 35 mm, respectively.<sup>(43, 47)</sup> Also, our results concur against *B. cereus*, with inhibition zones as high as 37.33 mm.<sup>(48)</sup> Another study reported excellent antibacterial activity for *Origanum vulgare* and *Thymus vulgaris* essential oils with MIC and MBC values of 160  $\mu\text{g/mL}$  and 320  $\mu\text{g/mL}$ , respectively.<sup>(49, 50)</sup> To understand the effectiveness of these extracts, further studies are necessary to characterize the role of individual phytochemicals (eugenol, cinnamaldehyde, thymol, and carvacrol), as well as their interactions.<sup>(51–53)</sup>

It is possible to highlight the hydroalcoholic extract of *Psidium guajava*, achieving inhibition zones of 21.87 and  $\pm 13.37$  mm for *S. aureus* and *B. cereus*, respectively. Earlier works have accounted for these activities in both the essential oil and the solvent extracts.<sup>(54–57)</sup> The hydroalcoholic extracts have better antibacterial activity than aqueous extracts against *S. aureus* (MIC dilution of 1:128 vs. 1:32).<sup>(58)</sup> This finding suggests that the hydroalcoholic extraction of *Psidium guajava* favors obtaining tannins, phenols, flavonoids, terpenoids, and glycosides. In contrast, saponins are soluble in water and, therefore, not present in ethanol or methanol extracts. Saponins are important as antibacterial drugs since they act as detergents and are considered cytotoxic.<sup>(59, 60)</sup>

On the other hand, the results observed in this work coincide with Biswas, who reported that two extracts of *Psidium guajava* obtained with methanol and ethanol had antibacterial activity against *B. cereus*, i.e., inhibition zones of 8.27 mm and 12.3 mm, respectively.<sup>(59)</sup> For the methanol and ethanol extracts vs. *S. aureus* inhibition zone values were 6.11 mm and 11 mm, respectively. It is important to note that none of the extracts of *Psidium guajava* showed activity vs. gram-negative bacteria, as previously reported by Nascimento when studying *Escherichia coli* and *Salmonella* spp.<sup>(61)</sup> As for the hydroalcoholic extracts, most of the extracts obtained by Soxhlet had higher antibacterial activity against *S. aureus* compared to the reference enrofloxacin ( $\pm 13.43$ ). In this phase, *Eysenhardtia polystachya* stands out, showing an inhibition zone of approximately 20.7 mm for *S. aureus* and 11.32 mm for *B. cereus*.

These findings are consistent with a previous study in which both the aqueous and ethanolic extract were compared and showed low activities when tested against *Phorphyromonas gingivalis* at a high MIC value for the ethanolic extract, while activity vs. *Streptococcus mutans* showed a lower MIC value for the aqueous extract.<sup>(62)</sup> The phytochemical analysis of *Eysenhardtia polystachya* has reported the isolation of chalcones, flavonoids, and phytosterols having antibacterial activity.<sup>(63–67)</sup> The inhibition zones observed with the hydroalcoholic extracts were significantly lower for *Escherichia coli* ATCC 10536 ( $\pm 14.85$  mm) and *Salmonella enterica* subsp. *Typhimurium* ATCC 14028; however, for *S. enterica*, both *Syzygium aromaticum* ( $\pm 15.3$  mm) and *Thymus vulgaris* ( $\pm 16.18$  mm) showed competitive zones of inhibition with enrofloxacin ( $\pm 14.37$  mm).

This is consistent with a study by El-Maati et al., which investigated the antioxidant and antibacterial power of *Syzygium aromaticum* extracts obtained with different solvents; their findings declare that the extract obtained with 80 % ethanol has a strong antibacterial power against *Salmonella enterica* serovar. *Enteritidis* PT4 with inhibition zones of 17 mm, determining that the antibacterial capacity is strongly related to the content of phenolic compounds in the extracts.<sup>(68)</sup> On the other hand, Rota et al., evaluated extracts from different varieties of thyme and demonstrated that *Thymus vulgaris* has a potential effect not only against gram-positive bacteria, but

can also have relevant activity against gram-negative bacteria such as *S. enteritidis* CECT 4155, *S. typhimurium* CECT 443, *E. coli* serovar. O157:H7 CECT 4267 and *E. coli* CECT 516, with inhibition zones of 32, 36, 19.6, and 28.3 mm, respectively.<sup>(69)</sup>

In contrast, the antibacterial activity of all aqueous extracts obtained by Soxhlet was nearly null vs. gram-negative bacteria.<sup>(70–72)</sup> It is tempting to speculate and attribute this difference to the cellular structure of gram-negative bacteria, whose outer membrane is composed of phospholipids, polysaccharides, and selective porins, acting as a protective barrier against a wide range of antibiotics and the plant extract.<sup>(44, 61)</sup> Despite the mesh-like peptidoglycan cell wall of gram-positive bacteria, they are more permeable to phytochemicals.<sup>(73–75)</sup> This complies well with reported studies claiming that gram-negative bacteria are more resistant to antimicrobials of plant origin as compared to gram-positive bacteria.<sup>(59, 75–77)</sup>

All extracts showed growth inhibition of *Bacillus subtilis* ATCC 6633. However, only the hydroalcoholic and aqueous extracts of *Origanum vulgare*, *Thymus vulgaris*, and *Syzygium aromaticum*, showed good antibacterial activity with inhibition zones close to that shown by enrofloxacin at a concentration of 2.5 µg/mL ( $\pm 1.003$  mm). The extract of *Syzygium aromaticum* showed good efficacy vs. *S. aureus* and *B. subtilis*; *Psidium guajava* vs. *B. subtilis*; *Rosmarinus officinalis* vs. *S. aureus*, and *Eysenhardtia polystachya* showed good efficacy against *S. aureus*, *B. subtilis*, and *B. cereus*, and was the only extract inhibiting *E. coli* and *S. enterica*. However, higher concentrations were required for this former plant to inhibit the growth of gram-negative bacteria.

In a study conducted by Alonso-Castro, they found that the antibacterial activity of the ethanolic extract of this plant tested against these gram-negative bacteria was good at low concentrations, i.e., with MIC values of 1.56 µg/mL and 0.78 µg/mL, respectively.<sup>(78)</sup> From a physicochemical perspective, both extraction techniques rendered consistent results as follows: to inhibit *S. aureus*, the best extracts were *Thymus vulgaris*, *Origanum vulgare*, *Psidium guajava*, and *Syzygium aromaticum*. For gram-negative bacteria, the hydroalcoholic extracts gave better results except for the aqueous extracts of *Eysenhardtia polystachya* against *E. coli*. For clove, the best results were obtained with the hydroalcoholic extract against *S. enterica* and *B. subtilis*. Finally, the hydroalcoholic extracts of *Origanum vulgare*, *Syzygium aromaticum*, *Psidium guajava*, and *Eysenhardtia polystachya*, were active against *B. cereus*.

It is clear that the extraction method influences the antibacterial properties of the tested plants and how they were assessed, i.e., aqueous extracts diffuse with difficulty in agar plates, but when in a liquid medium, the extract-bacteria interaction is favored. In contrast, the hydroalcoholic extracts diffuse well in agar plates, improving their performance. Also, variation in antibacterial activity can be explained by the quantities of secondary metabolites present in each plant and extracted with either method.<sup>(29, 79–82)</sup> As general antibacterial pharmacodynamics for phytobiotics extracted by non-polar substances, Odhav et al., suggested that the hydrophobic and hydrogen bonding of phenolic compounds to membrane proteins and destruction of electron transport systems are the main manner in which bacterial membranes are disrupted.<sup>(83)</sup>

In contrast, the antimicrobial activity of aqueous extracts could be due to anionic components such as thiocyanate, nitrate, chlorides, and sulfates, and many other compounds naturally present in plants.<sup>(84, 85)</sup> These results show that the complexity of the active compounds present in plants makes it necessary to evaluate the antibacterial capacity of their extracts obtained by more than one method.<sup>(82)</sup> Bubonja-Šonje et al., analyzed the different techniques to evaluate the antibacterial activity of plant extracts and phenolic compounds and reported that both the agar well diffusion method and the broth microdilution method to determine MIC and MBC are adequate. In addition, the CLSI guide suggests the use of complementary methods for the determination of antibacterial activity.<sup>(32, 86)</sup>

Finally, although some of the extracts presented in this study can be incorporated into veterinary clinical trials, it remains to be determined whether it is better to isolate the active ingredients with an additional extraction or simply use the primary extracts, alone or in combination with veterinary antibacterial drugs.

## Conclusions

Due to the wide diversity of bioactive phytochemicals medicinal plants possess, their potential as a source of new antibacterial agents is evident. Because of the antibacterial drug resistance observed worldwide, a significant increase in research is currently occurring. And the immediate practical application seems to be for veterinary use. The results obtained in this work allow us to conclude that the extracts obtained from *Origanum vulgare*, *Thymus vulgaris*, *Psidium guajava*, and *Syzygium aromaticum* obtained by any of the proposed methods could be promising candidates as antimicrobial agents, since both by the agar well diffusion method and in the determination of MIC and MBC, they proved to be effective, which provides a solid basis for future research aimed at the development of new drugs of plant origin. On the other hand, it is considered to continue evaluating the aqueous extract of *Eysenhardtia polystachya* since in this study, it was the only one that showed inhibition in gram-negative bacteria such as *E. coli* and *S. enterica*, which are considered resistant to many plant extracts.

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## Data availability

All relevant data are within the manuscript and its supporting information files.

## Acknowledgments

DGAPA-UNAM Posdoctoral Program. Postdoctoral fellowship SECIHTI. PASPA-DGAPA-UNAM. PA-PIIT-IT200425 UNAM y Ciencia Básica y de Frontera 2025-SECIHTI-1954.

## Conflicts of interest

The authors have no conflict of interest to declare regarding this publication.

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